EFFECT OF PEAT COMPONENTS ON MICROORGANISMS, WITH SPECIAL REFERENCE TO HUMIC ACIDS

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by

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ABSTRACT

Research was carried out to study the effect of humic substances, which are compounds that control or affect important environmental processes, on the metabolic activities of selected microorganisms. A yeast and a bacterium with potential economic importance were selected as test organisms. Given the potential of peat in pollution control applications and in other processes, humic substances from this material were employed in this study. *Phaffia rhodozyma*, a carotenoid producing yeast, was used as a test organism. The effect of different peat extracts on the growth and astaxanthin pigment production by *P. rhodozyma* was studied. Response Surface Methodology (RSM) was used to optimize the main operating conditions, fermentation time, temperature, and pH of media, for the experiments. At optimized growth conditions a maximum biomass productivity of 9.52 $g.L^{-1}$ was observed in peat hydrolysate (PH, non- modified peat hydrolysate), which was significantly higher over other peat hydrolysates tested (modified peat hydrolysates). The modified peat hydrolysates studied were: debituminised peat hydrolysate (DPH), peat hydrolysate with humic substances removed (PHR) and debituminised peat hydrolysate with humic substances removed (DPHR). The biomass productivity attained with non- modified peat hydrolysate was also significantly higher than that produced with yeast extract-malt extract (YM) broth. YM broth, the medium employed as control, produced biomass with the maximum astaxanthin content (487 $\mu g.g^{-1}$). Among the different peat hydrolysates, PHR produced a maximum astaxanthin content of 474 $\mu g.g^{-1}$, which was significantly higher than that produced in the other peat hydrolysates. The removal of humics contributed to increased pigment content. Also, the results indicated that peat bitumens promote the pigment production. When humic acids were added at different concentrations to YM broth, they affected the biomass concentration and yield significantly, compared to control experiments with no humic acids.

Studies were conducted with four strains of *Pseudomonas fluorescens*, a bacterium isolated from petroleum contaminated soils, to find the effect of humic acids on the biodegradation potential of polycyclic aromatic hydrocarbons (PAHs). These strains had been shown to degrade toluene and naphthalene. The optimum growth parameters temperature, pH of medium and agitation, were determined by applying RSM, and degradation studies were conducted at the optimized conditions. Humic acids, the main component of humic substances, from two sources (one isolated from peat and the other supplied by Aldrich Chemicals) were employed at concentrations between 0.02 to 0.1 %. Higher concentrations were inhibitory to all the four organisms and no degradation was observed. At lower concentrations, degradation was observed, though some inhibition of the degradation was also present.

This research indicates that humic substances have a definitive effect on the metabolism of the microbial species studied. The findings have implications for the use of humic substances-containing materials such as peat and soil in biodegradation / bioremediation operations. Peat and soil are commonly employed as support medium for biofilm development. It appears that the removal of humic substances or humic acids from these materials could result in better microbial degradation action.

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1.0 INTRODUCTION

1.1 Objectives

With the recent developments in industrialization and modernization, our environment is fast deteriorating and heading towards an irreparable state. To combat this deterioration, various preventive and treatment means are being adopted. The treatment of wastes from industries is mainly classified as physical, chemical and biological treatments. All the different treatment methods have their own benefits and limitations.

Peat, the organic soil, is available in plenty in several countries around the world, including Canada , the U.S.A. and Russia. Due to its properties, peat has been considered as an important resource in combating pollution problems. Peat has been used as a column pack material in waste gas and water systems. Apart from the ability of peat to adsorb and absorb contaminants, peat carbohydrate content allows the formation of biofilms. Biofilms play an important role in the biodegradation of contaminants (Martin, 1991).

The interactions of the components of peat with microorganisms are not completely understood, as both promoting and inhibiting effects of peat extracts on microbial metabolism have been reported (Kuster, 1963; Kuster *et al.*, 1968). Those effects have been mainly attributed to the presence of humic substances in peat (McLoughlin and Kuster, 1972). Humic substances are present in soil and sediments (Schnitzer, 1978), and their effect on the soil microbial population, which play a vital environmental role such as in the degradation of organic matter and contaminants, is not clear.

The present study was primarily aimed to better understand the role of humic substances from peat on test microorganisms with potential economic importance. To this effect, the carotenoid

producing yeast *Phaffia rhodozyma*, and the bacteria *Pseudomonas flourescens*, isolated from petroleum contaminated sites and able to degrade toluene and naphthalene, were selected.

The objectives set for the present study were :

a) Optimization of fermentation conditions for maximum biomass production by *P. rhodozyma* using Response Surface Methodology (RSM) in peat hydrolysates (PH), debituminised peat hydrolysates (DPH), peat hydrolysates with humic substances removed (PHR), debituminised peat hydrolysates with humic substances removed (DPHR) and Yeast extract-Malt extract broth (YM).

b) Comparison of biomass concentration, yield, astaxanthin content, protein, carbohydrate, lipids and ash content of *P. rhodozyma* grown in different peat hydrolysates at optimized conditions.

c) Study of the effect of added humic acids 1) supplied by Aldrich Chemicals and 2) isolated from peat, on the biomass productivity of *P. rhodozyma* grown in YM broth under optimized conditions.

d) Optimization of fermentation conditions for maximum biodegradation by the toluene and naphthalene degrading *Pseudomonas flourescens* strains grown in Hydrocarbon Degrading Medium (HDM)

e) Study of the effect of added humic acids on the biodegradation of toluene and naphthalene by the four strains of *P. flourescens*

1.2 Humic Substances in the Environment

Humic substances are present ubiquitously in the environment. They are distributed abundantly over the earth's surface, occurring in almost all terrestrial and aquatic environments.

Schnitzer (1978) reported that 60 -70% of the total soil organic carbon occurs in humic materials. The role of humic substances in the carbon cycle as a major source of CO_2 and as a carbon reservoir is very vital. As a class of compounds humic substances represent quantitatively important intermediates in the remineralization of biologically reduced carbon to CO_2. The genesis, pathways, chemical composition and effects on biota of humic substances is detailed further.

1.2.1 Definition

There are numerous definitions for humic substances, but the standard definitions arising from soil science research, as reported by Ertel *et al.*. (1988), are:

Fulvic acids are the base-extractable and acid soluble portion of the soil organic matter, while *humic acids* represent the base-soluble and acid-insoluble component. The base-insoluble residue is called *humin*. Other definitions require that humic substances be the end product of a specific transformation pathway or a uniform collection of singularly formed molecules.

1.2.2 Genesis of Humic substances

Humic substances arise from the chemical and biological degradation of plant and animal residues and from synthetic activities of microorganisms. The products thus formed tend to associate into complex chemical structures that are more stable than the starting materials.

The different hypotheses about the formation of humic substances proposed by Felbeck, as reported by Schnitzer (1978) are:

(a) *The plant alteration hypothesis* : Fractions of plant tissues which are resistant to microbial degradation, such as lignified tissues, are altered only superficially in the soil to form

humic substances. The nature of the humic substances formed is strongly influenced by the nature of the original plant material. During the first stages of the humification, high molecular weight humic acids and humins are formed. These are subsequently degraded into fulvic acids and ultimately to CO_2 and H_2O.

(b) *The chemical polymerization hypothesis* : Plant materials are degraded by microbes to small molecules which are then used by microbes as carbon and energy sources. The microbes synthesize phenols and amino acids, which are secreted into the surrounding environment where they are oxidized and polymerized to humic substances. The nature of the original plant material has no effect on the type of humic substances that is formed.

(c) *The cell autolysis hypothesis* : Humic substances are products of the autolysis of plant and microbial cells after their death. The resulting cellular debris (sugars, amino acids, phenols and other aromatic compounds) condenses and polymerizes *via* free radicals.

(d) *The microbial synthesis hypothesis* : Microbes use plant tissue as carbon and energy sources to synthesize intercellularly high-molecular weight humic materials. After the microbes die, these substances are released into the soil. Thus, high-molecular weight substances represent the first stages of humification, followed by extracellular microbial degradation to humic acid, fulvic acid and ultimately to CO_2 and H_2O.

1.2.3 Possible precursors for humic substance formation

All biopolymers, monomers or metabolites of algae, microorganisms or vascular plants have the potential of being precursors for becoming humic substances. The choice of potential precursors is highly dependent on the formation pathways. Since both constructive and

destructive pathways are possible for genesis of humic substances, biopolymers and their degradation products are all potential precursors and their likelihood of becoming humic substances depends on their reactivity under the environmental conditions present (Ertel *et al.*, 1988).

The biopolymer precursors include uncharacterized biochemical compounds like aliphatic polymers (vascular plant cuticles), apart from lignins, carbohydrates, protein and triglycerides. Choudhry (1984) reported the participation of 50 - 60 % of carbohydrates, 1 - 3 % of proteins and 10 - 30% of lignins and some phenolic compounds in the humification process. Certain types of biopolymers like uronic acids, proteins and some lignins, have the solubility behaviour of humic substances without modification and thus must be considered humic substances. These substances will contribute more towards humic substances where remineralization of biochemicals is slow. The refractory biopolymers have a higher probability of becoming humic substances, assuming that they naturally have or can be transformed to have the required solubility behaviour. Also the compounds that have been modified physically or chemically and have become stabilized to remineralization by microorganisms, as might occur due to abiotic condensations of metabolites, are generally more likely to be incorporated into humic substances. The presence of specific precursors in different environments may be due to their relative stability in the environment (Ertel *et al.*, 1988).

1.2.4 Possible pathways for humic substance formation

Ertel *et al.* (1988) have reported that both the constructive and destructive pathways, and biotic and abiotic processes may contribute simultaneously towards humic substances formation.

Thus the humic substance formation is not a linear, unidirectional, stepwise pathway, and there is no unidirectional precursor-product relationship between humic and fulvic acids, which are components of humic substances.

The chemical structure of biological precursors may influence pathways for humic substance formation. Highly refractory compounds like polyethylene biopolymers would have a high probability of forming humic substances directly or with a slight modification. Carbohydrates and proteins might be too liable to persist as humic substances but their highly reactive degradation products could react rapidly with other monomers or humic substances. Lignins, depending on the environmental conditions, can either be converted directly into humic substances or yield individual phenols which readily condense to form humic substances(Choudhry, 1984).

Environmental conditions also affect the relative importance of different processes. Photochemical condensations are clearly favoured more in the sea surface layers than in the soil. Biotic processes usually dominate humic substances formation in aerobic soils with high microbial activity. Microorganisms remove the liable biochemicals thereby resulting in a concentrated refractory humic substances. However at the same time metabolites may be formed which have the potential to react biotically or abiotically with co-existing compounds, possibly making them more refractory (Ertel *et al.*, 1988).

1.2.5 Molecular weights, elemental composition, components and functional groups of humic substances

Molecular weights of humic substances usually range from a few hundred to several millions. The major elements present in the humic substances are carbon (C), hydrogen (H),

nitrogen (N), sulfur (S) and oxygen (O) (Choudhry, 1984). The major components of humic substances are the humic and fulvic acids which are differentiated based on their solubility in acid. Whereas both the humic and fulvic acids dissolve in alkali solution, humic acids precipitate on acidification (i.e.) insoluble in acid. The third category fraction present in the humic substances is called humin.

Müller-Wegener (1988) categorised the system of humic substances into:

a) Parent substances b) Less well defined humic substances

a) **Parent substances** : well-defined chemical compounds, which are transformed by humification into humic substances. Two types to be distinguished :

1) *Primary parent substances*, which are compounds of aromatic character and reactive, and

2) *Secondary parent substances*, which are linked to already formed humic substances and then incorporated into their structure, losing their individuality.

b) **Humic substances which are less well defined** can be sub-divided into:

1) *humic acid precursors*, substances reacting to humic acids by humification with high reactivity,

2) *humic acids*, acids with a relatively high stability and distinct reactivity, and

3) *humines*, the final products of the humification with low reactivity and high stability.

Müller-Wegener (1988) defined fulvic acids as the special fraction of humic substances which is soluble in acids and bases.

The ranges of elemental content as reported by Schnitzer (1978) for humic and fulvic acids are,

HA : C = 53.8 - 58.7 %, O = 32.8 - 38.3 %, H = 3.2 - 6.2 %, N = 0.8 - 4.3 % and S = 0.1 - 1.5 %

FA : C = 40.7 - 50.6 %, O = 39.7 - 49.8 %, H = 3.8 - 7.0 %, N = 0.9 - 3.3 % and S = 0.1 - 3.6 %.

The major oxygen-containing functional groups in humic substances are the carboxyls, hydroxyls and carbonyls.

1.2.6 Interaction of humic substances with biological systems

The possible interactions of humic substances with biota can be classified into two distinct categories. First, the interactions where the organisms interacting are affected directly, *i.e.* humic substances act as active agents and affect specific biochemical interactions. Second, indirect effects, by their interaction with other abiotic systems, like pesticides or heavy metals, and may pose some effects. Table 1.1 depicts an overall chemical and biological processes effected by humic substances and their positive and negative impacts (Petersen, 1991).

1.2.6.A Direct Effects

1.2.6.1 Participation of humic substances in geochemical cycles

Humic substances actively participate in carbon and nitrogen cycles. As far as carbon cycle is concerned, humic acids exert a direct influence on soil microorganisms. Soil organic matter has a carbon content of about 50%, and this is relatively resistant to microbial degradation. Whereas the amount of carbon available for degradation by microbes (1.5×10^{12} t) is high, the rate of degradation amounts to only 2-5 x 10^{9} t.year^{-1}. Soil organic matter existing in slightly degraded state is not available as a carbon source, neither can it's degradation products be utilized by microbes. The main carbon source for soil microbes is litter, of which more than 50 x 10^{9} t.year^{-1} is mineralized (Müller-Wegener, 1988).

Nitrogen has a decisive influence on growth processes being a macronutrient. The release of nitrogen, due to degradation of humic substances can induce more intensive growth in microbes and stimulate plant life. Nitrogen release from humic substances is decided by the mode of nitrogen binding in the humic substances (Müller-Wegener, 1988).

1.2.6.2 Effect of humic substances on plant growth

The penetration of high molecular weight humic substances into the plant membrane limits their interaction. For instance, it is very unlikely for a humic substance of molecular weight 100,000 to enter a root unhindered. Nevertheless, the transfer of humic substances into the plant system is possible. Small particles such as humic acid precursors or humic acids manage to penetrate into the plant membranes and build up a new equilibrium of humic substances in the cell.

Different effects of humic acids on plant growth as summarized by Müller-Wegener (1988) is presented in Table 1.1. Rerabek (1960) observed and reported growth in length of roots of wheat when treated with humic acids alone, as well as with auxins. The reason was attributed to the auxinoid nature of the acids.

Fulvic acid, a component of humic substances, has been reported to initiate root in higher plants and tissue cultures by Schnitzer and Poapst (1967). Petrovic (1982) has reported gibberellin like effect exerted by humic acids, when hypocotyl test was demonstrated with green pepper in water culture. Studies by Vaughan *et al.*, as reported by Müller-Wegener (1988), showed an increase in cell elongation of excised root segments of pea seedlings, when treated with low concentrations of humic acids (up to 50 ppm).

1.2.6.3 Effect of humic substances on microorganisms

The study of the effect of humic substances on microorganisms has always been of interest, as the activities of microorganisms present in the soil and aquatic environment are definitely influenced by these substances. There has been contradictory reports about the effects of humic substances on microorganisms and some of the reports as summarized by Müller-Wegener (1988) is presented in Table 1.2.

Kuster (1963) observed antibacterial properties of humic extracts on *Escherichia coli*, *Bacillus subtilis* and *Cladosporium*, in the form of inhibition zones when paper discs soaked in humic substances were placed in spread plates. Studies on the effect of humic substances on the respiration and growth of *Candida utilis* by McLoughlin and Kuster (1972), revealed that there were no effects, neither inhibitory nor stimulatory, was exerted by the humic substances.

Humic acids were observed to interact with the cell walls of *Micrococcus luteus* and prevent them from disruption by lysozyme (Pflug and Ziechmann, 1982). Humic acids were added at concentrations of up to 500 ppm in growth medium of *M. luteus*. It was found that humic acids had no inhibitory effect on the growth, and a considerable decrease in the concentration of the incorporated humic acids was observed. When lysozyme was incorporated into the medium, it inflicted no effect on the cell wall of *M. luteus*. This resistance of the *M. luteus* cell wall for disruption was attributed to the interaction of the phenolic components of humic acids with the cell walls. The addition of molecular fractions of humic substances at a concentration of up to 30 $mg.L^{-1}$, to the nutrient solution has been reported to result in an increase in the number of soil microorganisms active within a particular physiological group (Visser, 1985).

Table 1.1 Overview of chemical and biological processes as effected by humic substances and their positive and negative impacts (Petersen, 1991).

No.	Mechanism	Positive	Negative
1	Protonation	Acts as a weak buffer	Lowers pH
2	Particle formation	Contributes mass	Stabilizes DOC
3	Complexation	More available or toxic	Less available or toxic
4	Surfactant	Improves permeability	Decrease permeability
5	Metabolism	As a carbon source	As a toxicant

Table 1.2 Effects of humic acids on plants and microorganisms (Muller-Wegener, 1988)

Effects of humic substances	
on plant growth	on microorganisms
Increase of biomass production	Metabolite
Increase of stem elongation	Co-metabolite
Increase of root formation	Indoleacetic acid like molecule
Influence of organogenesis	Detoxification agent
Cytokinin-like activity	Regulator of membrane permeability
Inhibition of indole acetic acid oxidase	Decoupler oxidative phosphorylation
Increase of phosphatase activity	Respiratory catalyst
Inhibition of peroxidase activity	
Inhibition of phosphorylase activity	

Humic substances were observed to induce a change in metabolism in certain microorganisms, thereby allowing them to proliferate on substrates which they could not utilize previously. At concentrations above 30 mg $.L^{-1}$ inhibition was observed . Similarly, fulvic acids at concentrations of up to approximately 50 $mg.L^{-1}$ appeared to have a more pronounced physiological effect than humic acids.

Bactericidal effect of humic acids has been reported by Hassett *et al.* (1987) on *Staphylococcus aureus* and *Serratia marcescens*. Artificial HA's polymerized from catechol or hydroquinone were used in this study. Exposure of *S. marcescens* to humic acids (from catechol) at 100 ppm concentration caused up to 95 % mortality, but neither species tolerated concentration of humic acids (from hydroquinone) higher than 30 $\mu g. ml^{-1}$. Martin *et al.* (1990) studied the effect of humic and fulvic acids isolated from peat on the biomass productivity of *Scytalidium acidophilum*. The results revealed stimulatory effects by humic acids up to a concentration of 0.2 % at pH 2, followed by a decline at higher concentrations. At pH 8, the growth was found to be stimulated up to 0.4 % level of humic acids. Fulvic acids showed a decrease in biomass productivity with increase in concentrations at both the pH tested, *i.e.* 2 and 8.

Several studies conducted with peat extracts as substrate for microbial growth have reported increased biomass production compared to the synthetic medium. Humic substances present in the peat extracts are released during the acid hydrolysis process. Some of the organisms which showed such stimulatory effects were *Agaricus campestris* (Martin and Bailey, 1985), *Candida utilis* (Quierzy *et al.*, 1979; Chang, 1985), *Pleurotus ostreatus* (Manu-Tawiah and Martin, 1987), *Scytalidium acidphilum* (Martin *et al.*, 1990), *Phaffia rhodozyma* (Martin *et al.*, 1993; Vázquez and Martin, 1998a).

Coates *et al.* (1998) have reported isolation of anaerobic microorganisms belonging to the family Geobacteraceae capable of reducing humic acids from a variety of environments like lake sediments, pristine and wet land sediments and marine sediments. All the isolates were found to oxidize acetate with highly purified soil humic acids as the sole electron acceptor. Benz *et al.* (1998) have reported about some iron-reducing bacteria to reduce humic acids and some low-molecular-weight quinones. The organisms studied, *Propionibacterium freudenreichii*, *Lactobacillus lactis* and *Enterococcus cecorum* all shifted their fermentation patterns to more oxidised products when humic acids were present. Their findings indicated that these fermenting bacteria in addition to iron-reduction, are capable of transferring electrons from anaerobic oxidations *via* humic acids towards iron reduction.

A comparative study on the antiviral properties of humic acids against humic-acid-like polymers on herpes simplex virus type 1 (HSV-1) was conducted by Klocking and Helbig (1991). They observed moderate antiviral activity exhibited by the humic acids at a minimum effective concentration of 20 $\mu g.ml^{-1}$.

1.2.6.4 Effect of humic substances on enzymes

The different influences of humic substances on biological systems are ultimately on the metabolism, thus they are related to the interaction of humic substances on enzymes.

The general interaction of humic substances with enzymes as summarized by Müller-Wegener (1988) are:

1) Humic substances and enzymes interact directly. Binding mechanisms from adsorptive or stearic effects to atomic bonds (*e.g.*, an acid amide or ester linkage). Humic substances are not

only able to modify the active centre by changing the quaternary or tertiary structure of the enzyme protein but can also act directly on the active sites.

2) Humic substances comprise a multitude of different structures and do not have a single molecular weight. Therefore, some humic molecules may act as analogous substrates and disturb the equilibrium of the enzymatic reaction.

3) The cation exchange properties of humic substances result in fixation of bivalent cations often used as cofactors for enzymatic catalysis or for stabilization of the structure of the protein molecule. This leads to the indirect interaction of humics on the microbial growth .

Studies by Mato *et al.* (1971) revealed that the parent humic acid and their three fractions used in the study by them, affected the indoleaceticacid (IAA) oxidase system. Earlier studies by Mato showed no effect by the ash of humic acids on the same enzyme system. Pflug and Ziechmann (1981) have reported inhibition of malate dehydrogenase from pig heart mitochondria by a number of humic acids extracted from different types of soil or prepared synthetically. The inhibitors were found to be competitive to both substrates of the forward reaction, whereas inhibition is of the mixed type with NADH in the reverse reaction. The study concluded that the effects were due to the formation of NAD^+ and humic acid complex.

Kubler (1988) reported the antithrombin like activity by humic acids. A strong inhibition of the intrinsic pathway of blood coagulation was observed with the addition of even 10 µg of humic acid to 0.1 mL citrated plasma. An effect on the extrinsic coagulation system was obtained only after addition of more than 30 µg per 0.1 ml plasma, and was confined to a slight increase in coagulation time. Humic acid at concentrations above 40 µg per 0.1 mL plasma acted like an antithrombin, by means of sudden inactivation of thrombin.

1.2.6.B Indirect effects

Indirect effects of humic substances on biota influence growth or other manifestations not as an active agent, but by interacting with other chemicals.

1.2.6.5 Binding of metal ions to humic substances

Many of the elements like Fe, Cu, Zn and Mn greatly influence growth, being essential as micronutrients for the metabolism of plants and a number of microorganisms. Humic substances, being good chelating agents support plants by transferring chelated metals to plant roots and besides they stimulate the translocation of these ions into the plant. The binding of soluble complexes is a precondition for the improved transport by humic substances. The precipitation of compounds is another possible reaction for metal ions with humic substances. A number of metals like Cd, Pb, Hg, Ba and Ca form insoluble complexes with water soluble fractions of humic substances. The complexes thus formed become unavailable to the plant and microbial life and causes deleterious effects due to accumulation (Müller-Wegener, 1988).

1.2.6.6 Binding of organic chemicals to humic substances

Only a few of the organic substances coming to the environment, in the form of agrochemicals or waste products, show no effect on the plant and animal kingdom. Soil acts as a filtering agent and the interaction of these organic substances with the humic substances present in the soil become vital. Cationic substances will react with humic substances, showing a high anionic charge, by electrostatic interaction. Non-polar compounds will react with less charged parts of the humic substances. Binding mechanisms range from van der Waals forces to covalent

bonds. The interaction of agrochemicals with humic substances will lead to more complex compounds and become unavailable for utilisation by microorganisms . They subsequently become lethal to biota. In some instances the interaction favour the biota, because of the unavailability of a toxic compound to plants or microbes. The adsorption of toxic chemicals from the water systems by the humic acids, which are not easily biodegradable, represents an important mechanism of detoxification (Müller-Wegener, 1988).

Lovley *et al.* (1996) have reported the involvement of humic substances as an electron acceptor for the anaerobic oxidation of organic compounds and hydrogen by some microorganisms like *Geobacter mettalireducens* and *Shewanella alga*. They identified that this electron transport yielded energy to support their growth. This reduction further enhanced the capacity of the microorganisms to reduce other, less accessible electron acceptors, such as insoluble Fe(III) oxides, since humic substances can shuttle electrons between the humic-reducing organisms and the Fe(III) oxide.

The potential of humic substances to serve as a terminal electron acceptor in microbial respiration and to function as an electron shuttle between Fe (III) oxides was investigated by Lovley *et al.* (1998). *Geobacter mettalireducens*, the Fe(III) reducing bacteria, which they used in their study, conserved energy to support growth from electron transport to humics. Growth of *G. metallireducens* with poorly crystalline Fe(III) oxide as the electron acceptor was greatly stimulated by the addition of as little as 100 μM of the humics analog, anthroquinone-2,6-disulfonate. They found that a wide phylogenetic diversity of microorganisms were also able to transfer electrons to humics.

1.3 *Phaffia rhodozyma*

Phaffia rhodozyma was first reported by Miller *et al.* (1976). They isolated it from the exudates of deciduous trees from Japan and Alaska. It was named after Herman Jan Phaff who has made significant contributions towards yeast taxonomy and ecology. It was classified under Deuteromycotina (Blastomycetes) and it's properties indicated a basidiomycetous origin.

1.3.1 Astaxanthin in *P. rhodozyma*

P. rhodozyma was found to have the carotenoid pigment astaxanthin, which gave it the pink colour. The chemical structure of astaxanthin is 3,3'-dihydroxy-ß,ß-carotene-4,4'-dione (Tangerås and Slinde, 1994). It was suggested that the presence of carotenoids within the yeast protects it against singlet oxygen damage (Schroedar and Johnson, 1995). Many methods have been proposed by different researchers to isolate astaxanthin from the yeast cells. One of the earlier methods, as suggested by Johnson *et al.* (1979) was to use extracellular enzyme, produced by the bacterium *Bacillus circulans*, which digests the cell wall and renders the pigment extractable by acetone and ethanol. This method was found to work most efficiently at pH 6.5 and lower concentrations of yeast. Okagbue and Lewis (1984) suggested a method, where distilled water and 0.02 molar citrate buffer at pH 7.0 were used to autolyse the yeast thereby rendering the pigment extractable. A mixed culture condition of *P. rhodozyma* and *B. circulans* was proposed by Okagbue and Lewis (1985), which led to the destruction of cell and rendered astaxanthin extractable. Gentles and Haard (1991) used the enzyme 'Funcelase' to break the cell walls and further extraction of astaxanthin was made with acetone and petroleum ether. The yeast cells were crushed with glass beads and extracted with acetone by An *et al.* (1989).

The French Press method was used to break the cell walls of *P. rhodozyma* to extract astaxanthin from *P. rhodozyma* by Sangha *et al.* (1995) which was found to yield three and a half times more pigments than extraction with funcelase enzyme. Astaxanthin was quantified using HPLC and spectrophotometric methods at 474 nm (Acheampong and Martin, 1995; Vázquez and Martin, 1998).

Increase in astaxanthin production was attempted with different chemical compounds and growing conditions. Mutation with UV radiation has resulted in paler cells which had lower astaxanthin content than the parent strain (An *et al.* ,1989). The chemicals ethyl methane sulfonate and N-methyl-N-nitro-N-nitroguanidine (NTG) generated mutations in *P. rhodozyma*, which resulted in increased astaxanthin production (An *et al.*, 1989). Exposure of the mutated cells again with NTG has resulted in a further increased astaxanthin content (Johnson and An, 1991). A general problem encountered with mutations was that not all the mutants generated were stable. Fang and Cheng (1993) have reported increased astaxanthin production by *P. rhodozyma*, almost by three times compared to parent cells, when mutated with NTG .

1.3.2 *P. rhodozyma* as a pigment source

Astaxanthin is the pigment responsible for the colouring of the salmonid fishes and in natural conditions they get their pigments from algae, crustaceans and krills. In pen reared conditions the pigment has to be incorporated into the feed. Biological sources have always been considered superior over synthetic pigments (Tangerås and Slinde, 1994).

Various studies were conducted by Johnson *et al.* (1977), Johnson *et al.* (1980) and Binkowski *et al.* (1993) have confirmed the superiority of using *P. rhodozyma* over synthetic

pigments for colouring salmon. They also reported the increased absorption of the biological source of pigment into the flesh by the fishes, over the synthetic pigments.

Johnson *et al.* (1980) have also studied the use of astaxanthin from the yeast *P. rhodozyma* for the pigmentation of egg yolks. The feed with broken yeast cells were found to deposit in egg yolks against the feed with whole yeast cells which resulted in no pigmentation.

1.3.3 Different carbon sources and *P. rhodozyma*

Although YM broth has been the synthetic medium used in most of the studies with *P. rhodozyma*, several other carbon sources have also been used for culturing *P. rhodozyma*. Some of the substrates used include alfalfa residual juice (Okagbue and Lewis, 1984), molasses (Haard, 1988), peat hydrolysate (Martin *et al.*, 1993), media from corn wet-milling co-products (Hayman *et al.* ,1995), diluted sugar cane juice, urea and sodium phosphate (Fontana *et al.*, 1996) and hemicellulosic hydrolysates of *Eucalyptus globulus* wood (Parajó *et al.*, 1998). These alternate substrates were used primarily to bring down the industrial production cost associated with the cultivation of *P. rhodozyma*.

Vázquez *et al.* (1997) studied the effect of different carbon sources on the carotenoid profiles of biomass produced by a few strains of *P. rhodozyma*. They found that the strain NRRL Y-17268, grown in xylose produced the maximum carotene content. Astaxanthin accounted to 82 % of the total carotenoids.

1.3.4 Growth studies of *P. rhodozyma* on peat hydrolysates

Peat hydrolysate is a good carbon source and has been used to grow several microorganisms with potential commercial importance. The study of the growth of *P. rhodozyma* in peat hydrolysates has been also attempted. The hydrolysis of peat with mild acid results in the release of carbohydrates into the hydrolysate. Besides, the hydrolysate has other growth promoting substances which has been confirmed by the increased biomass production and yield of the organisms grown in them. Martin *et al.* (1993) have studied and optimized different growth parameters for *P. rhodozyma* grown in peat hydrolysates. They found that the best conditions were: pH 7, 18 °C, 5-day culture time, and 200-rpm agitation rate. An astaxanthin concentration of 1279.82 $\mu g.g^{-1}$ was reported by these authors. Acheampong and Martin (1995) studied the kinetics of growth of *P. rhodozyma* and found the relationship between growth and astaxanthin production to be linear. The astaxanthin production was found to be growth associated and they reported the mean specific growth rate and doubling time to be 0.038 h^{-1} and 18.24 h for the accelerated growth phase.

Vázquez and Martin (1998b) used Response Surface Methodology (RSM) to optimize continuous culture conditions for *P. rhodozyma* grown in peat hydrolysates. Maximum biomass production was obtained by employing the predicted fermentation conditions: a dilution rate of 0.017/h and a pH level of 7.19. Confirmatory test produced a maximum biomass of 4.95 $g.L^{-1}$ and 0.52 $g.g^{-1}$ of biomass yield.

Kinetic parameters were calculated for the growth of the yeast, and biomass productivity was determined using mathematical models by Vázquez and Martin (1998a). A low maximum specific growth rate (μ_{max} = 0.08 h^{-1}) and a high Monod constant (Ks = 26.2 x 10^3) were obtained

for the substrates. Under selected conditions, a biomass productivity of 0.108 g $(lh)^{-1}$ and an astaxanthin productivity of 0.046 mg $(lh)^{-1}$ were obtained.

Ho *et al.* (1999) conducted a study to compare the growth and carotenoid production of *P. rhodozyma* grown in different feeding methods, *viz.*, constant feeding, exponential feeding, DO-stat (Dissolved Oxygen) and pH-stat fed-batch cultures. They recorded the highest biomass production of 17.4 $g.L^{-1}$ and the lowest carotenoid production of 307 $\mu g.g^{-1}$ of cell of *P. rhodozyma* from DO-stat fed-batch culture. The lowest biomass of 14.7 $g.L^{-1}$ and a highest carotenoid content of 412 $\mu g.g^{-1}$ of cell was obtained from the exponential, fed-batch culture.

1.4 Response Surface Methodology (RSM)

Response Surface Methodology (RSM) is a statistical procedure used to optimize experimental conditions, *i.e.* the interactive influence of some important factors on the main output of interest. In short, response surface experiments attempt to identify the output or response of a system as a function of the explanatory variables. The surface can be thought of as a surface over the explanatory variables' experimental space. RSM was first described by Box and Wilson as early as 1951. Though initially it was proposed for optimizing experimental conditions for chemical investigations, later it gained momentum in being used as a method for optimizing other experiments.

Hill and Hunter (1966) published a comprehensive literature survey on the RSM experimentations. Thompson (1982) summarized some important statistical models, experimental design and analysis methods for response surface experiments.

Off late RSM was started to be used as a methodology in optimizing fermentation experimental conditions. Maddox and Richert (1977) used RSM to optimize supplementary nutrient concentrations for a basal medium of whey filtrate. *Fusarium moniliforme* was grown in it and optimized for gibberellic acid production. They used central composite design and concluded that this experimentation resulted in saving time and experimental materials.

The optimization of experimental conditions for the bioconversion of citronellal to citronellol applying RSM was done by (Cheynier *et al.*, 1983). *Candida tenuis*, a yeast which was isolated from the gut of *Phoracantha semipunctata* was used in this study. They used a central composite experimental design with multiple linear regression to estimate the model coefficients of the factors influencing the bioconversion process. They too reported this procedure to be efficient and accurate. Marty (1985) used RSM to optimize glutaraldehyde activation of a support for enzyme immobilization. Simplex method was used to opitmize the parameters, glutaraldehyde concentration, pH and contact time. The study concluded that their experimental methods produced reliable and reproducible results.

Saval *et al.* (1993) used RSM to optimise culture conditions for streptomycin production. They used orthogonal-central composite experimental design to optimise the factors affecting streptomycin production like glucose, beer-autolyzate , sodium chloride and dibasic potassium phosphate. The organism used was *Streptomyces griseus* and their experiments resulted in a determination coefficient of 0.9384 for their second-order model, which showed their experimental results agreed with the model. Optimization of the culture medium for *Sclerotium rolfsii* and the enzymatic hydrolysis of lignocellulosic material was conducted by Haltrich *et al.* (1994). They used orthogonal central composite design and the factors studied to have influence

on the enzymatic hydrolysis were cellulose and peptone from meat. They tried five coded levels and their experimental results agreed very well with the second order model used.

Vázquez and Martin (1998b) optimized fermentation conditions like pH and dilution rate, which influences a continuous culture condition for *P. rhodozyma*. They used a factorial design to carry out the experiments and obtained optimal conditions. The data from the experimental design was subjected into a second order multiple regression equation. The dependent variables studied were biomass concentration, substrate concentration, biomass yield and biomass volumetric productivity. Their confirmatory tests proved the reliability of the prediction by the model. The regression coefficients for all the experiments were above 0.99 which proved that the experimental results agreed to the model selected.

Kim *et al.* (1998) used a factorial experimental design to optimize mixtures of six cellulases, five *Thermomonospora fusca* and one *Trichoderma reesei* to maximize the glucose produced from filter paper. Optimized mixtures A and B produced 8 and 1.5 times higher glucose than the sum of the activity of the individual cellulases. They reported that their glucose yield was dependent on total enzyme concentration.

Other applications of RSM include:

- Optimization of hexametaphosphate- assisted extraction of flaxseed proteins using RSM (Wanasundara and Shahidi, 1996) where a central rotatable composite design has beed used.

- Optimization of extraction of anthocyanin pigments from purple sunflower hulls using RSM by Gao and Mazza (1996) where face -centred cube design was used.

- Optimization of water washing process for sunflower heads before pectin extraction by Shi *et al.* (1996), where three-level three-factor experimental design was followed.

- Optimization of nitrogen and recovery in the enzymatic hydrolysis of *Squalus acanthias* protein (Diniz and Martin, 1997) where Box-Behnken factorial design was used.

- Optimization of reaction conditions for concentration of omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation by Wanasundara and Shahidi (1999) where a central composite rotatable design has been used.

Watts (1995) published a paper on canonical analysis which is an important part of the RSM which helps in predicting the optimum experimental conditions.

1.5 Hydrocarbons and their degradation in the environment

Hydrocarbons occur naturally in the environment, but they also enter the environment due to human activities. The hydrocarbons in the environment vary from simple methane to more complex aromatic hydrocarbons, like PAHs. These hydrocarbons persist in the environment and lead to health-related problems in all the biotic components of the environment. Some microorganisms possess the ability to utilize the complex hydrocarbons as sole carbon sources and thereby degrading them to simpler CO_2 and H_2O. These organisms mostly originate from soils and sediments (in case of aquatic environments).

The degradation of hydrocarbons is usually decided by the presence of enzymic apparatus acquired by microbes during the course of evolution. This in turn depends on two factors: first, the ability of the microbial enzymes to accept as substrate compounds having chemical structures similar to, but not identical with, those found in nature; and second the ability of these novel substrates, when in the presence of microbes, to induce or depress the synthesis of the necessary degradative enzymes. The study of biodegradability is linked inevitably with the study of both the

mechanisms of induction and the modes of action of those enzymes employed for degradation (Dagley, 1975).

Studies about degradation of hydrocarbons by microorganisms has been made as early as 1946 by Zobell, even there were a few works reported still earlier (Boyland and Levi, 1935; Strawinski and Stone, 1943). This author discussed the various mineral requirements of hydrocarbon oxidizers, apart from the environmental factors like oxygen tension, organic matter, temperature requirements and dispersion of hydrocarbons in culture media. Besides the occurrence of hydrocarbons oxidizers in nature, modification of petroleum products and activities of hydrocarbon oxidizers in the soil were also discussed.

The different metabolic pathways involved and the mechanisms of oxidation of hydrocarbons has been reviewed by McKenna and Kallio (1965). The first comparative study of petroleum degrading yeasts, fungi and bacteria and their ability to degrade a mixed hydrocarbon was reported by Walker *et al.* (1975). The mixed hydrocarbon substrate employed contained aliphatic, alicyclic, aromatic and polynuclear aromatic hydrocarbons. The yeasts which were studied included *Candida* sp., *Candida tropicalis*, *Hansenula beijerinckii*, *Aureobasidium pullulans*, *Rhodotorula glutinis* and *R. rubra* and the fungi *Cladosporium resinae*, *Aspergillus* spp., *Penicillium* spp. The bacterial spp. were *Pseudomonas* spp., *P. aeruginosa*, *Vibrio* spp. *Acinetobacter* spp., *Leucothrix mucor*, *Nocardia asteroides*, *N. corallina*, *Rhizobium meliloti* , *R. leguminosarum*, and a coryneform.

Their results showed that there was little correlation between the chain length of normal alkane and susceptibility to biodegradation. Cumene, naphthalene, phenanthrene, pristane, 1,2-benanthracene, perylene and pyrene were found to be degraded by microorganisms. They

observed the patterns of degradation to be similar for the bacteria, yeasts and fungi, though significant differences were observed between different isolates.

The microbial degradation of petroleum hydrocarbons as an environmental issue was discussed by Atlas (1981). In his publication, he reported the chemistry involved in the degradation of various hydrocarbons. Besides, the taxonomic relationships between the different organisms degrading petroleum hydrocarbons has been discussed. He has observed that the different organisms have been found to fall within a few phenetic groups. The different environmental factors influencing the biodegradation of petroleum compounds like physical state of pollutants, temperature, nutrients, oxygen, salinity and pressure have also been discussed.

Ribbons and Eaton (1982) reviewed the chemical transformations occurring to the aromatic hydrocarbons which supported microbial growth. They have discussed the chemical transformations occurring in various benzenoid compounds such as monoalkyl benzenes, dialkyl benzenes and fused poly nuclear hydrocarbons due to microbial degradation.

1.5. a) *Pseudomonas*

Pseudomonas spp., an aerobic soil organism, has always been reported to be a versatile organism, possessesing ability to degrade a variety of substrates, from simple glucose to more complex hydrocarbons. For instance one strain of *P. multivorans* can utilize up to 108 of the 146 diverse organic compounds preferred as growth substrates (Ornston, 1971). Ornston also has reviewed the regulation of catabolic pathways by which *Pseudomonas* degrades different complex organic compounds.

1.5.1 Degradation of Toluene

Toluene is an aromatic hydrocarbon (Fig. 1.1), it can be a natural product of diagenic origin or synthetically a starting material for complex synthesis. It is commonly used as a paint thinning agent. In some laboratory work, toluene is used as a surface sterilant over liquid media, to avoid microbial contamination from air. In spite of its resistance to biodegradation, some soil microorganisms have been identified to degrade toluene.

Galic and Vogel (1987) reported transformation of toluene by mixed methanogenic cultures derived from ferulic acid degrading sewage sludge enrichments. These authors reported that toluene was supplied semi-continuously as the carbon and energy source. After 60 days they found 50 % of the substrates to be reduced to CO_2 and methane. They observed 8 aromatic, 5 alicyclic and 10 aliphatic compounds after 64 day incubation period. Eventually the concentration of the intermediary compounds dropped. By the end of the incubation period all the aliphatic compounds had disappeared.

The degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 was conducted by Mars *et al.* (1996). *B. cepacia* was cultivated in a fed-batch bioreactor on toluene or trichloroethylene. Fries *et al.* (1997) isolated and characterized bacterial populations that grew in an aquifer, following three sequential field tests of phenol- or toluene-driven co-metabolism of trichloroethene (TCE). The toluene concentrations were reduced from 50 ppm to 5ppm. TCE was co-metabolized.

Tsao *et al.* (1998) studied the metabolism of benzene, toluene and xylene hydrocarbons in soil. Enrichment cultures obtained from soil exposed to benzene, toluene and xylene (BTX) mineralized benzene and toluene but cometabolized only xylene isomers. The authors used

C-labelled BTX hydrocarbons in soil either individually or as mixtures. At the end of each experiments they assessed the solvent extractable polymers, biomass and humic material. The results suggested that the catechol intermediates of the BTX degradation were preferentially polymerized into soil humus and that the methyl substituents of the catechols derived from toluene and xylene enhanced this incorporation.

Acuna *et al.* (1999) investigated the microbiological and kinetic aspects of a biofilter inoculated with a consortium of five bacteria and two yeasts adapted to remove toluene vapours. Initially the toluene sorption isotherm on peat and the effect of different environmental conditions on the toluene consumption rates of this consortium were measured. Bacterial counts up to 10 x 10^{11} cfu/g dry peat were found after 88 days, which were about 10^4 times higher than the inoculum. Further observations with SEM showed a nonuniform biofilm development on the support and presence of an extracellular material.

1.5.2 Degradation of Naphthalene

Naphthalene, an aromatic compound (Fig. 1.1), though complex has been reported to be degraded by a number of organisms. Walker and Wiltshire (1953) isolated naphthalene degrading organisms by enriching Rothamsted experimental station soils with naphthalene and then maintaining the organisms in plates with naphthalene as the sole carbon source. Later, Treccani *et al.* (1954) reported on the early stages of the oxidation of naphthalene by five organisms where D-trans-1:2-dihydro-1:2-dihydroxy naphthalene, salicylic acid and catechol appeared to be intermediate oxidation products.

Strawinski and Stone (1954) established some of the conditions for the oxidation of naphthalene by a pseudomonad based on the yield of the ether extractable substances. These authors observed vigorous aeration to yield approximately three times more degradation than unaerated controls. They also reported a higher pH to be better for degradation. Calcium and copper were identified to be essential for maximum degradation. It was also observed that the degraded part of naphthalene had more salicylic acid. Murphy and Stone (1955) reported the different intermediary compounds involved during the degradation of naphthalene. Naphthalene was found to degrade through salicylic acid, which was further oxidized through catechol to ß-ketoadipic acid. They also reported the evidence of a second pathway of naphthalene oxidation which produced 1,2-naphthoquinone, 1,2-dihydroxy naphthalene and was found to be non-enzymatically converted to1,2-naphthoquinone. Further, it was observed that the omission of $FeCl_2$ and $MgSO_4$ from the basal medium prevented the formation of salicylic acid, but no interference was observed with the production of 1,2-naphthoquinone.

The influence of different factors affecting the rate of assimilation of naphthalene by *Pseudomonas aeruginosa*, like aeration, pH, role of metallic ions, phosphate concentration in the medium have been conducted and reported by Klausmeier and Strawinski (1956). They reported that aeration, high pH, metallic ions and time to affect salicylate content of culture medium during naphthalene oxidation.

Davies and Evans (1964) proposed the ring-fission mechanism of degradation of naphthalene by soil pseudomonads. Their study revealed that naphthalene was oxidatively metabolized to 1,2-dihydroxynaphthalene, which then underwent ring cleavage. The immediate product of ring fission was found to be cis-o-hydroxybenzalpyruvate, isolated as a crystalline

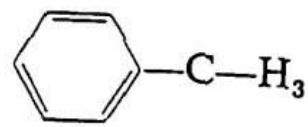
perchlorate. A NAD-specific dehydrogenase was reported to be present in the cell-free extract that oxidized salicylaldehyde to salicylate. Salicylate was then found to be oxidatively decarboxylated to catechol, which was then completely dissimilated through hydroxymuconic semialdehyde.

The regulation of naphthalene metabolism in *Pseudomonas* spp. was studied by Shamsuzzman and Barnsley (1974). They have reported the coordinated involvement of the enzymes naphthalene oxygenase, 1,2-dihydroxy naphthalene oxygenase and salicylaldehyde dehydrogenase during naphthalene degradation.

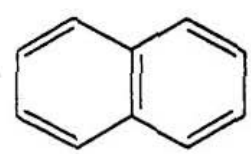
The metabolism of naphthalene by *Cunninghamella elegans* was studied by Cerniglia and Gibson (1977). They reported production of six metabolites, the major being 1-naphthol (67.9%) and 4-hydroxy-1-tetralone (16.7%). Minor metabolites produced were 1,4-naphthoquinone (2.8%), 1,2-naphthoquinone (0.2%), 2-naphthol (6.3%) ad trans-1,2-dihydroxy-1,2-dihydronaphthalene (5.3%). A detailed review on the pathway of naphthalene degradation by *Pseudomonas* spp. has been made by Ribbons and Eaton (1982).

With these background references the present study was planned and conducted. The emphasis was given on the effect of humic acids, among the different peat components, which apart from being a mojor component of peat, soil and sediments, also has controversial reports about it's effect on microorganisms.

Fig. 1.1 Molecular structure of toluene and naphthalene



Toluene



Naphthalene

2.0 MATERIALS AND METHODS

2.1 Effect of peat components on *Phaffia rhodozyma*

2.1.1 Organism and Inoculum

Phaffia rhodozyma, ATCC 24202 obtained from American Type Culture Collection (Rockville, MD, USA) was used. The organism was maintained on YM agar plates (DIFCO Chemicals, Detroit, Michigan, USA) and transferred every month. Inoculum was prepared by transferring a loopful of the organism from agar plates into 50 mL of YM broth in a 250 mL flask. It was then incubated at 22°C, in a water bath gyrating at 300 rpm for 24 h. One mL of this culture was subsequently inoculated to the experimental flasks containing 50 mL of the growth medium.

2.1.2 Substrates

2.1.2.1 YM Broth

The growth medium was prepared by dissolving 21 g of YM broth (DIFCO) in 1 L of water. The pH of the medium was adjusted to 7 with 0.1N sulphuric acid before sterilization.

2.1.2.2 Peat

Peat used in this study was collected from Sundew peat bog, St.John's, Newfoundland.

2.1.2.3 Peat hydrolysate

The air dried peat (dried at room temperature i.e. 20° C for 24 h) was mixed with 1.5 % sulphuric acid at 1:4 ratio (w:v) and autoclaved at 121 ± 1°C for 2 h. The hydrolysate was separated from the peat using a centrifuge sieve (International Equipment Co., Massachusetts)

with Whatman No.1 filter paper. The peat hydrolysate was stored at 4°C before further use (Martin *et al.*, 1990).

2.1.2.4 Debituminised peat hydrolysate

Bitumens from the peat were removed by extracting it with toluene:ethanol (1:1). The extraction was done by blending (Waring Blender) the peat with the solvent (1:4, w:v) for 5 min. The peat was separated from the solvent, washed with water and air dried at room temperature for 24 h. The dried peat was hydrolysed as above (section 2.1.2.3) to obtain the debituminised peat hydrolysate (Chavan and Shahidi, 1997; Martin *et al.*, 1990).

2.1.2.5 Peat hydrolysates with humic substances removed

Air dried peat (dried at room temperature i.e. at 20° C for 24 h) was treated with 1 % NaOH solution under a nitrogen atmosphere (the head space of the flask was replaced with nitrogen and closed air tight) for 24 h. The alkali solution carrying the humic substances was separated from the peat by filtering through a Whatman No.1 filter paper. The separated peat was washed 4-5 times with distilled water to ensure complete removal of humic substances. The peat was treated with 0.1 N sulphuric acid to bring down the pH to 6. The peat was air dried and subjected to acid hydrolysis as above (section 2.1.2.3) (Martin *et al.*, 1990).

2.1.2.6 Peat hydrolysates from peat debituminised and humic substances removed

The bitumen component of peat was removed initially with toluene : ethanol as described in section 2.1.2.4. The humic substances were removed following the method explained in section 2.1.2.5. The peat was dried and peat hydrolysates were obtained as detailed in section 2.1.2.3.

2.1.3 Growth medium and experimental setup

The total carbohydrate content of all the peat extracts was set to 20 $g.L^{-1}$. This was achieved by diluting the peat extracts with distilled water. The extracts were further supplemented with 0.68% yeast extract and 0.60% peptone. This was considered as the growth medium and the medium was adjusted to the experimental levels as per RSM design with 1.5 N NaOH. The experiments were conducted in shaker flasks. In 250 mL Erlenmeyer flasks, 50 mL of growth medium were sterilized in an autoclave at 121 ± 1°C for 20 min. Under aseptic conditions, 1 mL of inoculum was added and the flasks were incubated in a Gyrotory Shaker Water Bath at 300 rpm. Other operational conditions were followed from the experimental design.

2.1.4 RSM experimental design and analyses

Response Surface Methodology (RSM) was used to optimize experimental conditions for the experiments. Thus, after the operational conditions were optimized, the effect of peat components, particularly humic acids, were studied. The experimental design followed for the RSM experiments was face-centred cube design. The independent variables and the design levels are presented in Table 2.1. All the experiments were carried out in triplicates, and the mean and standard deviations of the results were calculated. The results were also subjected to a second order multiple regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. The analyses were performed using the Statistical Analysis System Software (SAS Institute Inc., Cary, NC).

Table 2.1 Independent variables and experimental design levels for response surfaces of *Phaffia rhodozyma* grown in different substrates.

Independent variables	Symbols		Levels		
	Coded	Uncoded	-1	0	+1
Fermentation time	X1	FT	24	72	120
Temperature	X2	T	12	18	24
Substrate pH	X3	pH	4	6	8

2.2 Effect of humic acids on *Phaffia rhodozyma*

Humic acids was obtained from Aldrich Chemicals (Milwaukee, Wisconsin, USA), and humic acids obtained from the peat, were tested for their effect on the biomass production and yield on *P. rhodozyma*.

2.2.1 Extraction of humic acids from peat

The humic acids from peat were separated following the method presented by Martin *et al.* (1990). To 10 g of air dried peat (dried at room temperature i.e. 20° C for 24 h) in a 250 mL flask, 100 mL of 0.1 N NaOH was added and agitated in a shaker for 24 h. The air in the flask were replaced by 100 % nitrogen. The alkali extract was separated from peat by filtering through a Whatman No.1 filter paper. The peat was further washed with distilled water and the washing was added to the alkali extract. The pH of the separated liquid was adjusted to 2.0 with 2 N HCl and was allowed to stand at room temperature for 24 h. The humic acids coagulate at the bottom and it was separated by centrifugation. The separated humic acids were freeze dried and stored before further use.

2.2.2 Growth of *P. rhodozyma* on different concentrations of humic acids

This experiment was primarily conducted to study the effect of humic acid component of the peat on the growth of *P. rhodozyma* on an ideal growth medium. To 50 mL of YM broth prepared with different concentrations of humic acids (Aldrich Chemicals, and humic acids separated from peat) *viz.*, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 % (w/v), 1 mL of

inoculum was added and incubated at optimum conditions for biomass production, pH, temperature and fermentation time (values adopted from RSM prediction for maximum biomass production in YM broth). The flasks were agitated at 300 rpm.

2.3 Gravimetric and chemical analyses

2.3.1 *Biomass concentration and* Yield

The biomass produced in the growth medium was centrifuged at 10,000 x g, and the separated biomass was transferred into a pre-weighed aluminium container. The biomass was then oven dried at 103°C for 18 h, stabilized in desiccator, weighed and expressed as $g.L^{-1}$.

Yield is calculated as the measure of the amount of biomass produced for a unit consumption of substrate. Substrate consumed was calculated as the difference between the initial and final carbohydrate concentrations.

2.3.2 Total carbohydrate content

The total carbohydrate content of the growth medium was determined by the anthrone reagent method (Morris, 1948). The method involves addition of 4 mL of anthrone reagent to 2 mL of the samples. The absorbance of the colour developed was measured at 540 nm using a *Milton Roy Spectrophotometer (Spectronic 601*, USA). The total carbohydrate is estimated from the standard curve of glucose.

2.3.3 Total nitrogen and protein content

The total nitrogen content in the biomass was determined by following the method reported by Martin *et al.* (1993). The method involved digestion, distillation and titration. To 0.5 g of the freeze dried biomass, in a Kjeldahl digestion tube, two Kjeltabs were added. To this, 25 mL of concentrated sulphuric acid was added and was digested in a Kjeltec Digestion System (Büchi 426, Rexdale, Ontario). The digestion was continued until the samples turned colourless. One hundred mL of water was carefully added as soon as the liquid reached room temperature. To each of the digestion tubes, 50 mL of 40 % NaOH was added before connecting it to a Ammonia Distillation Unit (Kjeltec Distillation System 315, Rexdale, Ontario). The distillate was collected in a 50 mL of 4 % boric acid solution with indicator. The distillation was continued until a total of 150 mL solution was collected. The flasks containing the distillate were capped tightly till it was titrated. The third step involved the titration of the distillate against a standard solution of 0.1 M sulphuric acid. The values obtained from the titration were used to calculate the % nitrogen values also. The protein content was calculated by the formula, Protein content = nitrogen content x 6.25.

2.3.4 Lipid content

To 50 g of freeze dried biomass sample, 100 mL of methanol and 50 mL of chloroform were added in a blender. The contents were then blended for 2 min. An aluminium foil cover was used to avoid spill overs. After, another 50 mL of chloroform was added and blended for 30 sec. To the blender (Waring blender), 50 mL of distilled water was added and blended for 30 sec. The blended contents were filtered using suction through a Büchner Funnel with Whatman filter paper

No.4. The blender was washed with chloroform / methanol (1:1) and the contents were transferred to the funnel. The filtrate was transferred into a 500 mL separatory funnel. The separatory funnel was left undisturbed till the chloroform layer separated and clarified. The chloroform layer was carefully drawn off into a graduated cylinder and the volume recorded. From this 10 mL of chloroform extract was transferred into pre-weighed aluminium dishes. The chloroform was allowed to evaporate for about 2 h and then the dishes were transferred to hot air oven set at 103°C for 1 h. The dishes were then cooled and weighed.

Lipid content was calculated using the formula:

$$F = [(W_2 - W_0)(V_1)(100)]/(V_2)(W_3)$$

F - lipid content (%)

W_0 - weight of empty aluminium dish (g)

W_2 - weight of aluminium dish with dried lipid residue (g)

W_3 - weight of sample taken (g)

V_1 - total volume of chloroform layer in graduated cylinder (mL)

V_2 - volume of chloroform extract removed and dried in aluminium weighing dish (mL)

(Bligh and Dyer, 1959)

2.3.5 Ash content

The content of ash in the biomass was determined according to the method used by Vázquez and Martin (1998a). It involved weighing 1 g of the freeze dried biomass into a previously dried, cooled porcelain crucible. The sample was ignited in a muffle furnace at 550°C for 24 h. The crucibles were then allowed to cool in a desiccator to room temperature. The crucible with the ash was weighed and the ash content was expressed in $g.kg^{-1}$.

2.3.6 Astaxanthin content

Astaxanthin content was estimated following the method of Martin *et al.* (1993), except for a modification. *Dimethylsulfoxide (DMSO) was used to break the freeze dried cells instead of* 'Funcelase'. To 200 mg of freeze dried cells, 5 mL of DMSO was added and they were vortexed for about 5 min. After digestion, the mixture was extracted with acetone and this procedure was repeated till the cells turned colourless. The acetone extract was transferred into a separatory funnel and to it equal volume of petroleum ether was added and the funnel was shaken well. The acetone part was separated and the absorbance of the ether was measured at 474 nm in a Milton Roy spectrophotometer (Spectronic 601, USA). The volume of petroleum ether was measured. Total astaxanthin content was calculated using 1 % extinction coefficient = 2100, by :

$$\text{Astaxanthin content} = \frac{\text{vol. of ether extract} \times \text{absorbance at 474 nm} \times 100}{\text{weight of the cells} \times \text{extinction coefficient}}$$

2.4 Humic acids - Toluene and Naphthalene degrading organisms interactions

2.4.1 Organisms, growth medium and inoculum

Toluene and naphthalene degrading organisms used in the present study were *Pseudomonas fluorescens* strains NC1, NC2, NC3 and NC4 which were isolated from a petroleum contaminated soil of Calgary, Alberta and was supplied by Nova Chemicals, Calgary.

Hydrocarbon degrading medium (HDM) listed in Table 2.2 was used for culturing these organisms. To 40 mL of HDM in a 100 mL vial, a loopful of organism was transferred, along with the respective carbon source and were incubated at the following conditions : temperature =

22°C, agitation = 150 rpm, for 24 h in case of toluene degraders. The inoculum was previously grown in HDM medium for 24 h at 150 rpm at 22°C.

2.4.2 *Optimization of operational conditions for growth of the soil microorganisms applying RSM*

The operational conditions for the growth of these bacterial cultures, namely, pH of the medium, incubation temperature and agitation, were optimized using RSM. The experimental design followed was face-centred cube design. The independent variables and the design levels are presented in Table 2.3. All the experiments were carried out in triplicates and their mean presented. The results from the experiments were subjected to a second order multiple regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. The analysis were performed using Statistical Analysis System Software (SAS Institute Inc., Cary, NC).

2.4.3 Effect of humic acids on the degradation of toluene and naphthalene by the soil microorganisms

The microorganisms were grown in 40 mL of HDM, in 100 mL vials, incorporated with different concentrations of humic acids - both Aldrich supplied humic acid-sodium salt, and the humic acids separated from peat - *viz.*, 0.02, 0.04, 0.06, 0.08 and 0.1 %, respectively. Earlier, concentrations above this was tried which produced no degradation.

2.4.4 GC-MS Analysis of toluene and naphthalene

Toluene and naphthalene in the vials were analyzed by injecting 20 µl of the head space gas drawn from the vial head space volume of 60 mL, into a Hewlett Packard gas chromatograph connected to a Finnagan 800 ion trap mass spectrophotometer (Model 1790, Avondale, PA, USA). The GC was fitted with a 30 m DB-5 column. The injection temperature was set at 100 °C and the oven temperature was isothermally controlled at 250 °C for toluene and 180 and 250 °C respectively for naphthalene. The mass spectrophotometer was operated in a selected ion monitoring mode. The peak areas of the generated single ion mass intensity were used and compared to head space concentration of standard solutions without the presence of bacteria. Uninoculated vials served as controls. The amount of toluene or naphthalene degraded was measured as a difference of the initial and final concentrations of toluene and naphthalene present in the head space.

2.5 Statistical analyses

All the experiments were conducted in triplicates and their means and standard deviations are presented. Statistical significance between the experimental values were tested using Tukey's Honest Significant Difference (HSD) method (Sprinthall, 1987).

Table 2.2 Composition of Hydrocarbon Degrading Medium

Component	Concentration ($g.L^{-1}$ of water)
KH_2PO_4	1.000
K_2HPO_4	1.000
NH_4NO_3	2.000
$MgSO_4.7H_2O$	0.300
$CaCl_2.2H_2O$	0.001
$FeSO_4.7H_2O$	0.001
Micronutrients	1 mL
Micronutrients Composition ($g.L^{-1}$):	
H_3BO_3	2.90
$MnCl_2.4H_2O$	1.80
$ZnSO_4.7H_2O$	0.20
$Na_2MoO_4.2H_2O$	0.40
$CuSO_4.5H_2O$	0.08
$Co(NO_3)_2.6H_2O$	0.05

Table 2.3 *Independent variables and experimental design levels for response surfaces of* degradation of toluene and naphthalene by the organisms isolated from soil.

Independent variable	Symbols		Levels		
	Coded	Uncoded	-1	0	+1
Agitation	X1	A	50	150	250
Agitation	X1	A	50	150	250
Temperature	X2	T	18	22	26
Medium pH	X3	pH	5	7	9

3.0 RESULTS AND DISCUSSION

3.0. a) Preliminary Studies

Before starting the studies with Response Surface Methodology (RSM), preliminary studies were conducted to identify the central points for the independent variables, which influence the dependent variable. Tests were also conducted to determine if the results from the preliminary studies on independent variables fit into linear or quadratic equations. The dependent variable studied, the biomass concentration, increased with increase in the independent variables and then decreased after reaching a maximum point, proving the trend to be quadratic. The independent variable value at which the dependent variable was maximum, was taken as the central point (0). Central points for the independent variables studied, namely fermentation time, pH and temperature, were 72 h, 6 and 18°C, respectively. The -1 and +1 levels were fixed equidistant from the central point. The levels and the codes used for the dependent and independent variables are presented in Table 2.1.

3.1 Effect of peat components on the growth and biomass composition of *Phaffia rhodozyma*

3.1.1. Optimization of biomass production of *P. rhodozyma* grown in different peat hydrolysates

The results obtained under different operational conditions with peat hydrolysate (PH), debituminised peat hydrolysate (DPH), peat hydrolysate with humic substances removed (PHR) and debituminised peat hydrolysate with humic substances removed (DPHR) are summarised in

Tables 3.1, 3.3, 3.5 and 3.7, respectively. The results were used to develop models in which the dependent variable was obtained as the sum of the contributions of the independent variables through first order, second order and interaction terms according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii} + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variable, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and crossproduct regression coefficients of the model. X_i and X_j represent the independent variables in coded values.

Tables 3.2, 3.4, 3.6 and 3.8 present the analysis of variance for the models obtained. The tables explain the response of the dependent variable, the biomass concentration, grown in PH, DPH, PHR and DPHR, respectively. The determination coefficients or R^2 value for the experiments were 0.97, 0.92, 0.96 and 0.97, respectively, which indicate that the second order model corresponded well to the experimental results. Only 3%, 8%, 4% and 3% of the total variation was not explained by the model according to the analysis of variance, respectively. The F values for the lack of fit test were insignificant and further confirmed that the models were appropriate.

The best explanatory equation for biomass production as predicted by the model is presented in Table 3.9. The polynomial model shows that the cross-product interaction contributes to the response. Canonical analysis of the response surface revealed maximum regions for the models. The stationary points presenting maximum biomass production for PH, DPH, PHR and DPHR are furnished in Table 3.10.

Table 3.1 Face-centered cube design matrix and response of dependent variables biomass concentration ($g.L^{-1}$) of *P. rhodozyma* cultivated in peat hydrolysate (PH) for the independent variables fermentation time, pH and temperature.

DP	X1	X2	X3	Biomass concentration
1	-1	-1	-1	1.0593
2	-1	-1	+1	1.1167
3	-1	+1	-1	2.5387
4	-1	+1	+1	2.5707
5	-1	0	0	2.8553
6	+1	-1	-1	3.8280
7	+1	-1	+1	4.6200
8	+1	+1	-1	5.4913
9	+1	+1	+1	5.2820
10	+1	0	0	6.6887
11	0	-1	0	6.4813
12	0	+1	0	7.1467
13	0	0	-1	8.8913
14	0	0	+1	8.0236
15	0	0	0	9.9640
16	0	0	0	9.7360
17	0	0	0	9.5360

DP - Design point

Table 3.2 Analysis of variance for response of dependent variable biomass concentration of *P. rhodozyma* cultivated in peat hydrolysate (PH).

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	28.3804	-	18.029
Quadratic	3	111.4314	-	70.787
Cross product	3	0.2084	-	0.132[a]
Total	9	140.0202	-	29.649
Residual				
Lack of fit	5	3.5813	0.7162	15.618[a]
Pure error	2	0.0917	0.0458	-
Total error	7	3.6730	0.5247	-
$R^2 = 0.97$				
Factor				
FT	4	83.8776	20.9694	18.411
T	4	16.0187	4.0047	3.516
pH	4	1.6669	0.4167	0.366[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.3 Face-centered cube design matrix and response of dependent variables biomass concentration ($g.L^{-1}$) of *P. rhodozyma* cultivated in debituminised peat hydrolysate (DPH) for the independent variables fermentation time, pH and temperature.

DP	X1	X2	X3	Biomass concentration
1	-1	-1	-1	0.2753
2	-1	-1	+1	0.6740
3	-1	+1	-1	3.3093
4	-1	+1	+1	2.8840
5	-1	0	0	1.5547
6	+1	-1	-1	9.1340
7	+1	-1	+1	8.1367
8	+1	+1	-1	7.1327
9	+1	+1	+1	4.2580
10	+1	0	0	7.3740
11	0	-1	0	8.0640
12	0	+1	0	2.6133
13	0	0	-1	8.9487
14	0	0	+1	8.0987
15	0	0	0	7.8880
16	0	0	0	6.5393
17	0	0	0	6.6180

DP – Design point

Table 3.4 Analysis of variance for response of dependent variable biomass concentration of *P. rhodozyma* cultivated in debituminised peat hydrolysate (DPH).

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	80.6969	-	14.968
Quadratic	3	35.7553	-	6.632
Cross product	3	18.2285	-	3.381[a]
Total	9	134.6807	-	8.327
Residual				
Lack of fit	5	11.4339	2.2868	3.991[a]
Pure error	2	1.1460	0.5730	-
Total error	7	12.5799	1.7971	-
$R^2 = 0.95$				
Factor				
FT	4	108.5340	27.1335	15.098
T	4	26.9941	6.7485	3.755
pH	4	11.6971	2.9243	1.627[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.5 Face-centered cube design matrix and response of dependent variables biomass concentration ($g.L^{-1}$) of *P. rhodozyma* cultivated in peat hydrolysate with humic substances removed (PHR) for the independent variables fermentation time, pH and temperature.

DP	X1	X2	X3	Biomass concentration
1	-1	-1	-1	0.6620
2	-1	-1	+1	2.0193
3	-1	+1	-1	3.1353
4	-1	+1	+1	3.4233
5	-1	0	0	5.2567
6	+1	-1	-1	7.3600
7	+1	-1	+1	7.4593
8	+1	+1	-1	6.2703
9	+1	+1	+1	6.2887
10	+1	0	0	7.1407
11	0	-1	0	6.7193
12	0	+1	0	6.9557
13	0	0	-1	7.3067
14	0	0	+1	7.3107
15	0	0	0	8.0207
16	0	0	0	8.7267
17	0	0	0	7.9953

DP - Design point

Table 3.6 Analysis of variance for response of dependent variable biomass concentration of *P. rhodozyma* cultivated in peat hydrolysate with humic substances removed (PHR).

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	40.7454	-	26.830
Quadratic	3	36.0759	-	23.755
Cross product	3	5.1658	-	3.402[a]
Total	9	81.9872	-	17.995
Residual				
Lack of fit	5	3.1989	0.6398	3.712[a]
Pure error	2	0.3447	0.1723	-
Total error	7	3.5435	0.5062	-
$R^2 = 0.96$				
Factor				
FT	4	53.4079	13.3520	26.376
T	4	8.5975	2.1494	4.246
pH	4	1.9081	0.4770	0.942[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.7 Face-centered cube design matrix and response of dependent variables biomass concentration ($g.L^{-1}$) of *P. rhodozyma* cultivated in debituminised peat hydrolysate with humic *substances removed* (DPHR) for the independent variables fermentation time, pH and temperature.

DP	X1	X2	X3	Biomass concentration
1	-1	-1	-1	1.1760
2	-1	-1	+1	0.7140
3	-1	+1	-1	5.2730
4	-1	+1	+1	5.2620
5	-1	0	0	6.8893
6	+1	-1	-1	6.7500
7	+1	-1	+1	6.5207
8	+1	+1	-1	6.9280
9	+1	+1	+1	5.6173
10	+1	0	0	8.2347
11	0	-1	0	6.4987
12	0	+1	0	8.2493
13	0	0	-1	7.3973
14	0	0	+1	8.3047
15	0	0	0	9.0640
16	0	0	0	8.7567
17	0	0	0	9.1953

DP - Design point

Table 3.8 Analysis of variance for response of dependent variable biomass concentration of *P. rhodozyma* cultivated in debituminised peat hydrolysate with humic substances removed (DPHR)

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	31.1897	-	25.847
Quadratic	3	49.9212	-	41.370
Cross product	3	11.1675	-	9.225[a]
Total	9	92.2784	-	25.490
Residual				
Lack of fit	5	2.7143	0.5429	10.713[a]
Pure error	2	0.1013	0.0507	-
Total error	7	2.8157	0.4022	-
$R^2 = 0.97$				
Factor				
FT	4	38.4039	9.6010	23.869
T	4	27.4936	6.8734	17.088
pH	4	3.8750	0.9688	2.408[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.9 Prediction equation obtained for the biomass concentration ($g.L^{-1}$) of *P. rhodozyma* grown in PH, DPH, PHR and DPHR

Substrate	Best explanatory equations	R^2	P Level
Peat hydrolysate (PH)	$Y = 9.12 + 1.57 X_1 + 0.59 X_2 - 0.01 X_3 - 3.88 X_1^2 - 0.07 X_1X_2 - 1.84 X_2^2 + 0.06 X_1 X_3 - 0.13 X_2 X_3 - 0.20 X_3^3$	0.95	0.01
Debituminised peat hydrolysate (DPH)	$Y = 6.97 + 2.73 X_1 - 0.61 X_2 - 0.47 X_3 - 2.48 X_1^2 - 1.39 X_1X_2 - 1.6 X_2^2 - 0.48 X_1 X_3 - 0.34 X_2 X_3 + 1.58 X_3^3$	0.92	0.01
Peat hydrolysate with humic substances removed (PHR)	$Y = 8.08 + 2.00 X_1 + 0.19 X_2 + 0.18 X_3 - 1.76 X_1^2 - 0.77 X_1X_2 - 1.12 X_2^2 - 0.19 X_1 X_3 - 0.14 X_2 X_3 - 0.65 X_3^3$	0.96	0.01
Debituminised peat hydrolysate with humic substances removed (DPHR)	$Y = 9.00 + 1.47 X_1 + 0.97 X_2 - 0.11 X_3 - 1.44 X_1^2 - 1.17 X_1X_2 - 1.63 X_2^2 - 0.13 X_1 X_3 - 0.07 X_2 X_3 - 1.15 X_3^3$	0.97	0.01

Table 3.10 Canonical analysis and predicted parameters for maximum biomass production ($g.L^{-1}$) of *P. rhodozyma* grown in PH, DPH, PHR and DPHR

Parameters	PH	DPH	PHR	DPHR
FT	91.15	104.28	100.40	94.11
T	19.81	14.99	17.26	18.80
pH	6.39	6.40	6.12	5.84
Predicted response	9.6087	7.9994	8.6700	9.4126
Stationary point	Maximum	Saddle	Maximum	Maximum

Fig. 3.1 (a) Three dimensional graph - Optimisation of biomass concentration of *P. rhodozyma* grown in peat hydrolysate

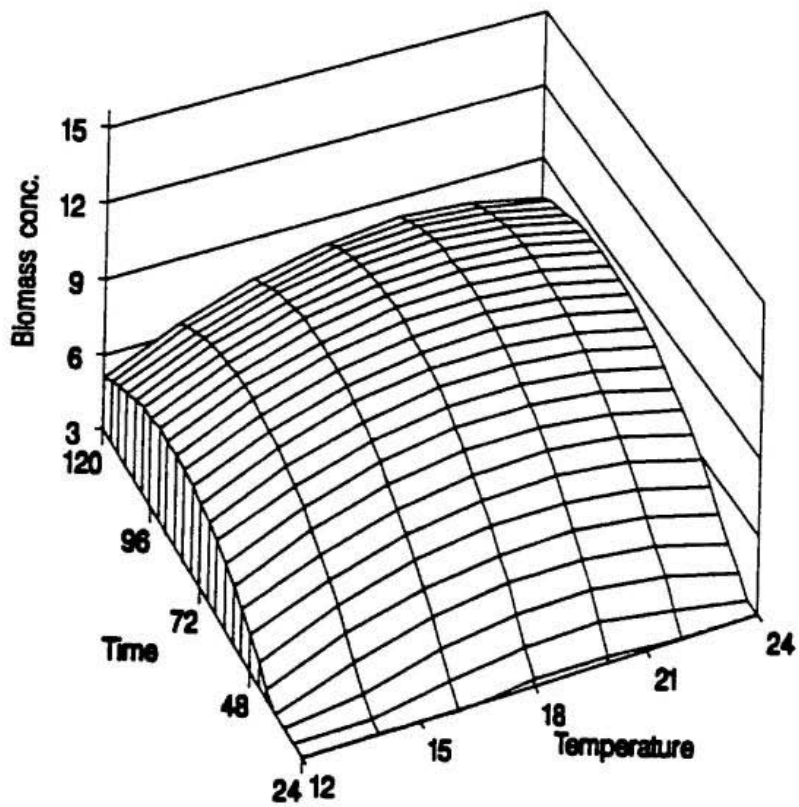


Fig. 3.1 (b) Contour graph - Optimisation of biomass concentration of *P. rhodozyma* grown in peat hydrolysate

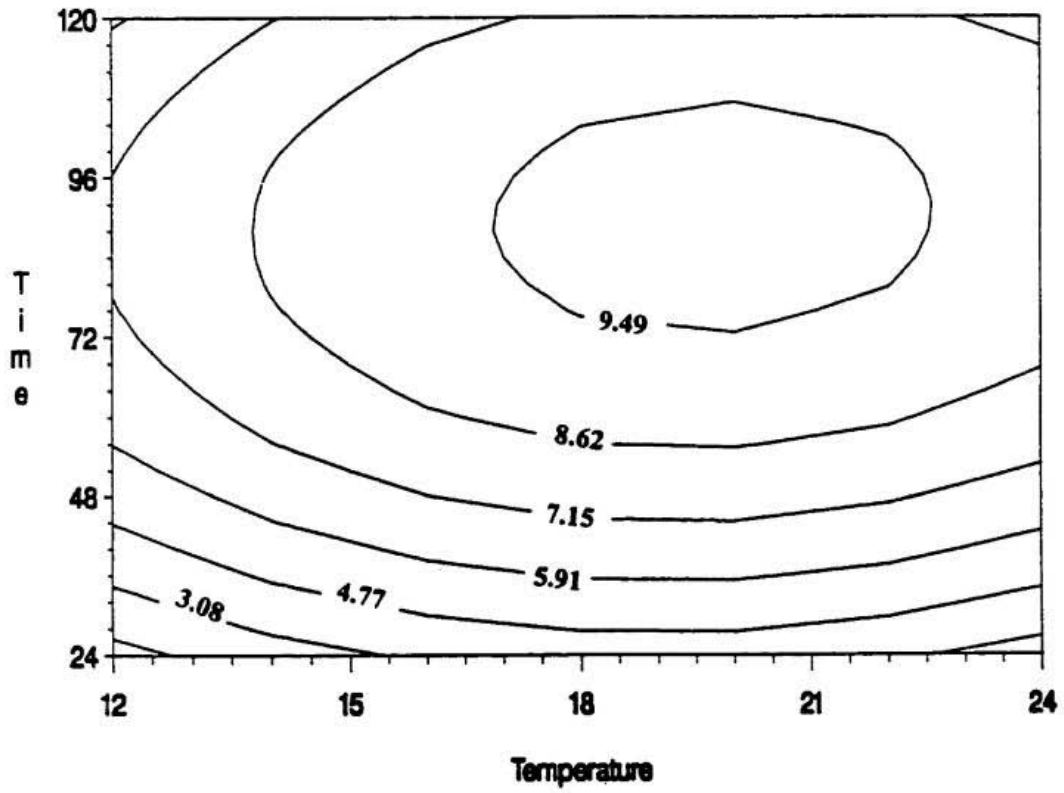


Fig. 3.2 (a) Three dimensional graph - Optimisation of biomass concentration of *P. rhodozyma* grown in debituminised peat hydrolysate

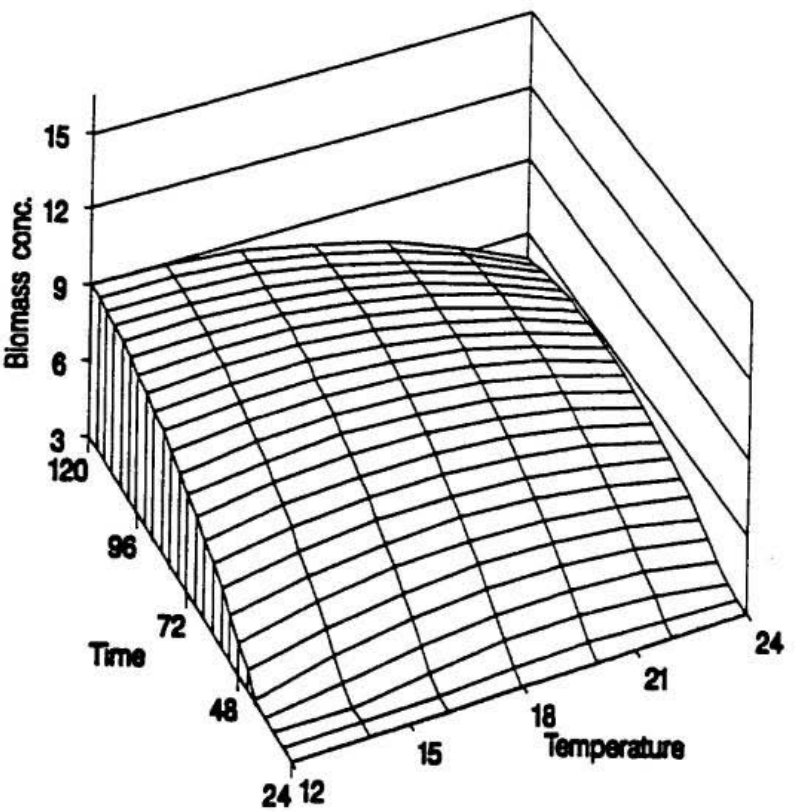


Fig. 3.2 (b) Contour graph - Optimisation of biomass concentration of *P. rhodozyma* grown in debituminised peat hydrolysate

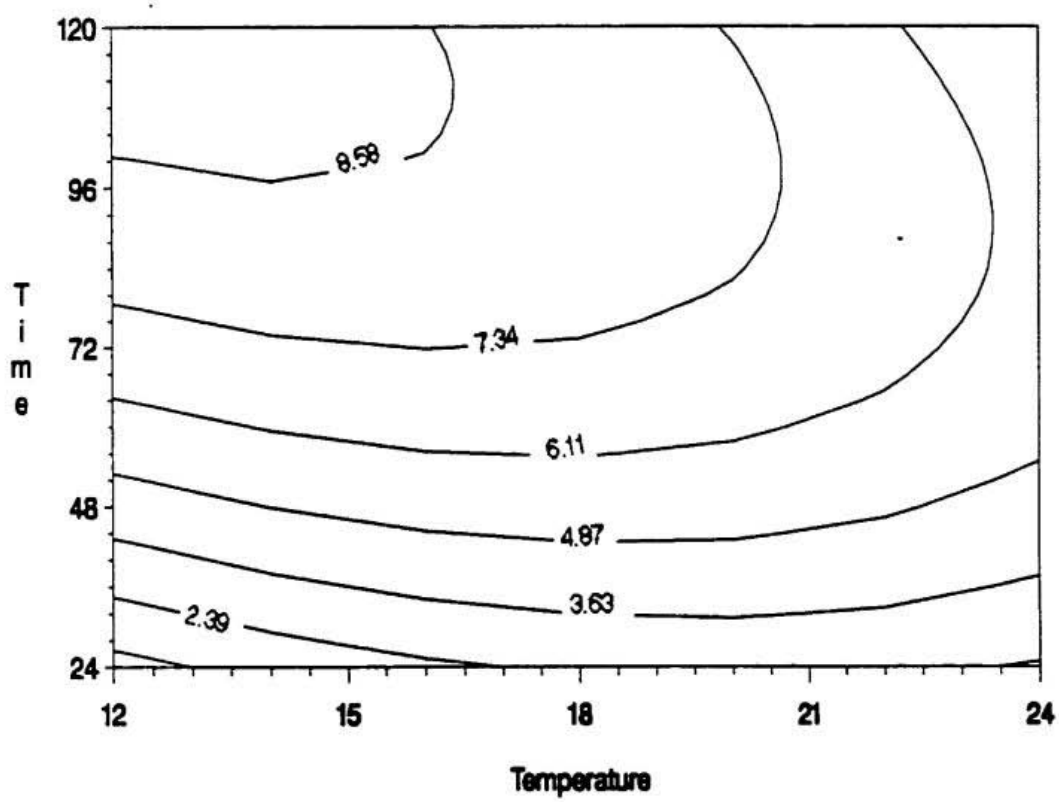


Fig. 3.3 (a) Three dimensional graph - Optimisation of biomass concentration of *P. rhodozyma* grown in peat hydrolysate with humic substances removed

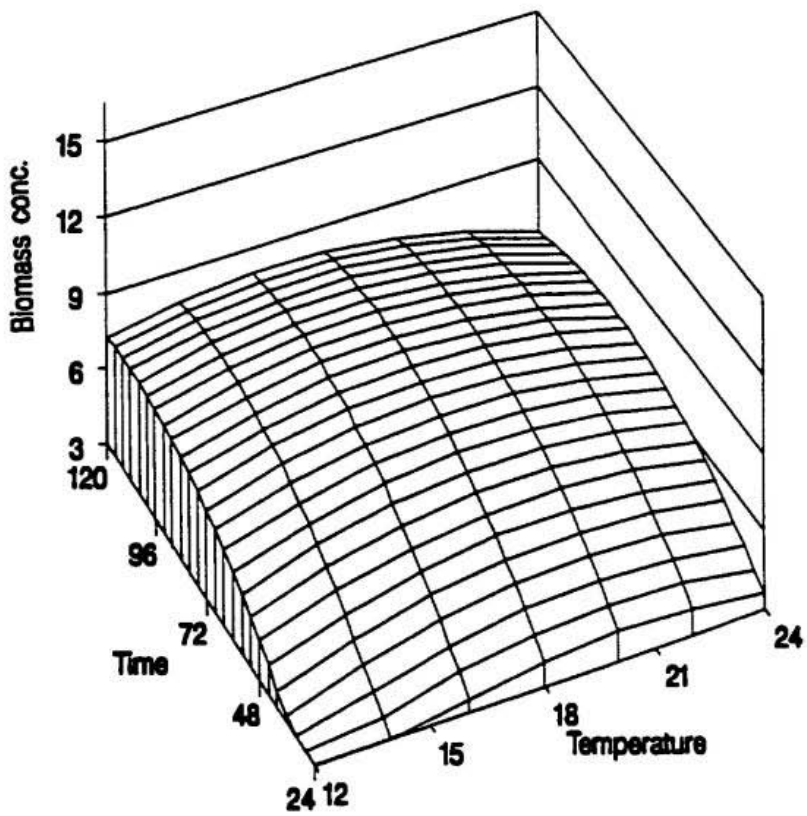


Fig. 3.3 (b) Contour graph - Optimisation of biomass concentration of *P. rhodozyma* grown in peat hydrolysate with humic substances removed

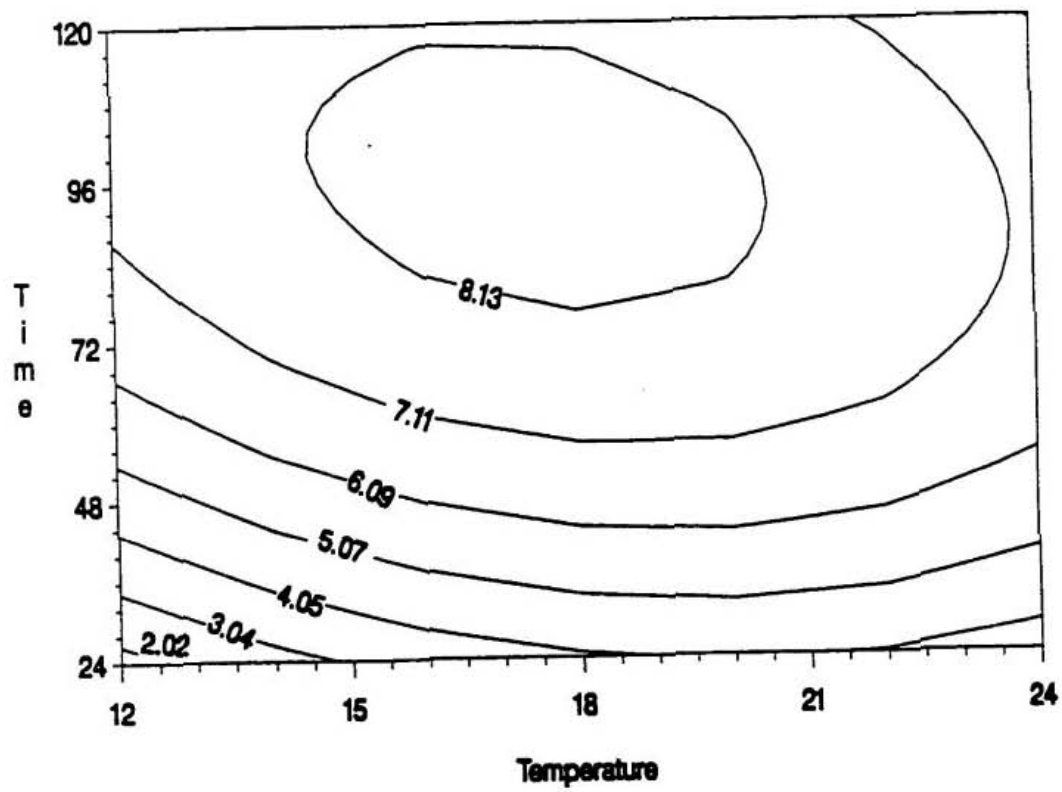


Fig. 3.4 (a) Three dimensional graph - Optimisation of biomass concentration of *P. rhodozyma* grown in debituminised peat hydrolysate with humic substances removed

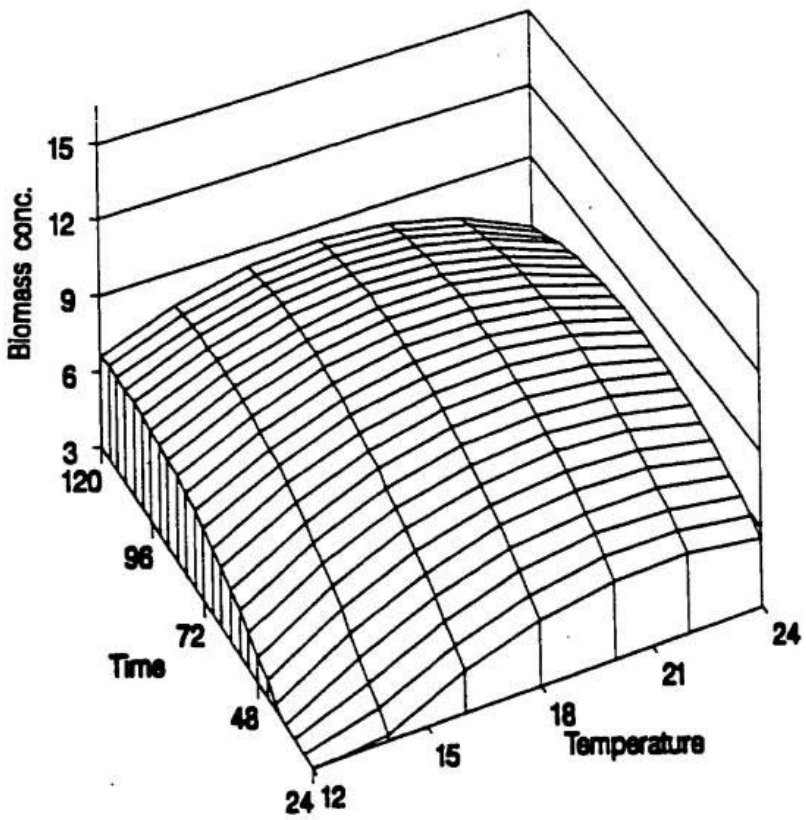


Fig. 3.4 (b) *Contour graph* - Optimisation of biomass concentration of *P. rhodozyma* grown in debituminised peat hydrolysate with humic substances removed

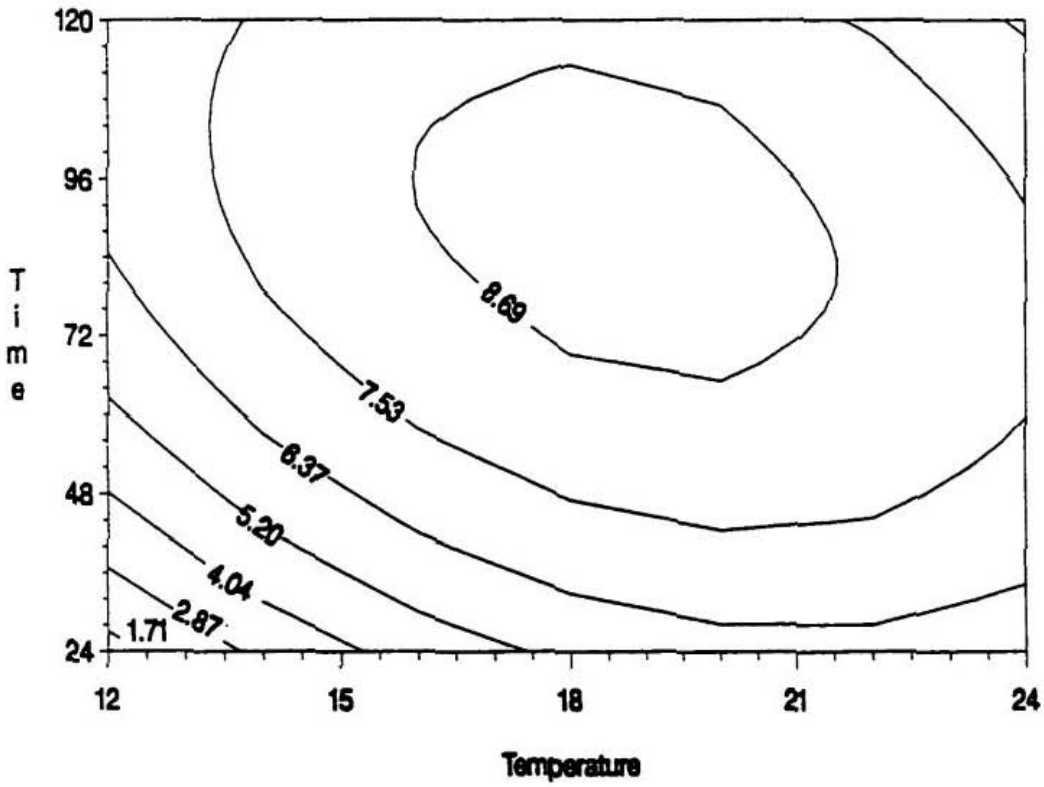


Table 3.11 Biomass concentration ($g.L^{-1}$) and yield ($g.g^{-1}$) of *P. rhodozyma* grown in PH, DPH, PHR and DPHR grown in conditions optimised by RSM.

Parameters	PH	DPH	PHR	DPHR
Biomass concentration ($g.L^{-1}$)	9.52 ± 0.08^{b}	7.97 ± 0.09^{a}	8.39 ± 0.43^{a}	9.28 ± 0.24^{b}
Yield ($g.g^{-1}$)	0.51 ± 0.02^{a}	0.45 ± 0.06^{a}	0.38 ± 0.02^{a}	0.41 ± 0.03^{a}

* Data with different superscripts in the same row are statistically significant (Tukey's honest significant difference test)

Three-dimensional graphs and contour plots showing the influence of each variable on biomass production in PH, DPH, PHR and DPHR are presented in Figs. 3.1 to 3.4. The accuracy of the model was further tested by conducting a set of experiments using the critical values for maximum biomass production. Table 3.11 gives the biomass and yield (amount of biomass produced for the amount of substrate utilized) of *P. rhodozyma* grown under the optimum operational conditions in all the four substrates. The biomass concentration produced in PH and DPHR were significantly higher than that produced in DPH and PHR. Maximum biomass was produced in *P. rhodozyma* grown in PH. The decrease in biomass in DPH, PHR and DPHR may be due to the removal of growth promoting substances which were lost during the extraction of bitumens and humic substances before hydrolysis.

A significant difference in the biomass produced by DPHR over DPH and PHR could be attributed to the simultaneous absence of both bitumens and humic substances, which may synergistically inhibit biomass production. When both inhibitory substances are jointly removed, the resultant effect on the yeast growth is more important than that produced by the removal of growth promoting substances during extraction procedures.

Earlier studies by Martin *et al.* (1993) and Vázquez and Martin (1998b) on the biomass productivity of *P. rhodozyma* on peat hydrolysates in batch and continuous culture experiments have showed maximum production of 5.5 $g.L^{-1}$ and 10.5 $g.L^{-1}$, respectively, whereas the current study resulted in a maximum biomass concentration of 9.52 ± 0.08 $g.L^{-1}$ under batch culture conditions.

Regarding yields, there was a decrease in their values in the experiments with DPH, PHR and DPHR. But Tukey's honest significant difference test showed that there was no significant

difference in the yield values obtained from different peat hydrolysates. A previous study by Martin *et al.* (1993) has reported a maximum yield of 0.57 by *P. rhodozyma* grown in peat hydrolysates. This agreed well with the results from the present study which has recorded a maximum of 0.51. Vázquez and Martin (1998b) have reported a maximum biomass yield of 0.52 at optimised parameters in a continuous culture condition.

Martin *et al.* (1990) reported the biomass production and yield of *Scytalidium acidophilum* grown in PH, DPH, PHR and DPHR. There was a significant decrease in the biomass produced in DPH, PHR and DPHR, compared to that produced in PH. In the present study there was a significant decrease in biomass observed when *P. rhodozyma* was grown in DPH and in PHR, against that produced in PH. However the biomass produced in DPHR did not decrease significantly compared to PH . A significant fall in the biomass yield was observed by Martin *et al.* (1990) for *S. acidophilum* grown in DPH, PHR and DPHR, compared to that grown in PH. In the present study there was no significant difference in the biomass yield of *P. rhodozyma* grown in different hydrolysates.

3.1.2 Biomass composition of *P. rhodozyma* cells grown in PH, DPH, PHR and DPHR

The summary of the results of the biochemical analysis done, namely the total carbohydrate, protein, lipid, ash and astaxanthin contents, is presented in Table 3.12. The carbohydrate content was calculated as (100 - (protein + lipid + ash)). There was significantly higher carbohydrate present in DPH and DPHR compared to other hydrolysates. Protein content was not significantly different among the biomass produced from the four different substrates. Lipid content was significantly lesser in DPH and DPHR compared to PH and PHR. The reason

could be attributed to the removal of bitumens, which have been reported to be present in peat hydrolysates in the form of lipids (Martin and Manu-Tawiah, 1989). The absence of these fatty acids in DPH and DPHR might have contributed to the decrease in lipid content of *P. rhodozyma* cells.

There was no significant difference in the ash content of the biomass produced in the different peat hydrolysates, except for the ash content of the biomass produced in DPHR, which was significantly lesser than the ash in the other three peat hydrolysates.

Vázquez and Martin (1998a) have reported 32, 49, 13 and 5 % of total carbohydrate, protein, lipid and ash contents, respectively, in the biomass produced in peat hydrolysates grown in peat hydrolysates under continuous culture conditions.

There was a significant difference in the astaxanthin content produced by *P. rhodozyma* grown in PH and PHR over DPH and DPHR. This difference could be due to the removal of bitumens before acid hydrolysis, which are reported to have 2.6 % of carotenes and xanthophylls and to be precursors for astaxanthin production (Fuchsman, 1980; Acheampong and Martin, 1995). PHR produced maximum astaxanthin, which was significantly higher than that produced by PH. This might be due to the absence of humic substances, acting as inhibitors, in PHR.

Although the astaxanthin concentration produced in this work was below that of 1567 $\mu g.g^{-1}$ reported by Martin *et al.* (1993) in PH under batch culture condition, results from the present study are comparable with the concentration of 544 $\mu g.g^{-1}$ reported by Vázquez and Martin (1998b) under continuous culture conditions.

Table 3.12 Biomass composition of *P. rhodozyma* grown in PH, DPH, PHR and DPHR in conditions optimized by RSM.*

Parameters	PH	DPH	PHR	DPHR
Carbohydrate	507.1 ± 10.8^{a}	578.2 ± 11.5^{b}	514.6 ± 12.4^{a}	585.5 ± 5.5^{b}
Protein	315.8 ± 8.0^{a}	308.1 ± 8.8^{a}	308.2 ± 12.0^{a}	305.2 ± 3.1^{a}
Lipid	96.0 ± 3.4^{b}	29.3 ± 2.1^{a}	95.3 ± 4.8^{b}	34.7 ± 7.3^{a}
Ash	81.0 ± 0.4^{a}	84.4 ± 3.0^{a}	81.9 ± 0.4^{a}	74.7 ± 0.6^{b}
Astaxanthin	423 ± 21^{b}	345 ± 17^{a}	474 ± 14^{c}	347 ± 12^{a}

* Data with different superscripts in the same row are statistically significant (Tukey's honest significant difference test). All components are given in $g.L^{-1}$, with the exception of astaxanthin ($\mu g.g^{-1}$).

3.2 Optimization of the growth and astaxanthin production of *P. rhodozyma* cultivated in YM broth

The results of the various experiments conducted with face-centered cube design for optimizing biomass and astaxanthin production by *P. rhodozyma* are presented in Tables 3.13 and 3.15. The values obtained were used to develop models, in which each dependent variable was obtained as the sum of the contributions of independent variables through first order, second order and interactive terms according to the equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii} + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variable, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and crossproduct regression coefficients of the model, and X_i and X_j represent the independent variables in coded values.

Tables 3.14 and 3.16 present the analysis of variance for the models obtained that explains the biomass and astaxanthin production, respectively. The determination coefficients or R^2 value for both the experiments were 0.96 and 0.99 respectively, which indicates that the second order model corresponded well to the experimental results, and only 4% and 1% of the total variations were not explained by the model. The F values for the lack of fit test were insignificant which confirmed the appropriateness of the model.

The best explanatory equation for biomass and astaxanthin production as predicted by the model is presented in Table 3.17. The polynomial model shows that the cross-product interaction contributes to the response. Canonical analysis was also conducted which revealed maximum regions for the models.

Table 3.18 shows the stationary point predicted for maximum biomass and astaxanthin production by the analysis. Contour plots and three-dimensional graphs showing the influence of each variable on biomass and astaxanthin production are presented in Figs. 3.5 and 3.6. Further, a confirmatory test was conducted using the critical values for maximum biomass production. The confirmatory tests produced a maximum of 7.22 ± 0.14 g.L $^{-1}$ and 487 ± 9 $\mu g.g^{-1}$ for biomass and astaxanthin production, respectively which substantiated the predictability of the model.

The fermentation conditions predicted by the model for both biomass and astaxanthin production were near equal, which further confirms the earlier report of astaxanthin production as growth related by Acheampong and Martin (1995). There was a gradual increase in the production of astaxanthin with increase in growth, followed by a decrease after the optimum conditions. This pattern is almost the same as that of biomass concentration.

The present study has affirmed the relationship between astaxanthin production and biomass production and has also suggested the best fermentation conditions for maximum astaxanthin yield. Besides, this study will help in deciding the time of harvest of the yeast cells to obtain maximum yield of astaxanthin, which will be of interest to the aquaculture industries.

Table 3.13 Face-centered cube design matrix and response of dependent variables biomass concentration ($g.L^{-1}$) of *P. rhodozyma* for the independent variables fermentation time, pH and temperature.

DP	X1	X2	X3	Biomass conc. ($g.L^{-1}$)
1	-1	-1	-1	1.1540
2	-1	-1	+1	1.3753
3	-1	+1	-1	3.4380
4	-1	+1	+1	3.3887
5	-1	0	0	3.5513
6	+1	-1	-1	4.8020
7	+1	-1	+1	4.1173
8	+1	+1	-1	5.4873
9	+1	+1	+1	5.8893
10	+1	0	0	5.2213
11	0	-1	0	6.5440
12	0	+1	0	6.3760
13	0	0	-1	6.7053
14	0	0	+1	6.5367
15	0	0	0	7.1233
16	0	0	0	6.9693
17	0	0	0	7.5547

DP - Design point

Table 3.14 Analysis of variance for response of dependent variable biomass concentration

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	20.2472	-	19.627
Quadratic	3	38.1444	-	36.976
Cross product	3	0.5323	-	0.516[a]
Total	9	58.9240	-	19.040
Residual				
Lack of fit	5	2.2229	0.4446	4.828[a]
Pure error	2	0.1842	0.0921	-
Total error	7	2.4070	0.3439	-
$R^2 = 0.96$				
Factor				
FT	4	33.9768	8.4942	24.702
T	4	5.4916	1.3729	3.993[a]
pH	4	0.4091	0.1023	0.297

[a] - significant at 1% level, DF - degrees of freedom

Table 3.15 Face-centered cube design matrix and response of dependent variables astaxanthin production ($\mu g.g^{-1}$) of *P. rhodozyma* for the independent variables fermentation time, pH and temperature

DP	X1	X2	X3	Astaxanthin content($\mu g.g^{-1}$)
1	-1	-1	-1	38
2	-1	-1	+1	42
3	-1	+1	-1	69
4	-1	+1	+1	123
5	-1	0	0	160
6	+1	-1	-1	218
7	+1	-1	+1	305
8	+1	+1	-1	319
9	+1	+1	+1	345
10	+1	0	0	397
11	0	-1	0	407
12	0	+1	0	414
13	0	0	-1	428
14	0	0	+1	424
15	0	0	0	489
16	0	0	0	491
17	0	0	0	474

DP - Design point

Table 3.16 Analysis of variance for response of dependent variable astaxanthin production

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	142259.0000	-	14.968
Quadratic	3	269523.0000	-	6.632
Cross product	3	498.3750	-	3.381[a]
Total	9	412281.0000	-	8.327
Residual				
Lack of fit	5	4162.6935	832.5387	9.643[a]
Pure error	2	172.6667	86.3333	-
Total error	7	4335.3602	619.3371	-
$R^2 = 0.99$				
Factor				
FT	4	231643.0000	57911.0000	93.504
T	4	16426.0000	4106.5419	6.631
pH	4	8414.0959	2103.5240	3.396[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.17 Prediction equation obtained for biomass concentration ($g.L^{-1}$) and astaxanthin production ($\mu g.g^{-1}$) grown in YM broth

Response	Best explanatory equation	R^2	P Level
Biomass conc. ($g.L^{-1}$)	$Y = 7.06 + 1.26 X_1 + 0.65 X_2 - 0.03 X_3 - 2.56 X_1^2 - 0.23 X_1X_2 - 0.49 X_2^2 - 0.06 X_1 X_3 + 0.1 X_2 X_3 - 0.33 X_3^3$	0.96	0.01
Astaxanthin content ($\mu g.g^{-1}$)	$Y = 476.40 + 115.20 X_1 + 26.00 X_2 - 16.70 X_3 - 191.69 X_1^2 + 3.63 X_1X_2 - 59.69 X_2^2 + 6.87 X_1 X_3 - 1.38 X_2 X_3 + 44.19 X_3^3$	0.99	0.01

Table 3.18 Canonical analysis and predicted combined parameters condition for maximum biomass concentration ($g.L^{-1}$) and astaxanthin content ($\mu g.g^{-1}$), and the confirmatory test results

Parameters	Biomass conc. ($g.L^{-1}$)	Astaxanthin Content ($\mu g.g^{-1}$)
FT	82.44	86.71
Temp.	21.74	19.35
pH	6.07	6.42
Predicted max. response	7.4065	499
Stationary point	Maximum	Maximum
Confirmatory test results	7.22 ± 0.14	487 ± 9

Fig. 3.5 (a) Three dimensional graph - Optimisation of biomass concentration of *P. rhodozyma* grown in YM broth

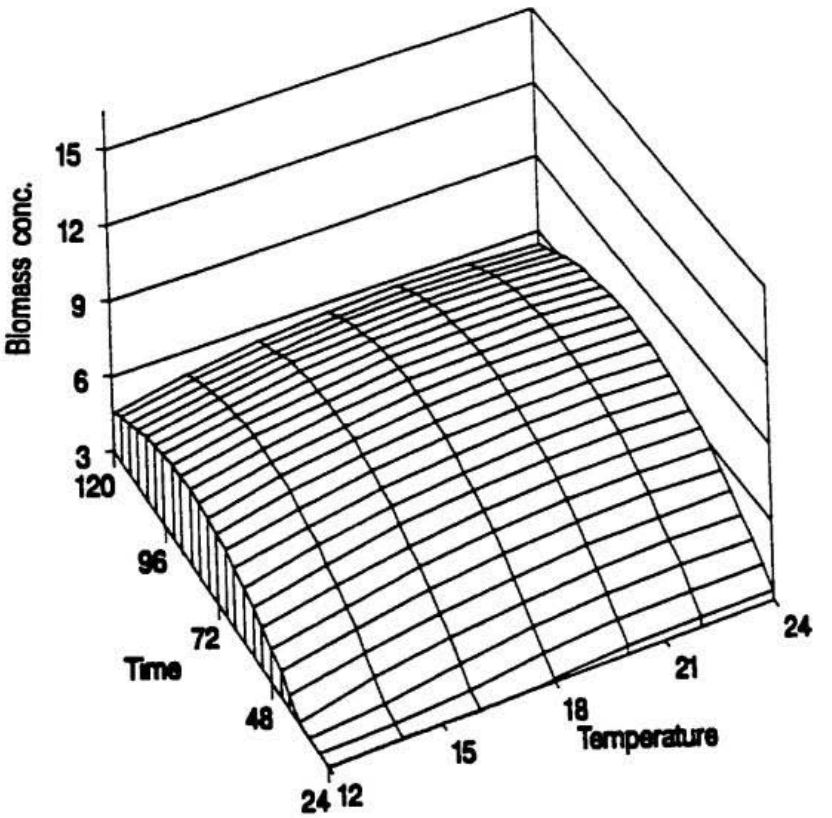


Fig. 3.5 (b) Contour graph - Optimisation of biomass concentration of *P. rhodozyma* grown in YM broth

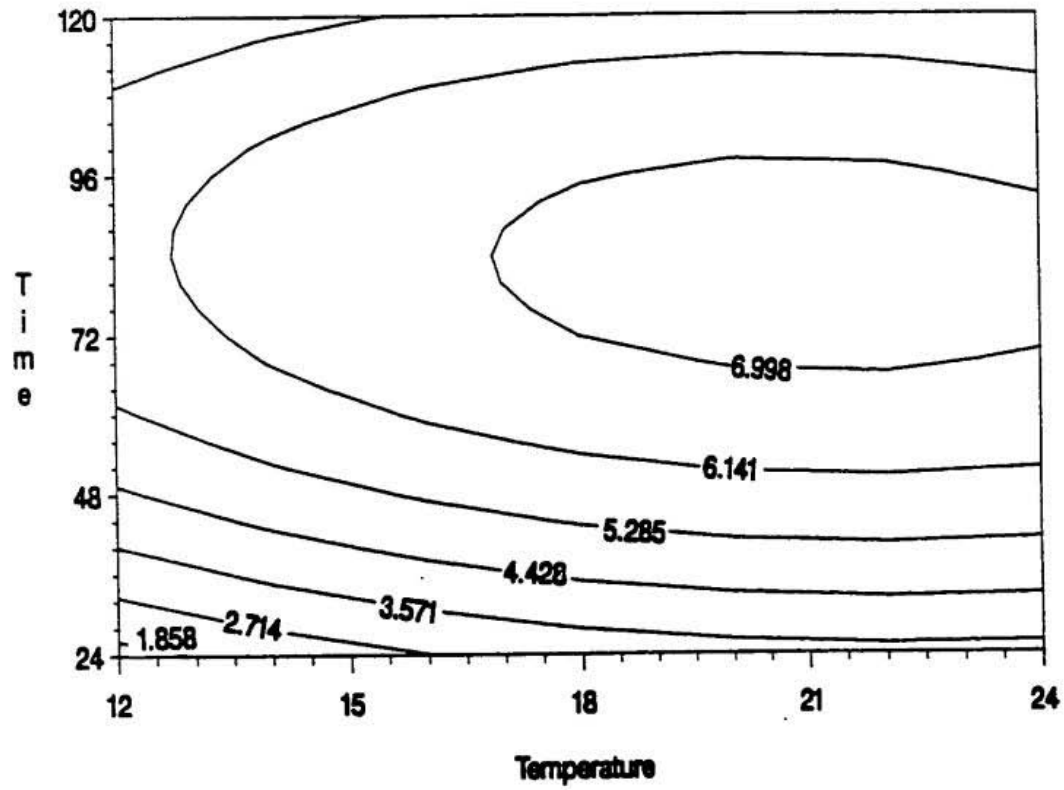


Fig. 3.6 (a) Three dimensional graph - Optimisation of astaxanthin production of *P. rhodozyma* grown in YM broth

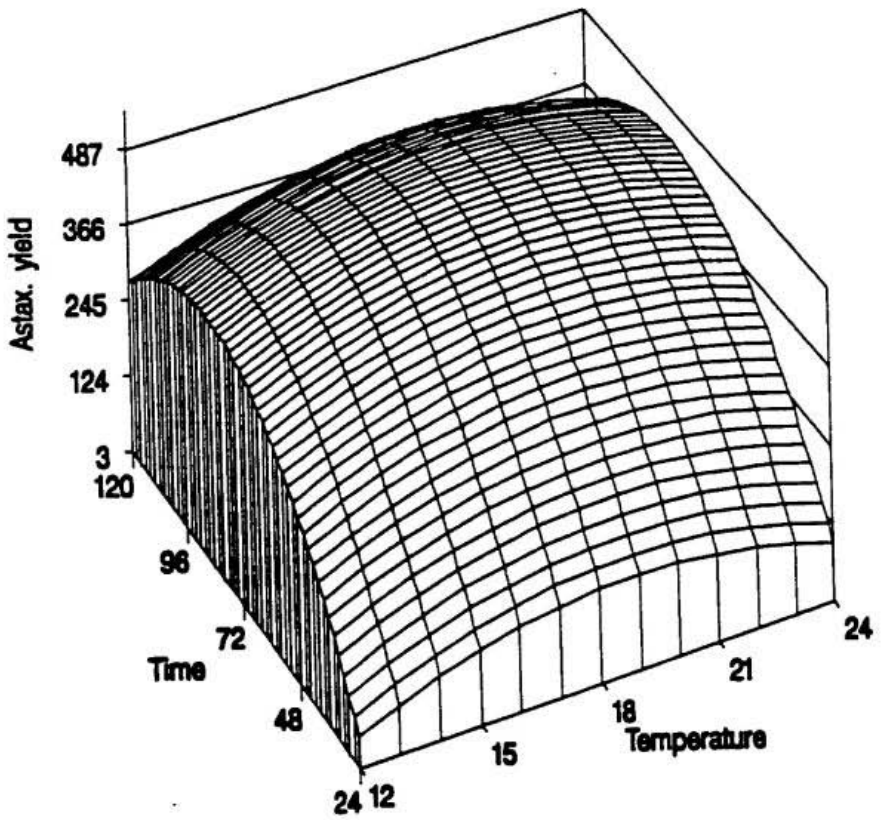
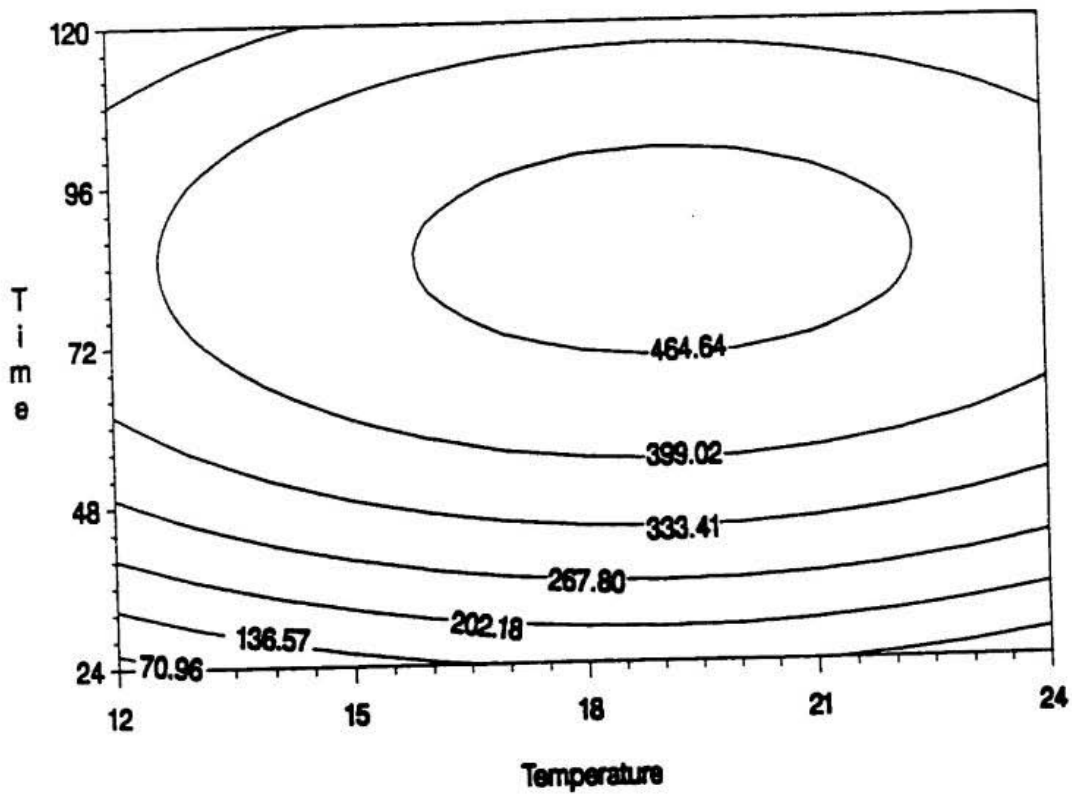


Fig. 3.6 (b) Contour graph - Optimisation of astaxanthin production of *P. rhodozyma* grown in YM broth



3.3 Effect of humic acids on the biomass concentration and yield of *Phaffia rhodozyma* grown in YM broth.

Phaffia rhodozyma was grown in YM broth under optimised conditions adopted from RSM (Table 3.18) with humic acids supplied by Aldrich Chemicals, and with humic acids isolated from peat. The humic acids were incorporated into YM broth at concentrations 0.02, 0.04, 0.06, 0.08 and 0.10 %. The addition of humic acids to the cultures resulted in decreases in biomass production as well as yield. Tukey's HSD test disclosed that there was a statistically significant difference ($P < 0.05$) between the treatments with humic acids and the control experiment without humic acids. The biomass concentrations of *P. rhodozyma* grown in YM broth with 0.02, 0.04 and 0.06 % of humic acids were significantly lesser than that produced in the control experiment. Moreover, the biomass concentrations of *P. rhodozyma* grown in 0.08 and 0.10 % concentration of humic acids were significantly lesser than those produced in the control experiment and with smaller humic acid concentrations. The biomass yield showed a decreasing trend. Significant differences for the yield were observed between the control and treatments, but not within the treatments, according to the Tukey's HSD test.

Similar trends were observed when *P. rhodozyma* was grown in YM broth with humic acids supplied by Aldrich, and with humic acids isolated from peat humic substances. The decrease in biomass production may be due to the inhibition of the yeast growth by humic acids. Martin *et al.* (1990) reported increase in biomass productivity of *Scytalidium acidophilum* grown in synthetic media with different concentrations of humic acids starting from 0.12 to 0.30 %, which dropped after that. In the present study *P. rhodozyma* was observed to be sensitive to increasing concentrations of humic acids at concentrations starting from 0.02 %.

Visser (1985) has observed increase in growth and physiological activity of microroganisms isolated from organic soil when grown in medium with humic substances of concentration up to 3 %, beyond which there was a decrease. Whereas, microorganisms from sandy soils exhibited inhibition even at a lesser concentration.

Table 3.19 Effect of humic acids (Aldrich chemical) at different concentrations on the biomass production and yield of *P. rhodozyma*.

Conc. of humic acid (%)	Biomass production ($g.L^{-1}$)	Yield ($g.g^{-1}$)
Control	7.22 ± 0.14^{c}	0.39 ± 0.040^{b}
0.02	6.82 ± 0.09^{b}	0.12 ± 0.011^{a}
0.04	6.68 ± 0.02^{b}	0.10 ± 0.005^{a}
0.06	6.66 ± 0.10^{b}	0.09 ± 0.018^{a}
0.08	5.85 ± 0.38^{a}	0.07 ± 0.004^{a}
0.10	5.69 ± 0.18^{a}	0.07 ± 0.006^{a}

* Data on the same column with different alphabetical superscripts are significantly different (Tukey's honest significant difference test).

Table 3.20 Effect of humic acids isolated from peat at different concentrations on the biomass production and yield of *P. rhodozyma.*

Conc. of humic acid (%)	Biomass production ($g.L^{-1}$)	Yield ($g.g^{-1}$)
Control	7.22 ± 0.14^{b}	0.39 ± 0.030^{c}
0.02	6.78 ± 1.08^{a}	$0.17 \pm 0.011^{a,b}$
0.04	6.55 ± 0.39^{a}	0.11 ± 0.048^{a}
0.06	6.44 ± 0.72^{a}	0.09 ± 0.022^{a}
0.08	6.11 ± 0.10^{a}	0.06 ± 0.031^{a}
0.10	5.55 ± 0.12^{a}	0.05 ± 0.009^{a}

* Data in the same column with different alphabetical superscripts are significantly different (Tukey's honest significant difference test).

3.4 Optimization of toluene degradation by *Pseudomonas fluorescens* strains NC1 and NC2.

Response surface methodology was used to optimise the fermentation conditions to obtain maximum biodegradation of toluene by *Pseudomonas fluorescens* strains NC1 and NC2. The biodegradation (in per cent) of toluene was chosen as the dependent variable. The independent variables, *i.e.* the fermentation conditions studied, were temperature, pH and agitation. Time was not taken as an independent variable as in *P. rhodozyma*, since for degradation studies the trend will not be quadratic. At a particular time, degradation is maximum *i.e.* all the substrate is utilized and there will not be a trend from then. The central points for the parameters, 22°C, a pH of 7, and 150 rpm, respectively, were obtained from previous studies conducted at Memorial University (Diegor, E., 1999). The factorial design, a face centered cube, helped in identifying the combined best parameter conditions. The fermentation time was maintained constant, at 48 h.

The per cent biodegradation of toluene obtained under the different combined operational conditions for strains NC1 and NC2 cultured in HDM medium for are summarised in Table 3.21 and 3.23, respectively. The results were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through first order, second order and interaction terms according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii} + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variable, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and crossproduct regression coefficients of the model and X_i and X_j represent the independent

Table 3.21 Face-centered cube design matrix and response of the dependent variable

biodegradation of toluene by *P. fluorescens* strain NC1 for the independent variables temperature, pH and RPM.

DP	X1	X2	X3	% Biodegradation
1	-1	-1	-1	0.00
2	-1	-1	+1	3.76
3	-1	+1	-1	12.90
4	-1	+1	+1	21.45
5	-1	0	0	26.87
6	+1	-1	-1	31.36
7	+1	-1	+1	35.06
8	+1	+1	-1	42.57
9	+1	+1	+1	51.66
10	+1	0	0	61.47
11	0	-1	0	68.16
12	0	+1	0	77.46
13	0	0	-1	85.72
14	0	0	+1	91.05
15	0	0	0	96.98
16	0	0	0	98.07
17	0	0	0	99.12

DP - Design point

Table 3.22 Analysis of variance for response of dependent variable *per cent* toluene degraded strain NC2.

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	3020.22545	-	68.814
Quadratic	3	15794.00000	-	359.900
Cross product	3	13.94890	-	0.318[a]
Total	9	18829.00000	-	143.000
Residual				
Lack of fit	5	100.118940	20.023788	17.448[a]
Pure error	2	2.290067	1.145033	-
Total error	7	102.409007	14.629858	-
$R^2 = 0.99$				
Factor				
T	4	8575.860514	2143.965129	146.500
pH	4	1449.376291	362.344073	24.767
RPM	4	138.819451	34.704863	2.372[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.23 Face-centered cube design matrix and response of dependent variable biodegradation of toluene by *P. fluorescens* strain NC2 for the independent variables temperature, pH and RPM.

DP	X1	X2	X3	% Biodegradation
1	-1	-1	-1	0.51
2	-1	-1	+1	2.14
3	-1	+1	-1	9.98
4	-1	+1	+1	14.87
5	-1	0	0	17.11
6	+1	-1	-1	18.94
7	+1	-1	+1	27.16
8	+1	+1	-1	34.14
9	+1	+1	+1	38.68
10	+1	0	0	46.40
11	0	-1	0	61.33
12	0	+1	0	69.36
13	0	0	-1	78.62
14	0	0	+1	84.30
15	0	0	0	97.87
16	0	0	0	94.04
17	0	0	0	100.00

DP - Design point

Table 3.24 Analysis of variance for response of dependent variable per cent toluene degraded by *P. fluorescens* strain NC2.

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	1843.72082	-	9.624
Quadratic	3	17293.00000	-	90.268
Cross product	3	7.44305	-	0.039[a]
Total	9	19144.00000	-	33.310
Residual				
Lack of fit	5	428.763670	85.752734	9.401[a]
Pure error	2	18.242467	9.121233	-
Total error	7	447.006137	63.858020	-
$R^2 = 0.98$				
Factor				
T	4	8709.798970	2177.449742	34.098
pH	4	1235.192988	308.798247	4.836
RPM	4	81.327922	20.331980	0.857[a]

[a] - significant at 1% level, *DF - degrees of freedom*

variables in coded values.

Tables 3.22 and 3.24 present the analysis of variance for the models obtained that explains the response of the dependent variable. The determination coefficient or R^2 value for both the experiments were 0.99 and 0.98, respectively, which indicates that the second order model corresponded well to the experimental results, and only 1% and 2% of the total variations were not explained by the model according to the analysis of variance. The F values for the lack of fit test were insignificant and further confirmed that the models were appropriate.

The best explanatory equation for biodegradation of toluene as predicted by the model is presented in Table 3.25. The polynomial model shows that the cross-product interaction contributes to the response. Canonical analysis of the response surface revealed maximum regions for the models. The stationary points presenting maximum biodegradation of toluene by *P. fluorescens* strains NC1 and NC2 are furnished in Table 3.26.

Three-dimensional graphs and contour plots showing the influence of each variable on biodegradation of toluene by *P. fluorescens* strains NC1 and NC2 are presented in Figs. 3.7 and 3.8. The accuracy of the model was further tested by conducting a set of experiments using the critical values for maximum biodegradation. The confirmatory tests produced a maximum degradation of 100 % for both the strains. The operational conditions obtained from the models were used in the experiments followed, where the influence of humic acids were studied on the biodegradation of toluene by *P. fluorescens* strains NC1 and NC2.

Many studies have been made earlier on the biodegradation of hydrocarbons by microorganisms. These microorganisms possess the ability to utilize toluene as the sole carbon source. The different factors which affect the degradation process like mineral requirements of hydrocarbon oxidizers, effect of oxygen tension, effect of organic matter, temperature

requirements and dispersion of hydrocarbons in culture media have been elaborately discussed by Zobell as early as 1946.

Toluene has been reported to be degraded under both aerobic and anaerobic conditions. . Mars *et al.*. (1995) studied the degradation of toluene by an aerobic organism ***Burkholderia cepacia*** G4 which was cultured in a growth-limited fed batch reactor.Toluene degradation by a mixed methanogenic culture has been studied by Galic and Vogel (1986). Fries *et al.* (1997) isolated and characterized bacterial populations from an aquifer and found that they were able to degrade toluene. *Pseudomonas putida* is the organism commonly referred to possess the ability to degrade petroleum compounds. The catabolic pathways involved in the degradation of different petroleum compounds by *P. putida* has been published by Ornston (1971).

Table 3.25 Prediction equation obtained for percent toluene degradation by *P. fluorescens* - strains NC1 and NC2.

Response	Best explanatory equation	R^2	P Level
Percent toluene degraded (strain NC1)	$Y = 94.54 + 15.71\ X_1 + 6.77\ X_2 + 3.04\ X_3 - 47.74\ X_1^2 - 0.35\ X_1X_2 - 19.09\ X_2^2 + 0.06\ X_1\ X_3 + 1.27\ X_2\ X_3 - 3.52\ X_3^3$	0.99	0.01
Percent toluene degraded (strain NC2)	$Y = 89.56 + 12.07\ X_1 + 5.70\ X_2 + 2.49\ X_3 - 52.00\ X_1^2 + 0.57\ X_1X_2 - 18.41\ X_2^2 + 0.78\ X_1\ X_3 - 0.05\ X_2\ X_3 - 2.30\ X_3^3$	0.98	0.01

Table 3.26 Canonical analysis and predicted combined parameters condition and confirmatory results for maximum biodegradation of toluene by *P. fluorescens* strains NC1 and NC2.

Parameters	Toluene biodegradation	
	Strain NC1	Strain NC2
T	22.66	22.48
pH	7.38	7.31
RPM	197	206
Predic. max.(%)	97.19	91.44
Stationary point	Maximum	Maximum
Confirmatory test	100.00	100.00

Fig. 3.7. (a) Three dimensional graph - Optimisation of degradation of toluene by *P. fluorescens* strain 1 cultured in HDM medium

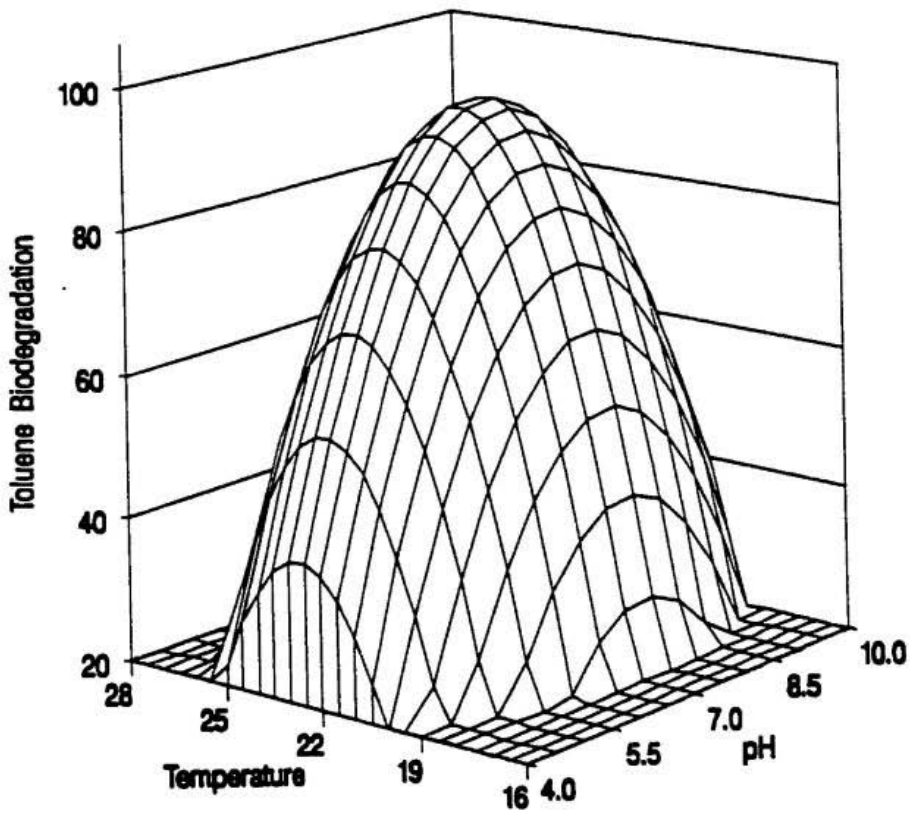


Fig. 3.7. (b) Contour graph - Optimization of toluene degradation by *P. fluorescens* strain 1 cultured in HDM medium

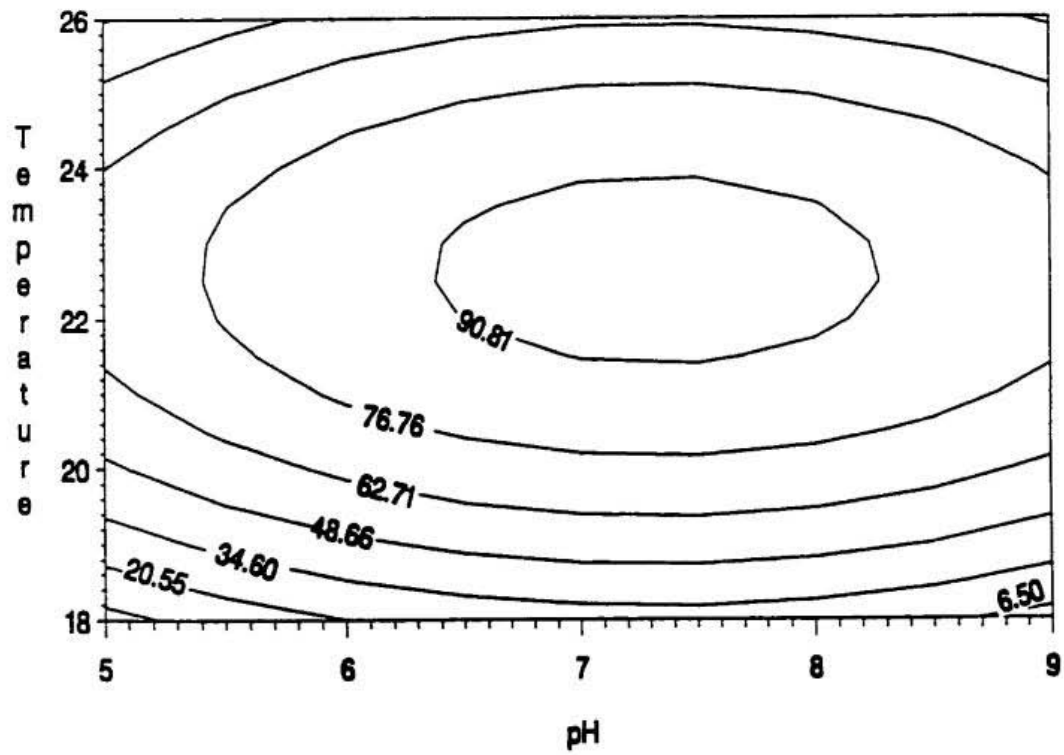


Fig. 3.8. (a) Three dimensional graph - Optimisation of degradation of toluene by *P. fluorescens* strain 2 cultured in HDM medium

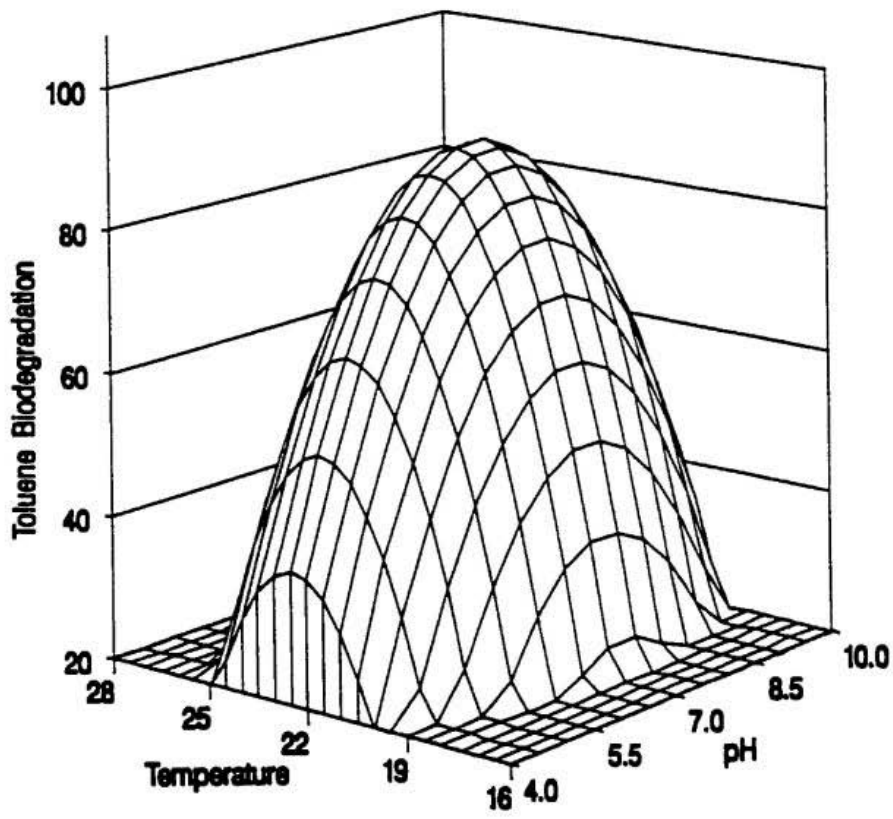
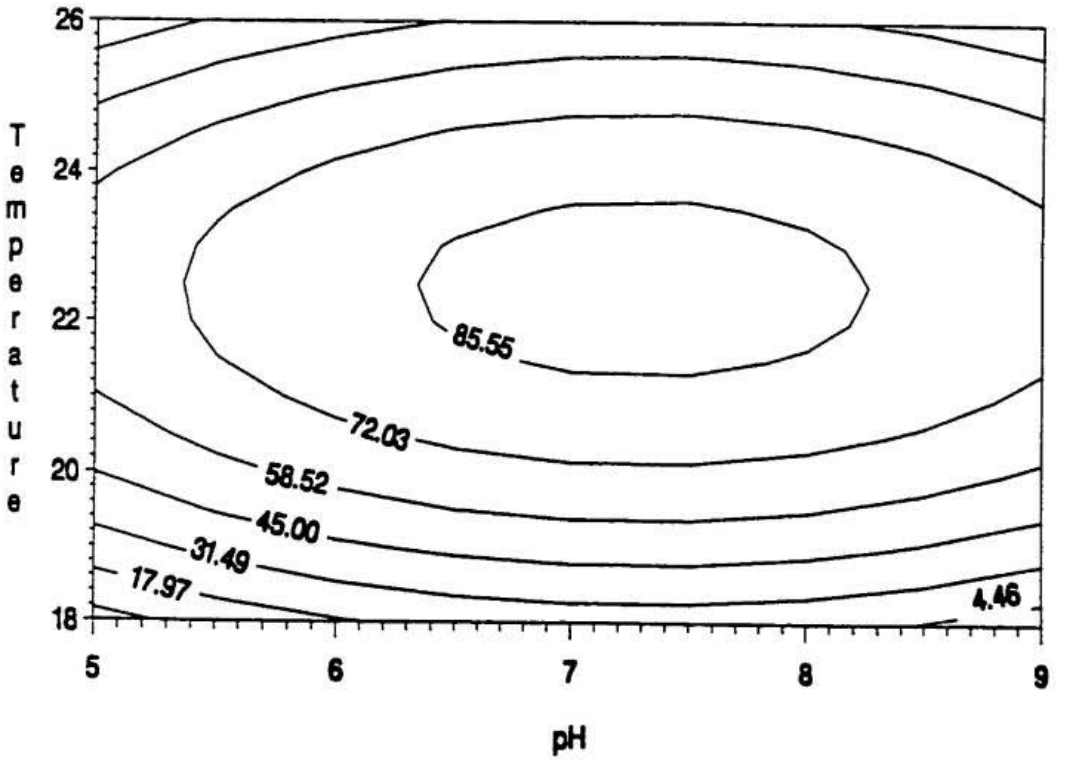


Fig. 3.8. (b) Contour graph - Optimization of toluene degradation by *P. fluorescens* strain 2 cultured in HDM medium



3.5 Effect of humic acids on toluene degradation

The head space of the control and inoculated vials were analysed and the toluene present (%) after degradation by *P. fluorescens* strains NC1 and NC2, at different concentrations of humic acids, is presented in Tables 3.27 to 3.30. Both sources of humic acids *i.e.* Aldrich salt and humic acids isolated from peat were used, and the amount of toluene present was recorded at 12 h intervals.

The present study showed that increasing concentrations of humic acids inhibited the degradation of toluene by *P.fluorescens*, as shown in the Tables 3.27 to 3.30, against control with no humic acids, for both the strains and both kinds of humic acids employed. Strain NC1 was observed to degrade toluene faster than strain NC2. Though degradation was observed until certain concentrations of humic acids in all the experiments, the rate of degradation was slower than that in the control samples, which can be attributed to the inhibition of the growth of *P. fluorescens* by humic acids. With strain NC1, the effect of humic acids from both sources were nearly the same. After 48 h, in vials with concentrations up to 0.06 % humic acids, there was no toluene present. All other concentrations of humic acids had significantly higher amount of toluene present in the vials, at all the time intervals, than the control experiments with no humic acids.

Studies with strain NC2 showed that humic acids from both sources exhibited varied effects on the degradation of toluene, unlike strain NC1 for which humic acids from both sources had similar effects. Though degradation was observed until 0.06 % of humic acids concentration, the rate of degradation was slower than that observed in the previous experiments. There was a statistically significant difference ($P < 0.05$) in the amount of toluene present in the vials after 48

h, for all the experiments with different concentrations of humic acids in it. Whereas zero degradation of toluene was observed with strain NC2 in vials with humic acids supplied by Aldrich Chemicals from 0.08 % concentration, in vials with humic acids isolated from peat, degradation started from a concentration of 0.06 %. This may be due to the presence of higher concentration of inhibitory compounds in humic acids from peat compared to the humic acid supplied by Aldrich chemicals. In the vials with 0.02 % humic acids, there was no toluene present after 48 h, though slower degradation was observed until 36 h. This can be explained by the significantly higher amount of toluene present in the vials with 0.02 % humic acids, compared to the control vials. In vials, with humic acids concentration above 0.02 % there were significantly higher amount of toluene present, at all the time intervals, than control experiments with no humic acids.

Pseudomonas spp. have been studied widely as degrading organisms of petroleum compounds (Atlas, 1981; Leahy and Colwell, 1990; Mars *et al.*, 1996). The two strains used in the study were *Pseudomonas* spp. isolated from petroleum contaminated sites of Calgary and has been screened as a toluene degrading organism.

Previous reports on the role of humic acids on microorganisms is controversial. Benz *et al.* (1998) and Coates *et al.* (1998) have reported oxidation and reduction of humic acids by *Propionibacterium freudenreichii* and *Geobacter* spp., respectively. Meanwhile, Pflug and Ziechmann (1982) and Hassett *et al.* (1987) have reported bactericidal action of humic acids on species like *Micrococcus luteus*, *Staphylococcus aureus* and *Serratia marcescens*. The different possible reasons for the inhibitory or stimulatory effects have been discussed by Müller-Wegener (1988). The present study with *P. fluorescens* exhibited clear reduction in degradation of toluene which is an effect of the inhibition of the microbial growth.

Table 3.27 Effect of humic acids (Aldrich chemical) at different concentrations on toluene biodegradation by *P. fluorescens* strain NC1 at 12 h intervals

% HA	% Toluene present in head space				
	0 hour	12 hours	24 hours	36 hours	48 hours
Control	100.00[a]	1.20 ± 0.08[a]	0.08 ± 0.02[a]	0.00[a]	0.00[a]
0.02	100.00[a]	17.29 ± 1.12[b]	0.95 ± 0.05[a]	0.00[a]	0.00[a]
0.04	100.00[a]	27.80 ± 0.98[c]	3.32 ± 0.86[a]	0.00[a]	0.00[a]
0.06	100.00[a]	91.92 ± 4.21[d]	48.00 ± 2.22[b]	11.26 ± 0.72[b]	0.00[a]
0.08	100.00[a]	93.37 ± 2.99[e]	56.96 ± 4.09[c]	25.03 ± 1.17[c]	4.46 ± 0.91[b]
0.10	100.00[a]	100.00[f]	92.36 ± 1.33[d]	90.85 ± 2.71[d]	84.54 ± 2.58[c]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at $P > 0.05$ %.

Table 3.28 Effect of humic acids (isolated from peat) at different concentrations on toluene biodegradation by *P. fluorescens* strain NC1 at 12 h intervals

% HA	% Toluene present in head space				
	0 hour	12 hours	24 hours	36 hours	48 hours
Control	100.00[a]	1.80 ± 0.07[a]	0.90 ± 0.03[a]	0.00[a]	0.00[a]
0.02	100.00[a]	38.42 ± 0.18[b]	6.59 ± 0.78[b]	0.00[a]	0.00[a]
0.04	100.00[a]	48.82 ± 0.09[c]	11.10 ± 1.18[c]	0.00[a]	0.00[a]
0.06	100.00[a]	60.88 ± 2.21[d]	16.62 ± 0.90[d]	6.55 ± 0.98[b]	0.00[a]
0.08	100.00[a]	100.00[a]	100.00[a]	28.38 ± 2.21[c]	2.08 ± 0.66[a]
0.10	100.00[a]	100.00[a]	100.00[a]	91.10 ± 4.52[d]	85.80 ± 6.27[b]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at $p > 0.05$ %

Table 3.29 Effect of humic acids (Aldrich chemical) at different concentrations on toluene biodegradation by *P. fluorescens* strain NC2 at 12 h intervals

% HA	% Toluene present in head space				
	0 hour	12 hours	24 hours	36 hours	48 hours
Control	100.00[a]	71.42 ± 3.98[a]	10.11 ± 2.06[a]	3.69 ± 0.29[a]	0.00[a]
0.02	100.00[a]	100.00[b]	30.05 ± 0.16[b]	5.41 ± 0.40[b]	0.00[a]
0.04	100.00[a]	100.00[b]	34.90 ± 2.61[b]	12.93 ± 0.13[c]	3.29 ± 0.06[b]
0.06	100.00[a]	100.00[b]	59.11 ± 4.24[c]	42.47 ± 1.04[d]	39.15 ± 1.89[c]
0.08	100.00[a]	100.00[b]	100.00[d]	100.00[e]	100.00[d]
0.10	100.00[a]	100.00[b]	100.00[d]	100.00[e]	100.00[d]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at $P > 0.05$ %

Table 3.30 Effect of humic acids (isolated from peat) at different concentrations on toluene biodegradation by *P. fluorescens* strain NC2 at 12 h intervals

% HA	% Toluene present in head space				
	0 hour	12 hours	24 hours	36 hours	48 hours
Control	100.00[a]	100.00[a]	66.05 ± 2.33[a]	3.10 ± 0.72[a]	0.00[a]
0.02	100.00[a]	100.00[a]	88.42 ± 3.90[b]	50.12 ± 2.54[b]	30.10 ± 3.66[b]
0.04	100.00[a]	100.00[a]	100.00[c]	93.26 ± 3.66[c]	58.61 ± 4.17[c]
0.06	100.00[a]	100.00[a]	100.00[c]	100.00[d]	100.00[d]
0.08	100.00[a]	100.00[a]	100.00[c]	100.00[d]	100.00[d]
0.10	100.00[a]	100.00[a]	100.00[c]	100.00[d]	100.00[d]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at $p > 0.05$ %

3.6 Optimization of biodegradation of naphthalene by *P. fluorescens* strains NC3 and NC4

Response surface methodology was used to optimise the fermentation conditions to obtain maximum biodegradation of naphthalene by *P. fluorescens* strains NC3 and NC4. The dependent variable selected was the biodegradation per cent of naphthalene. The independent variables were temperature, pH and agitation. Preliminary studies revealed that the central points for the parameters were 22°C, a pH of 7 and 150 rpm, respectively. The factorial design, a face centered cube, helped in identifying the combined best parameter conditions. The fermentation time was maintained constant at 72 h.

The per cent biodegradation of naphthalene for strains NC3 and NC4 cultured in HDM medium, obtained under the different combined operational conditions, are summarised in Table 3.31 and 3.33, respectively. The results were used to develop models in which each dependent variable was obtained as the sum of the contributions of the independent variables through first order, second order and interaction terms according to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_{ii} + \sum_{i=1}^{2} \sum_{j=2}^{3} \beta_{ij} X_i X_j$$

where Y is the dependent variable, β_0, β_i, β_{ii} and β_{ij} are constant, linear, quadratic and crossproduct regression coefficients of the model and X_i and X_j represent the independent variables in coded values.

Tables 3.32 and 3.34 present the analysis of variance for the models obtained that explains the response of the dependent variable. The determination coefficient or R^2 value for both experiments were 0.96 and 0.98 respectively, which indicates that the second order model

Table 3.31 Face-centered cube design matrix and response of dependent variables biodegradation of naphthalene by *P. fluorescens* strain NC3 for the independent variables temperature, pH and RPM

DP	X1	X2	X3	% biodegradation
1	-1	-1	-1	0.90
2	-1	-1	+1	5.45
3	-1	+1	-1	9.83
4	-1	+1	+1	13.11
5	-1	0	0	18.06
6	+1	-1	-1	23.85
7	+1	-1	+1	26.22
8	+1	+1	-1	31.31
9	+1	+1	+1	36.70
10	+1	0	0	44.16
11	0	-1	0	53.85
12	0	+1	0	63.25
13	0	0	-1	75.51
14	0	0	+1	81.70
15	0	0	0	99.70
16	0	0	0	97.02
17	0	0	0	93.45

DP - Design point

Table 3.32 Analysis of variance for response of dependent variable per cent naphthalene degraded by *P. fluorescens* strain NC3.

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	1560.39254	-	5.410
Quadratic	3	16042.00000	-	55.612
Cross product	3	0.61124	-	0.002[a]
Total	9	17603.00000	-	20.341
Residual				
Lack of fit	5	653.394163	130.678833	13.292[a]
Pure error	2	19.663267	9.831633	-
Total error	7	673.057430	96.151061	-
$R^2 = 0.96$				
Factor				
T	4	7601.114146	1900.278536	19.763
pH	4	1372.647377	343.161844	3.569[a]
RPM	4	50.101886	12.525472	0.130[a]

[a] - significant at 1% level, DF - degrees of freedom

Table 3.33 Face-centered cube design matrix and response of dependent variables biodegradation of naphthalene by *P. fluorescens* strain NC4 for the independent variables temperature, pH and RPM.

DP	X1	X2	X3	% Biodegradation
1	-1	-1	-1	0.50
2	-1	-1	+1	1.79
3	-1	+1	-1	6.91
4	-1	+1	+1	9.19
5	-1	0	0	16.41
6	+1	-1	-1	29.39
7	+1	-1	+1	35.52
8	+1	+1	-1	41.62
9	+1	+1	+1	47.72
10	+1	0	0	53.98
11	0	-1	0	60.65
12	0	+1	0	71.36
13	0	0	-1	77.70
14	0	0	+1	82.17
15	0	0	0	91.33
16	0	0	0	98.61
17	0	0	0	95.85

DP - Design point

Table 3.34 Analysis of variance for response of dependent variable per cent naphthalene degraded by *P. fluorescens* strain NC4

Source	DF	Sum of squares	Mean Square	F Ratio
Regression				
Linear	3	3288.00876	-	21.474
Quadratic	3	14975.00000	-	97.804
Cross product	3	23.63834	-	0.154[a]
Total	9	18287.00000	-	39.811
Residual				
Lack of fit	5	330.256453	66.051291	4.890[a]
Pure error	2	27.015467	13.507733	-
Total error	7	357.271919	51.038846	-
$R^2 = 0.98$				
Factor				
T	4	9166.369627	2291.592407	44.899
pH	4	1032.038938	258.609734	5.055
RPM	4	76.535476	19.133869	0.375[a]

[a] - significant at 1% level, DF - degrees of freedom

corresponded well to the experimental results, and only 4% and 2% of the total variations were not explained by the model according to the analysis of variance. The F values for the lack of fit test were insignificant and further confirmed that the models were appropriate.

The best explanatory equation for biodegradation, as predicted by the model, is presented in Table 3.35. The polynomial model shows that the cross-product interaction contributes to the response. Canonical analysis of the response surface revealed maximum regions for the models. The stationary points presenting maximum biodegradation of toluene by *P. fluorescens* strains NC3 and NC4 are furnished in Table 3.36. Three-dimensional graphs and contour plots showing the influence of each variables on biodegradation of naphthalene by *P. fluorescens* strains NC3 and NC4 are presented in Figs. 3.9 and 3.10. The accuracy of the model was further tested by conducting a set of experiments using the critical values for maximum biodegradation. The confirmatory tests produced a maximum degradation of 100 % for both the strains. The operational conditions obtained from the models were used in the experiments followed, where the influence of humic acids were studied on the biodegradation of toluene by *P. fluorescens* strains NC3 and NC4.

Pseudomonas sp. have been reported to oxidize naphthalene. Studies on naphthalene degradation by pseudomonads date back to as early as 1955 by Strawinski and Stone. These authors indicated that salicylic acid was formed as a result of dissimilation of naphthalene by *Pseudomonas aeruginosa*. Later, Klausmeier and Strawinski (1957) studied the naphthalene oxidising system by *P. aeruginosa* and the factors related to the formation of the intermediary compound salicylic acid.

Naphthalene was found to be oxidatively metabolized by soil pseudomonads through D-

trans-1,2-dihydro 1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene, which then was observed to undergo ring cleavage by Davies and Evans (1963). The degradation pathways and the different enzymes involved in naphthalene oxidation were further unravelled by Barnsley (1975), Williams *et al.* (1975) and Jeffrey *et al.* (1975).

Table 3.35 Prediction equation obtained for percent naphthalene degradation by *Pseudomonas fluorescens* - strains NC3 and NC4

Response	Best explanatory equation	R^2	P Level
Percent naphthalene degraded (strain NC3)	$Y = 86.90 + 11.49\ X_1 + 4.39\ X_2 + 2.18\ X_3 - 48.42\ X_1^2 + 0.17\ X_1X_2 - 20.98\ X_2^2 - 0.008\ X_1\ X_3 + 0.22\ X_2\ X_3 - 0.92\ X_3^3$	0.96	0.01
Percent naphthalene degraded (strain NC4)	$Y = 88.28 + 17.34\ X_1 + 4.89\ X_2 + 2.02\ X_3 - 47.85\ X_1^2 + 1.33\ X_1X_2 - 17.04\ X_2^2 + 1.08\ X_1\ X_3 + 0.12\ X_2\ X_3 - 3.11\ X_3^3$	0.98	0.01

Table 3.36 Canonical analysis and predicted combined parameters condition and confirmatory results for maximum biodegradation of naphthalene by *P. fluorescens* strains NC3 and NC4.

Parameters	Naphthalene biodegradation	
	Strain NC3	Strain NC4
T	22.47	22.75
pH	7.22	7.30
RPM	269	186
Predic. max.(%)	89.12	90.65
Stationary point	Maximum	Maximum
Confirmatory test	100.00	100.00

Fig. 3.9. (a) Three dimensional graph - Optimisation of degradation of naphthalene by *P. fluorescens* strain 3 cultured in HDM medium

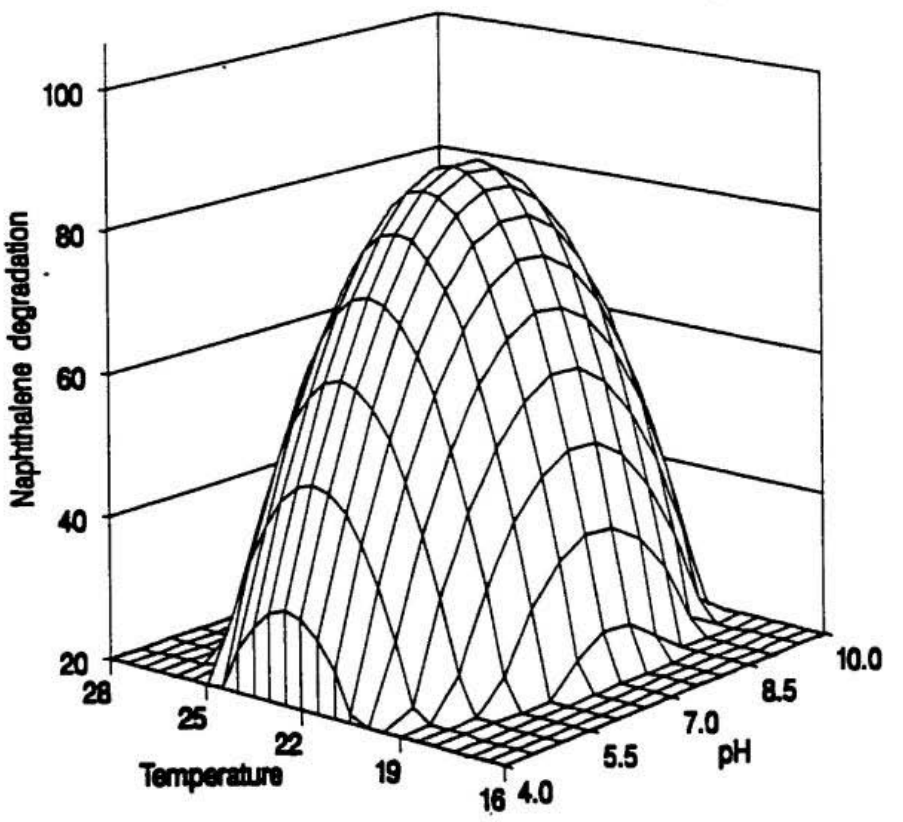


Fig. 3.9. (b) Contour graph - Optimization of naphthalene degradation by *P. fluorescens* strain 3 cultured in HDM medium

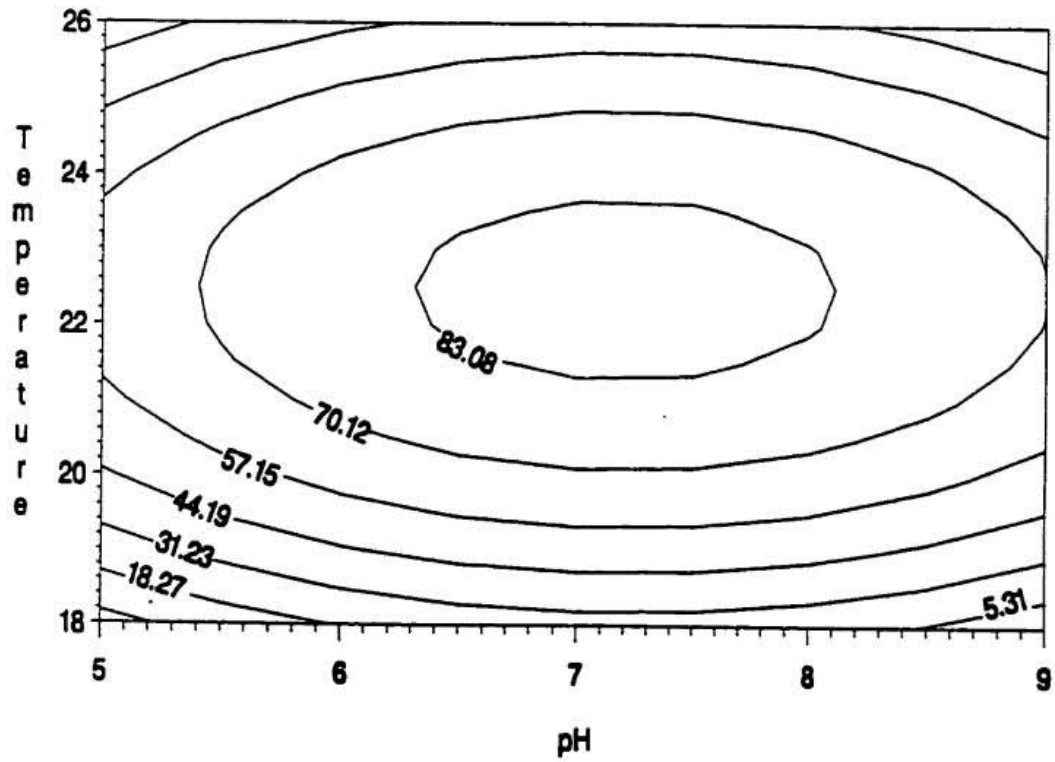


Fig. 3.10. (a) Three dimensional graph - Optimisation of degradation of naphthalene by *P. fluorescens* strain 4 cultured in HDM medium

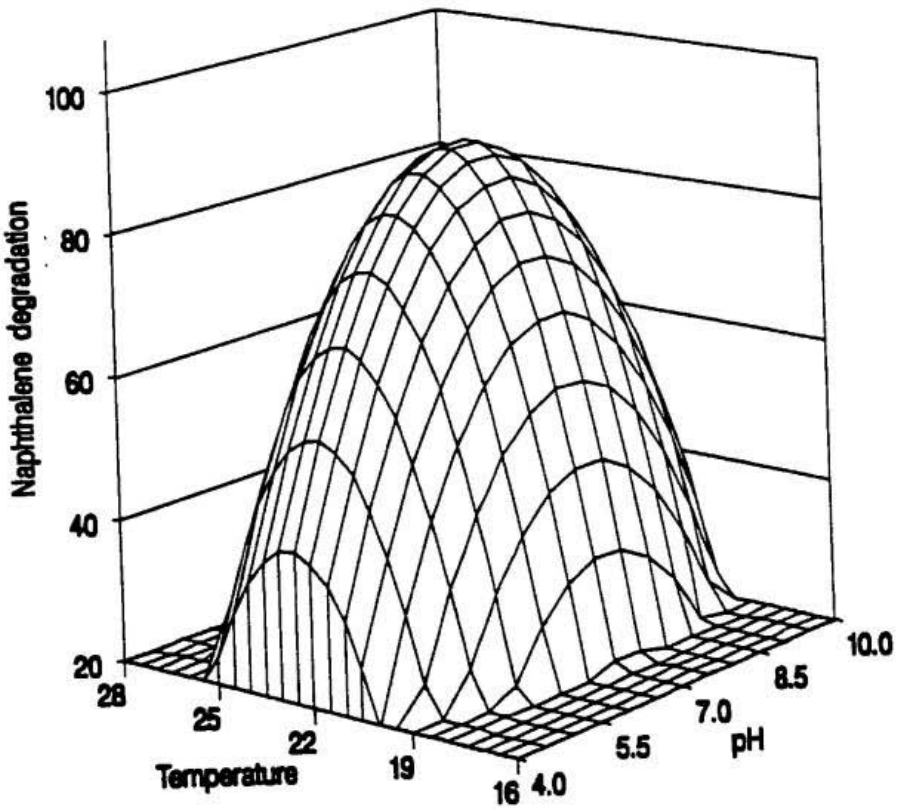
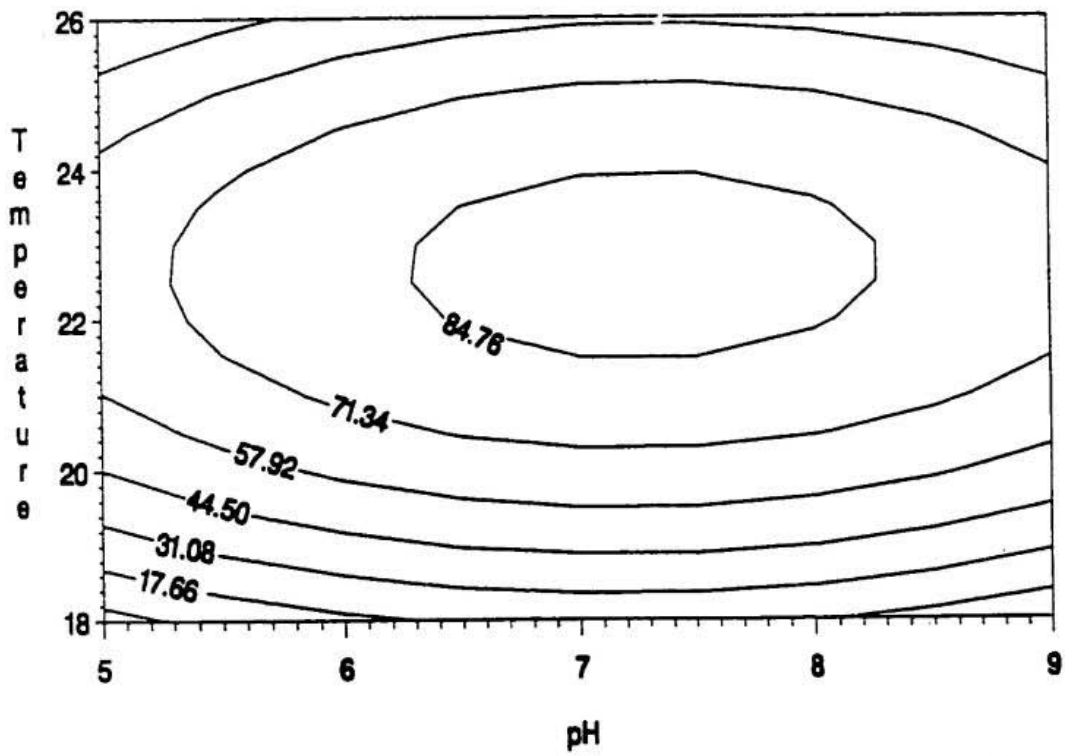


Fig. 3.10. (b) Contour graph - Optimization of naphthalene degradation by *P. fluorescens* strain 4 cultured in HDM medium



3.7 Effect of humic acids on naphthalene degradation

The head space of the control and inoculated vials was analysed and the naphthalene present (%) after degradation by *P. fluorescens* strains NC3 and NC4 are presented in Tables 3.37 to 3.40. Both sources of humic acids, *i.e.* isolated from peat and humic acid supplied by Aldrich chemicals, were used at different concentrations, and the amount of toluene present was recorded at 12 h intervals, for up to 72 h.

The present study showed that increasing concentrations of humic acids inhibited the degradation of naphthalene by *P. fluorescens*. This is explained by the higher amount of naphthalene present in the head space of the vials with humic acids compared to control, with no humic acids. Naphthalene degradation was slower than toluene degradation. In the control vials, with no humic acids, it took 72 h for the complete degradation of naphthalene. With *P. fluorescens* strain NC3, grown in vials with humic acid supplied by Aldrich, except at 0.02 % concentration, where there was a little reduction of naphthalene, no reduction was observed with other concentrations of humic acid. Similar results were observed with *P. fluorescens* grown in vials incorporated with humic acids isolated from peat, except that there was an insignificant reduction at 0.04 % concentration after 72 h. These results clearly indicate the sensitiveness of these naphthalene degrading *Pseudomonas* strains to humic acids.

The degradation rate of naphthalene by *P. fluorescens* strain NC4 was relatively faster compared to strain NC3. Though complete degradation was observed only after 72 h, at 12, 24, 36, 48 and 60 h the amount of naphthalene assimilated by strain NC4 was higher. There was a significant difference in the effect of humic acids with increasing concentrations . Biodegradation of naphthalene was observed with the concentrations 0.02 and 0.04 % supplied by Aldrich

chemicals. But degradation was observed only with 0.02 % in vials incorporated with humic acids isolated from peat. Zero degradation of naphthalene was observed with other higher concentrations. These results showed a definite inhibitory effect of *P. fluorescens* by humic acids.

The effect of humic substances has always been controversial. Both stimulatory and inhibitory effects have been reported. Tolpa and Czyzewski (1963) reported increased biomass production by *Saccharomyces cerevisiae*, *Torula utilis* and *Candida tropicalis* when grown in media containing humic extracts. A study on the physiological effect of humic substances on the physiological groups of amylolytic and proteolytic microorganisms and denitrifiers of soil revealed stimulatory effect (Visser, 1985 a). Mishra and Srivastava (1986) identified *Aspergillus awamori*, *Penicillium* sp. and *Humicola insolens* which could use humic acids as their sole carbon source. A study on the effect of humic acids on *Scytalidium acidophilum* by Martin *et al.* (1990) revealed stimulatory effect of humic acid on the biomass production upto a concentration of 0.2 % at pH 2 and 0.4 % at pH 8.

Contradictory reports on the inhibitory effects of humic acids are also available. McLoughlin and Kuster (1972) observed inhibitory effects due to presence of humin molecule, affecting nutrient status and permeability of microbial cells. Batericidal action of humic acids on *Serratia marcescens* and *Staphylococcus aureus* has been reported by Hassett *et al.* (1987). Elsewhere in this work, inhibitory effects of humic substances on the growth of the yeast *Phaffia rhodozyma* are discussed.

The present study found definite inhibitory effects of humic acids on the growth of the four strains of *Pseudomonas fluorescens* tested.

Table 3.37 Effect of humic acids (Aldrich chemical) at different concentrations on naphthalene biodegradation by *P. fluorescens* strain NC3 at 12 h intervals

	% Naphthalene present in head space						
% HA	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours	72 hours
Control	100.00[a]	100.00[a]	100.00[a]	88.15 ± 3.45[a]	36.83 ± 0.86[a]	7.08 ± 0.71[a]	0.00[a]
0.02	100.00[a]	100.00[a]	100.00[a]	100.00[b]	89.29 ± 2.26[b]	79.15 ± 3.48[b]	75.28 ± 1.52[b]
0.04	100.00[a]	100.00[a]	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]
0.06	100.00[a]	100.00[a]	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]
0.08	100.00[a]	100.00[a]	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]
0.10	100.00[a]	100.00[a]	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at p > 0.05 %

Table 3.38 Effect of humic acids (isolated from peat) at different concentrations on naphthalene biodegradation by *P. fluorescens* strain NC3 at 12 h intervals

	% Naphthalene present in head space						
% HA	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours	72 hours
Control	100.00[a]	100.00[a]	100.00[a]	88.15 ± 3.45[a]	36.83 ± 0.86[a]	7.08 ± 0.71[a]	0.00[a]
0.02	100.00[a]	100.00[a]	100.00[a]	92.69 ± 1.87[b]	85.74 ± 2.88[b]	75.52 ± 1.22[b]	70.19 ± 1.35[b]
0.04	100.00[a]	100.00[a]	100.00[a]	100.00[c]	100.00[c]	100.00[c]	97.83 ± 1.80[c]
0.06	100.00[a]	100.00[a]	100.00[a]	100.00[c]	100.00[c]	100.00[c]	100.00[c]
0.08	100.00[a]	100.00[a]	100.00[a]	100.00[c]	100.00[c]	100.00[c]	100.00[c]
0.10	100.00[a]	100.00[a]	100.00[a]	100.00[c]	100.00[c]	100.00[c]	100.00[c]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at P > 0.05 %

Table 3.39 Effect of humic acids (Aldrich chemical) at different concentrations on naphthalene biodegradation by *P. fluorescens* strain NC4 at 12 h intervals

% HA	% Naphthalene present in head space						
	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours	72 hours
Control	100.00^{a}	82.74 ± 1.12^{a}	67.85 ± 1.74^{a}	48.58 ± 0.82^{a}	19.13 ± 0.90^{a}	8.69 ± 0.72^{a}	0.00^{a}
0.02	100.00^{a}	100.00^{b}	100.00^{b}	78.75 ± 3.24^{b}	75.91 ± 1.24^{b}	54.73 ± 1.06^{b}	41.68 ± 0.46^{b}
0.04	100.00^{a}	100.00^{b}	100.00^{b}	100.00^{c}	83.96 ± 2.72^{c}	71.25 ± 0.94^{c}	64.93 ± 0.74^{c}
0.06	100.00^{a}	100.00^{b}	100.00^{b}	100.00^{c}	100.00^{d}	100.00^{d}	100.00^{d}
0.08	100.00^{a}	100.00^{b}	100.00^{b}	100.00^{c}	100.00^{d}	100.00^{d}	100.00^{d}
0.10	100.00^{a}	100.00^{b}	100.00^{b}	100.00^{c}	100.00^{d}	100.00^{d}	100.00^{d}

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

*** Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at P > 0.05 %**

Table 3.40 Effect of humic acids (isolated from peat) at different concentrations on naphthalene biodegradation by *P. fluorescens* strain NC4 at 12 h intervals

	% Naphthalene present in head space					
% HA	0 hour	12 hours	24 hours	36 hours	48 hours	60 hours
Control	100.00[a]	82.74 ± 1.12[a]	67.85 ± 1.74[a]	42.60 ± 0.73[a]	11.20 ± 0.84[a]	1.53 ± 0.12
0.02	100.00[a]	82.13 ± 0.69[a]	77.28 ± 0.99[b]	69.00 ± 1.49[b]	31.75 ± 1.57[b]	13.45 ± 1.9
0.04	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]	100.00[c]
0.06	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]	100.00[c]
0.08	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]	100.00[c]
0.10	100.00[a]	100.00[b]	100.00[c]	100.00[c]	100.00[c]	100.00[c]

% HA - percent humic acid added in different treatments. All experimental values given are mean of three replicates

* Data with different alphabetical superscripts within a column are significantly different (Tukey's honest significant difference test) at $P > 0.05$ %

4.0 SUMMARY AND CONCLUSIONS

Humic substances are present ubiquitously in nature and are formed during the decomposition of plants and animals. They are present in peat, soils and sediments, and take part in different environmental reactions. Their influence on the biota has been controversial. The present study was primarily conducted to unravel their effects, particularly humic acids, a major component of humic substances, on microorganisms.

Studies were conducted with a yeast, *Phaffia rhodozyma*, and a bacteria, *Pseudomonas fluorescens*. Initially, the effect of components of peat were studied on the biomass production and biochemical characteristics of *P. rhodozyma*, to get an overview of the effect of humic substances, as well their interactive effects with other peat components. RSM was used to optimize the biomass production of *P. rhodozyma* in each of the substrates. The results of these experiments showed higher productivity by *P. rhodozyma* grown in PH and DPHR, than when grown in DPH and PHR. This could be attributed to individual and synergistic inhibitory effects of bitumens and humic substances, and to the decrease in nutrients resulting from the extraction of bitumens and humic substances. Astaxanthin production by *P. rhodozyma* grown in PHR, where bitumens were present after the removal of humic substances, was the maximum among the different peat hydrolysates studied. The result validated the contribution of bitumens to astaxanthin production. Significantly lesser amounts of lipids were observed in biomass grown in both DPH and DPHR, where bitumens were removed before hydrolysis. This finding indicates the possibility of bitumen being a contributing factor towards the lipid content of the microbial biomass. The astaxanthin content produced by YM broth was higher than those in peat

hydrolysates, both modified or non-modifed. However, the use of YM broth as substrate resulted in less biomass than produced by the peat hydrolysates. When humic acids were added to the YM broth, there was a definite inhibitory effect on the biomass production and yield.

The four *Pseudomonas fluorescens* species isolated from petroleum contaminated sites were optimized for maximum biodegradation in HDM medium using RSM. The effect of humic acids at different concentrations were studied on the biodegradation potential of the organisms. Addition of humic acids showed significant reductions in the biodegradation potential of the four strains. At lower concentrations of humic acids, slow degradation was observed at 12 and 24 h . The results of these studies with *P. rhodozyma* and *P. fluorescens* strains demonstrate the inhibitory effect of humic acids on microbial metabolism.

The present work suggests that care should be taken about the type of support material to be used in biodegradation operations. The presence of humic acids in the support material, peat or soil, may contribute to the inefficiency of the system, humic acids inflicting an inhibitory effect on the microorganisms involved in the biodegradation. Steps to remove the humic acids, using mild alkali, could be a solution to this issue. Though in natural systems microbial reactions occurs in peat and soil systems, removal of humic acids may result in enhanced biodegradation.

5.0 REFERENCES

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LIST OF ABBREVIATIONS

DOC	-	Dissolved Oxygen Content
DPH	-	Debituminised peat hydrolysate
DPHR	-	Debituminised peat hydrolysate with humic substances removed
FT	-	Fermentation Time
HA	-	Humic acids
PAH	-	Polycyclic aromatic hydrocarbon
PH	-	Peat hydrolysate
PHR	-	Peat hydrolysate with humic substances removed
RSM	-	Response surface methodology
YM	-	Yeast extract– Malt extract broth