CHARACTERIZATION OF *ASPERGILLUS CARBONARIUS* MUTANT IN RELATION TO XANTHIN PRODUCTION, TOXICITY STUDIES AND FERMENTATION CONDITIONS FOR PIGMENT PRODUCTION

THESIS

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By

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(2009)

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Date: 28th August 2009
Place: Mysore

CERTIFICATE

I hereby certify that this thesis entitled **"Characterization of *Aspergillus carbonarius* mutant in relation to xanthin production, toxicity studies and fermentation conditions for pigment production"** submitted by **Mr. K. R. Sanjay** for the award of Doctor of Philosophy in Microbiology to the University of Mysore, is the result of research work carried out by him in the Food Microbiology Department, Central Food Technological Research Institute, Mysore, under my guidance during the period July 2004 to July 2009.

G. Vijayalakshmi

(Guide)

DECLARATION

I hereby declare that the thesis entitled "**Characterization of *Aspergillus carbonarius* mutant in relation to xanthin production, toxicity studies and fermentation conditions for pigment production"** submitted to the University of Mysore, for the award of Doctor of Philosophy in Microbiology, is the result of research work carried out by me in the Department of Food Microbiology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. G. Vijayalakshmi,** during the period of July 2004 to July 2009.

I further declare that the results of this work have not been previously submitted for any degree or fellowship.

Place: Mysore

Date: 28th August 2009 **(K. R. SANJAY)**

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Mysore

Date: ***K. R. SANJAY***

Dedicated to my beloved Parents, Teachers,

Family members and to my lovely daughter

"SINCHANA"

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INTRODUCTION

All *Aspergillus* species are major agents of decomposition and decay and thus possess the capability to produce a broad range of enzymes. The activities of several *Aspergillus* species have their fermentative powers harnessed for commercial purposes. They are common sources of extra cellular enzymes and organic acids used in food processing (Raper and Fennell 1965).

THE GENUS *ASPERGILLUS*

Aspergillus, belongs to the class Ascomycetes, family Eurotiaceae and is a widely distributed saprophytic fungus. Genus *Aspergillus* contains 132 species, subdivided into 18 groups. *Aspergillii* that exhibit sexual stage are classified as;

Class: Ascomycetes

Order: Eurotiales

Family: Eurotiaceae

Form genus: *Aspergillus* (*Eurotium, Sartorya and Emerciella*).

Most of the *Aspergillus* species can be readily identified when cultivated on czapekdox and malt extract agar media. For telomorphic species, cornmeal agar and oatmeal agar were recommended. The characteristic of *Aspergillus* conidiophores is having aseptate stripe terminating in a vesicle, on which the conidiogenous cells (phialides and metulae) are borne. The color of the conidial heads is also an important character, together with characteristic of sclerotia. Many species produce sclerotia on agar media when freshly isolated.

ASPERGILLUS NIGER GROUP

Members of *Aspergillus* section *nigri* are widely studied for industrial purposes (Raper and Fennell 1965). Products produced by strains of *Aspergillus niger* holds the GRAS (**G**enerally **R**ecognized **A**s **S**afe) status from the FDA (Bigelis and Lasure 1987). Until now, the taxonomy of black *Aspergillii* is not clear, because it is primarily based on morphological criteria and in some cases the differences between the species are very subtle.

A. niger cultures were grown on czapeckdox agar and 2% malt extract agar at 25ºC for 7 and 14 days respectively. Members of the *A. niger* aggregate were characterized by their typical blackish colonies, more or less ornamented conidia with globose to subglobose shape with 6 µm diameters. After analysis of characters, five readily distinguishable (*A. heteromorphus, A. japonicus, A. carbonarius, A. ellipticus* and *A. tubigensis*) and an *A. niger* aggregate which subdivided into seven varieties were recognized (Kester and Visser 1990).

ASPERGILLUS CARBONARIUS

A. carbonarius (Bainier) Thom was placed under the *A. niger* group since it formed black coloured colonies and globose radius conidial heads. *A. carbonarius* is characterized by the production of black, brownish-black and purplish-brown conidial heads. Members of this group are the most explored in industries for the production of gallic acid, citric acid and many other enzymes (Raper *et al.* 1945).

Colonies of *Aspergillus carbonarius* on czapeckdox agar reach 2.5 to 3.5 cm diameter with in 10 days of incubation. On maturation of conidia, surface of colonies turn

black and granular, except for a 1 to 2 mm wide margin. Reverse phase of the colony is colourless. Prolonged incubation induces light yellow to blackish color in the middle of the colony. Some instances of exudates were described which were initially colourless and later became brown. Conidiophores of *A. carbonarius* which measure 5 to 6 μm in length are much bigger than that of *A. niger*. The conidial heads are usually 500 to 600 μm in dia and are radially arranged. They form spherical vesicles that measured 60 to 80 μm in diameter. The phialides measured 25 to 50 X 8 to11 μm in length. Secondary sterigamata are usually smaller and measured 8 to15 μm X 6 to 8 μm in size. Conidia are globose, with hyaline spines in young culture (Rapper and Fennell 1965).

NATURAL VARIENCE IN *ASPERGILLUS*

Species of *Aspergillus* form a very large proportion of all the molds utilized in industrial work. They are cosmopolitan and adapted to wide environmental conditions. Naturally isolated molds show variations that differ in degree rather than in basic characters. Natural variants can be frequently obtained in laboratory by selective isolation from a sector of areas of a typical growth or by single spore isolations. Raper and Fennell (1965) have reported the isolation of natural mutants *A. fumigatus mut. helvola* and *A. nidulans mut. albus* that have originated in the absence of artificial stimuli. Some species and strains are subjected to continuous variation in physiological character, which will alter the characteristic of whole culture such as overgrowths or appearing abruptly. Mangin (1990) was the first to make a comparative study of a number of isolates of *Aspergillus* form various sources. Greene (1933) isolated 448 single spore cultures form *A. fischeri*, among which, one group produced very large, scattered perithecia against

typical small perithecia formers and the other groups produced conidial structures in profusion, but forming few perithecia. In *A. terreus* var. *boedijni* colonies are much brighter and xnathine orange in colour (Blochwitz 1934). In *A. terreus var. floccosus*, formation and colour of conidial heads are typical but their number is greatly reduced and they are born upon aerial hyphae. *A. fumigatus* type culture showed heavily sporulating velvety colonies, whereas other variants isolated produced flocked colonies with comparatively less conidial heads. Hence, variants arise through gradual changes from well identifiable strains of species (Raper and Fennell 1965).

INDUCED VARIENCE IN *ASPERGILLUS*

Variation in *Aspergillus* mainly occurs due to mutation. There are several reports on induced variation in *Aspergillus* for strain improvement. By subjecting conidia to low voltage cathode ray bombardment, Whelden (1940) has obtained mutants of *A. niger* that possessed brown shaded heads rather than black and larger conidial structures. Tan coloured conidial head were produced from a mutant of *A. terreus* by irradiating spores with ultra violet rays (Raper *et al.* 1945). Various authors have studied heterokaryosis in *A. niger* and suggested that ultraviolet radiation induces multinucleated conditions in conidia. The explanation was that due to blockage in different steps of enzymatic reactions by mutation, resulted in nuclei of two mutants enter the same conidiophore to complement the blocked enzymatic step (Ravi kumar, 2004). Gordon *et al.* (2000) found that subjecting spores of *A. niger* to UV mutagenesis and selecting against temperature, mutants of affected general secretary pathway can be isolated.

Strain improvement for various fermentation processes can be accomplished using physiological mutations. In strain improvement process, mutations were mainly induced with ultra violet rays, ionizing radiations and chemicals like lithium chloride, camphor, nitrous oxide etc.

CARBON METABOLISM IN *ASPERGILLUS*

Aspergillii are capable of utilizing a wide range of organic compounds as source of carbon and energy for growth (Cochrane 1958). Carbon metabolism by **E**mbden – **M**eyerhoff – **P**arnas (EMP) and **P**entose **P**hosphate (PP) pathway is well documented in *Aspergillii* (Blumenthal 1965). Niderpruem (1965) reported the presence of enzymes of the tricarboxylic acid cycle. The increased activity of the enzymes of the EMP and PP pathways with increased rate of supply of glucose resulted in faster growth rate of the fungus. Mutants play important role in the study of carbon metabolism mainly in relation to enzymes of physiological importance. Some mutants isolated for deficiencies in carbon metabolism showed differences in growth on nitrate or ammonium (Hankinson 1974). However nitrogen regulation is dependent on nature of carbon source. Some amino acids serve both as nitrogen source as well as moderate or poor carbon source for the growth of fungus. Both inducible and constitutive uptake systems are present in *Aspergillus. Aspergillii* are known to utilize amides as carbon source (Hynes and Pateman 1970). The regulation of main catabolic enzymes or specific uptake systems was due to growth of *Aspergillii* on different carbon source (Romono and Kornberg 1968).

Two types of regulations for carbon utilization involving enzymes and permeases were demonstrated. They are induction of enzymes involved in carbon metabolism and

the carbon catabolic repression, where certain carbon sources, generally the better ones prevent the synthesis of enzymes and permeases required for the utilization of other poor carbon sources (Romano and Kornberg 1968).

NITROGEN METABOLISM IN *ASPERGILLUS*

Extensive studies of nitrogen metabolism and its regulation have been conducted with *Aspergillus nidulans* and *Neurospora crassa* (Azuine, *et al.* 1992). Compounds such as ammonia, glutamine and glutamate are preferentially used as nitrogen sources by these fungi. However, when these primary nitrogen sources are not available, other nitrogen sources like, nitrate, nitrite, purines, amides, amino acids and proteins were used by fungi (Cooper 1996). Utilization of various secondary nitrogen sources is highly regulated and requires the synthesis of a set of pathway-specific catabolic enzymes and permeases which are otherwise subject to nitrogen catabolite repression (Caddick 1992). Inorganic nitrate serves as an excellent nitrogen source for *Aspergillus*, *Neurospora*, and many other fungal species. Utilization of nitrate requires the de novo synthesis of nitrate reductase and nitrite reductase. Nitrate reductase, a large homodimeric multi-redox protein, catalyzes the conversion of nitrate to nitrite (Campbell and Kinghorn 1990). Conversion of nitrate to nitrite involves transfer of electrons derived from NADPH in a stepwise manner to a carboxy-terminal flavin domain which contains FAD, then to a central heme-containing domain and finally to amino-terminal molybdopterin containing domain (Campbell and Kinghorn 1990). Mutational studies on *A. nidulans* at many loci resulted in chlorate resistance and loss of nitrate reductase. These loci include the structural gene which encodes the nitrate reductase polypeptide (Crawford and Arst

1993). Mutants affected in utilization of nitrate (*niiA*) as a sole nitrogen source was unable to grow on nitrite. These *niiA* mutants produced nitrate reductase but not nitrite reductase. Mutants that lacked nitrate and nitrite reductases were not able to utilize both nitrate and nitrite (Pateman *et al.* 1964). *A. clavatus* utilized ammonium in preference to other nitrogen sources due to the repression of systems by ammonium that were required for the utilization of other nitrogen sources (Robinson *et al.* 1974).

BIOTECHNOLOGICAL IMPORTANCE OF *ASPERGILLUS*

Black *Aspergillii* are widely used for industrial applications for production of enzymes, organic acids, fermented foods and beverages (Yokotsuka and Sasaki 1998). The property of filamentous fungi like *Aspergillus niger*, *Aspergillus oryzae* to secrete a large diversity of biopolymer degrading enzymes in significant quantities has stimulated the industrial application of these filamentous fungal strains. *A. niger* has been primarily used for production of enzymes and organic acids from long time in food industry, without any apparent adverse effects on human health. A wide-range of fungal enzymes produced by *Aspergillus niger* and other black *Aspergillus* species such as *Aspergillus aculeatus* are used in food processing (Verdoes *et al.* 1993).

Other application of *Aspergillus* is production of fermented foods like, Soy sauce, Temphae and Cheese. Because of its great secretion capacity *A. niger* and *A. oryzae* are attractive organisms for the production of non-fungal (e.g. mammalian) proteins, such as the bovine milk-clotting enzyme and (pro-) chymosin or human BRM's (biological response modifiers like interferons and interleukins) (Beppu 1995). The enzyme producing strains have been evaluated for safety before entering the market. The safety of

A. niger for industrial use has been reviewed recently (Schuster *et al.* 2002; Pariza and Jonson 2001).

CAROTENOIDS

Many microorganisms produce a wide range of natural products as primary and secondary metabolites. These compounds include antibiotics, carotenoids, fatty acids and plant growth hormones (Smith and Berry 1975). Biosynthesis of secondary metabolites is usually associated with cell differentiation or development and most of the secondary metabolites are produced by organisms that exhibit filamentous growth with relatively complex morphology (Bu'Lock 1961).

Carotenoids (tetra terpenoids) are a group of fat-soluble pigments with 40-carbon atoms backbone. Carotenoids produced in plants, algae, fungi, yeast and photosynthetic bacteria comprise the largest group of pigments such as β-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, canthaxanthin and astaxanthin (Bertram *et al.* 1991). Carotenoids contain the largest number of colours that occur naturally and are available for colouring food (Britton *et al.* 1995). Carotenoids impart yellow to orange colours to foods and are fairly stable to heat and pH changes (Lewis *et al*. 1977).

Carotenoids are polyisoprenoid compounds that are divided into two main groups: Carotenes or hydrocarbon carotenoids composed of carbon and hydrogen atoms and xanthophylls or oxygenated hydrocarbon derivatives containing oxygen functional group such as, hydroxy, keto, epoxy, methoxy or carboxylic acid (Almeida and Penteado 1988). Carotenoid's structural characteristic is a conjugated double bond system, which influences their chemical, biochemical and physical properties. They are made up of isoprene units and differ in the length of the middle chain and substitution at each end.

Carotenoids are readily soluble in organic solvents such as acetone, ethanol, diethyl ether, and chloroform (Shahidi *et al.* 1998). The major carotenoids which impact colours in food and feed are β-carotene, astaxanthin, zeaxanthin, lycopene, leutin, capsanthin, α-carotene, violaxanthin, bixin, canthaxanthin and norbixin. However, the great interest in studying these compounds is due to their physiological and biological functions (Anindita Roy 1998).

ASTAXANTHIN

Astaxanthin (3,3$^{!}$-dihidroxy-ß,ß-carotene-4,4$^{!}$-dione) is a oxycarotenoid imparts characteristic orange flesh coloration in fish with strong antioxidant activity (Kurashige *et al.* 1990). Astaxanthin is a naturally occurring pigment in the crest, egg yolk and in various tissues in the domestic chicken (Fletcher 1989). Therefore astaxanthin is used as a valuable feed additive for farmed fish to maintain health and flesh color. There has been growing interest in the use of astaxanthin as a pigment for aquaculture and poultry industry (Choubert and Storebakken 1989).

The structure of astaxanthin is similar to ß-carotene but a slight change in the end rings makes the molecule more effective antioxidant than ß-carotene. Astaxanthin has two chiral or asymmetric centers that are carbon numbered 3 and 3$^{!}$ on the two rings in the structure. These chiral centers has given rise to three stereoisomers; 3S 3$^{!}$S, 3R 3$^{!}$S, and 3R 3$^{!}$R. The 3S 3$^{!}$S and 3R 3$^{!}$R stereoisomerisms are mirror images of each other and are called enantiomers. The 3R 3$^{!}$S form is called as a meso and it is optically inactive. Astaxanthin has two hydroxyl groups, one on each terminal ring. These are free to react with acids, such as fatty acids to form esters (Bertram *et al.* 1991).

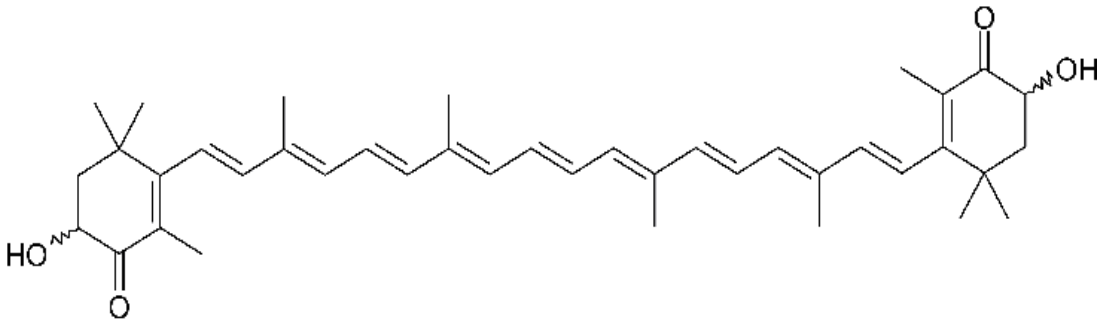


Figure: 1. Chemical structure of astaxanthin (3,3^{l}-dihydroxy- β, β -carotene-4,4^{l}-dione)

CAROTENOID BIOSYNTHESIS

General carotenoid biosynthetic pathway consists of following steps.

Formation of phytoene from Iospentenyl diphosphate (IPP)

C40 carotenoids are made in thousands of plant and microbial species, starting with the synthase-catalyzed condensation of two molecules of geranyl-geranyl diphosphate (C_{20}PP) to form phytoene. Geranyl-geranyl pyrophosphate (GGPP) is the immediate precursor of carotenoids. It is a C_{20} compound derived from four C_5 isoprene units. In order to initiate chain elongation, IPP (C_5) is isomerised to its allelic isomer dimethylallyl diphosphate (DMAPP). Condensation of IPP and DMAPP in a head to tail fashion generates the C_{10} molecule geranyl pyrophosphate (GPP). Addition of IPP to GPP results in farnesyl pyrophosphate (FPP, C_{15}) formation and further IPP molecule yields GGPP (Poulter *et al.* 1981). Two molecules of GGPP are condensed in a head to head manner to form the intermediate pre-phytoene pyrophosphate (PPPP). Subsequent elimination of the diphosphate group and stereospecific proton abstraction results in phytoene (C_{40}) formation **(Figure-2**). Phytoene (7, 8, 11, 12, 70, 80, 110, 120-octahydro-cc-carotene) is a colourless, symmetrical hydrocarbon containing three conjugated double

bonds. Its C_{40} skeleton forms the basis from which all carotenoids are derived (Bouvier *et al.* 2000).

Desaturation reaction

A series of conjugated carbon–carbon double bonds constitute the characteristic chromophore of carotenoid pigments. These are introduced into phytoene by a series of desaturation reactions. Four desaturation steps occurs sequentially, phytofluene (7, 8, 11, 12, $7^{!}$, $8^{!}$-hexahydro-ψ,ψ-carotene), ζ-carotene (7, 8, 11, 12, 70, 80-terahydro-ψ,ψ-carotene), neurosporene (7, 8-dihydro-ψ,ψ-carotene) and lycopene (ψ,ψ-carotene). The extended chromophore converts colourless phytoene into red-coloured lycopene. The enzyme responsible for the desaturation of phytoene to ζ -carotene is phytoene desaturase (PDS). The removal of two hydrogen atoms during each desaturation step suggests the involvement of an electron transport chain for regeneration of reductants. ζ-Carotene desaturase catalyses the desaturation of ζ -carotene into lycopene via neurosporene .

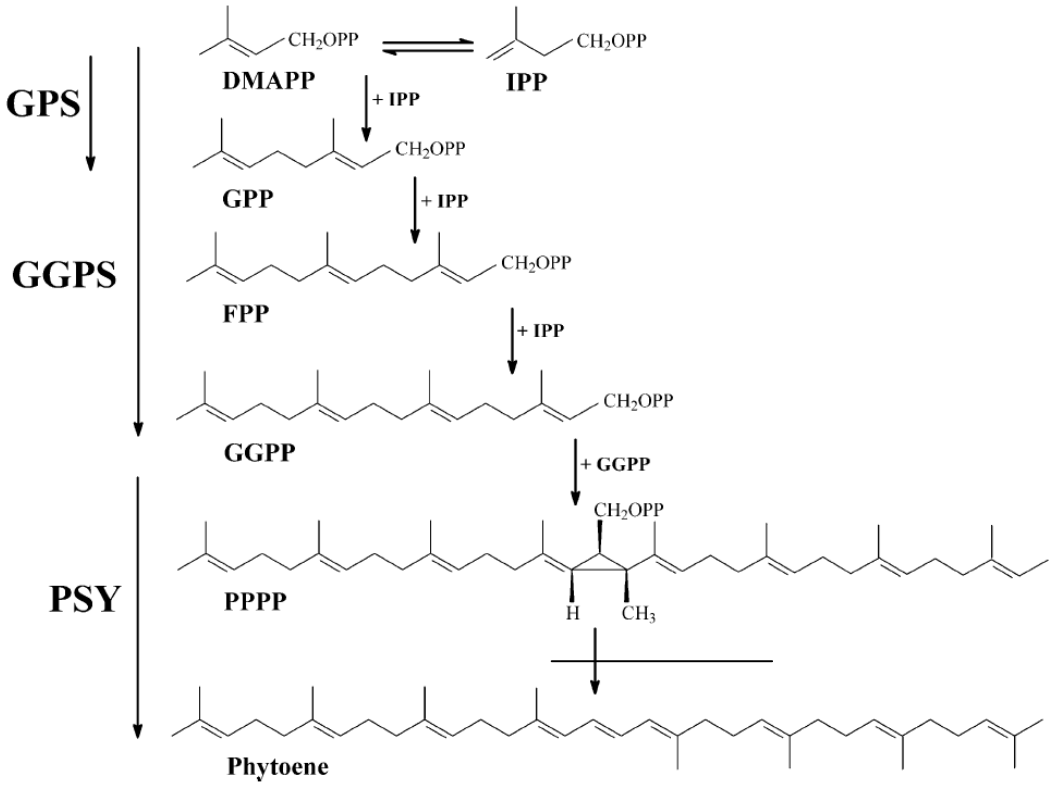


Figure-2. Phytoene synthesis from IPP and DMAPP. Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; PPPP, prephytoene diphosphate; GPS, geranyl diphosphate synthase; GGPS, geranylgeranyl diphosphate synthase and PSY, phytoene synthase (Source: Fraser and Bremley, 2004)

Cyclisation reaction

Carotenoid cyclisation is limited to the formation of a six-membered ring at one or both ends of the acyclic precursor and lycopene is the precursor for cyclisation. Formation of cyclic end groups is initiated by proton attack at the C-2 of the terminal C-1, 2, double bond which is isolated from the polyene chain. α and β-ionone rings are the two types of cyclic end groups. These end groups differ by the position of a double bond within the cyclohexane ring. The type of end group depends on the nature of the cyclase

enzyme (Beyer *et al.* 1991). Two major cyclase enzymes are lycopene-β-cyclase (LCY-b), which introduces β –rings and the lycopene-ε-cyclase (LCY-e) that introduces ε–rings (Pecker *et al.* 1996). Hydroxylation of the C-3 and C-30 positions of α-carotene and β-carotene results in the formation of lutein and zeaxanthin via α-cryptoxanthin and β-cryptoxanthin respectively. The introduction of hydroxyl moieties into β–ring carotenes is catalyzed by the enzyme hydroxylases (Paul *et al.* 2004).

Formation of xanthophylls

Xanthophylls are oxygenated carotenoids formed by series of cyclisation and hydroxylation reactions of lycopene molecule via γ carotene, β carotene, echinenone, and phoenicaoxanthin (**Figure-3**.) (Wang *et al.* 1999).

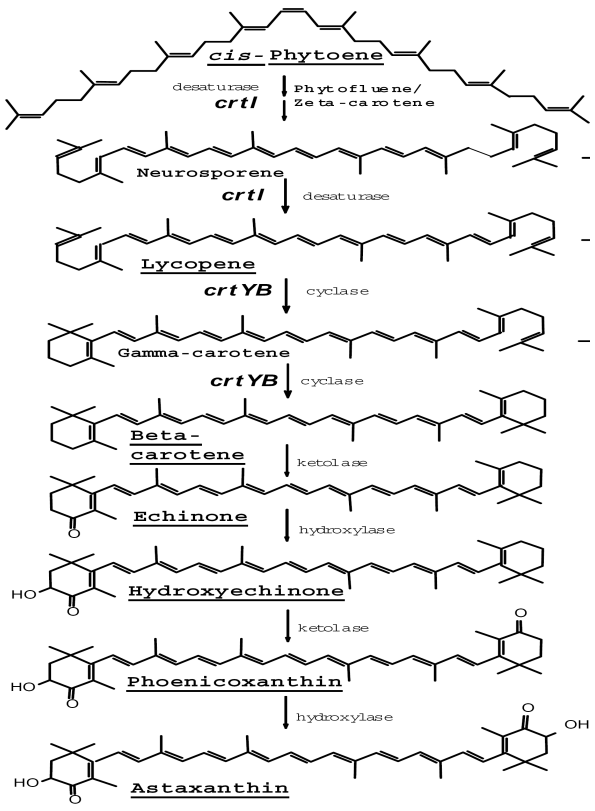


Figure:3. Dicyclic and monocyclic carotenoid-biosynthetic pathways (Source: Fraser and Bremley 2004)

MICROBIAL PRODUCTION OF CAROTENOIDS

The production of carotenoid from biological sources has been an area of intensive investigation. Because of the inherent biosynthetic capacity of a variety of different organisms, there has been a considerable effort to develop systems to produce carotenoids commercially from biological source. With the new trend of biotechnology and recombinant technology, the biosynthetic capability of organisms to produce carotenoids can be enhanced (Bohm *et al.* 1997). Carotenoids are intra-cellular compounds and cannot be excreted into medium in a fermentation process. The key productivity parameters for biological production are the cost of biomass production, the concentration of interested carotenoid accumulation inside the cell and the metabolic activity of the cells producing the carotenoids.

There are several commercial operations currently used to produce carotenoids for human as well as animal consumption. The production of β-carotene by the alga *Dunaliella sp*, is a well developed technology (Finkelstein *et al.* 1995). The production of astaxanthin from *Phaffia rhodozyma* has also been extensively studied and considerable research has been performed to increase the productivity of astaxanthin synthesis in *Phaffia rhodozyma* (Jacobson *et al.* 1995). The genes responsible for the synthesis of carotenoids such as lycopene, β-carotene and astaxanthin have been isolated from the epiphytic *Erwinia* species, marine bacteria *Agrobacterium aurantiacum* and *Alcaligenes* sp. (Misawa *et al.* 1990). The ubiquitous nature of fernesyl pyrophoshate among yeasts has been utilized in the microbial production of lycopene and β -carotene by the yeast *Saccharomyces cerevisiae* carrying the *Erwinia uredovora* carotenogenic genes (Yamano *et al.* 1994). The yeast *Candida utilis* has the potential to produce a large amount of

carotenoids by redirecting the carbon flux for the ergosterol biosynthesis into the nonendogenous pathway for carotenoid synthesis (Miura *et al.* 2004). Cells of *Phaffia rhodozyma* and *Haematococcus pluvialis* were found as suitable delivery systems of astaxanthin for aquaculture (Furubayashi 1991; Mewid *et al.* 1995). Researchers have developed varieties of *Adonis aestivalis* plant which produces astaxanthin in the petals with an increased astaxanthin content (Mawson 1995). *Monascus purpureus* is another fungus well studied for the production of polyketides pigments which are non-toxic and are used in oriental countries as food colorants (Martin *et al.* 2003). *Phycomyces blakesleanus* yet another fungus studied for the production of β-carotene (Cerda-Olmedo *et al.* 1993).

Many bacterial species were also known to produce carotenoids. A new strain of *Corynebacterium sp,* was found to produce canthaxanthin (Tsubokura *et al.* 1996). Two new bacterial species *Altermonus* and *Flexibacter* were reported for the production of zeaxanthin. *Flavobacterium multivorum* has been known for production of zeaxanthin. An improved zeaxanthin producing strain of this bacterium was developed by Gierhart (1995). *Agrobacterium aurantiacum* a marine bacterium and *Pheniophora sp*, of the aphyllophorales are also known to produce astaxanthin.

There are considerable research has been carried out to develop strains of organisms to produce a variety of carotenoids. The potential commercial interest for the production of carotenoids and the cloning of genes encoding biosynthetic enzymes has led to all kinds of examples of metabolic pathway engineering. These examples include the over expression of a gene encoding a rate-limiting enzyme (Hoshino *et al.* 1994), the expression of carotenogenic genes in noncarotenogenic heterologous hosts (Farmer *et al.*

2000), increase of the carbon flux into the carotenoid biosynthetic pathway (Albrecht *et al.* 1999) and the combination of genes and modification of catalytic activities in order to improve and/or modify carotenoid biosynthetic pathways (Mann *et al*. 2000).

BIOLOGICAL ACTIVITIES OF CAROTENOIDS

The activity of carotenoids as nutrients and drugs are related to their ability to scavenge active free radicals in living systems (Edge *et al*. 1997). One of the most widely discussed roles of the carotenoids is the interaction with free radicals that initiate harmful reactions such as lipid peroxidation (Qian *et al.* 2000). Carotenoids can react with ferric ions in metal-induced lipid peroxidation to form carotenoid radical cations (Wei *et al.* 1997). Addition of carotenoids to either plasma or isolated Low Density Lipid (LDL) fractions demonstrates the protective action against peroxidation (Panasenko *et al.* 2000).

Growth inhibition of a variety of tumor cell lines, including GOTO (neuroblastoma), PANC-1 (pancreatic cancer), HGC27 (stomach cancer) and HeLa (cervical cancer) have been reported using an emulsion of palm oil carotenoids (Nishino *et al.* 1992). Canthaxanthin at higher concentration (100 μM), inhibits the growth of three tumor cell lines and stimulates the growth of 3T3 cells (a non-tumor cell line) (Huang *et al.* 1992). This effect is due to a secreted cytokine when carotenoids are added directly to human peripheral blood mononuclear cells (Abril *et al.* 1989).

Astaxanthin and canthaxanthin, two relatively minor dietary carotenoids can protect liposomes against Cu^{+}-initiated lipid peroxidation (Rengel *et al.* 2000). Studies have demonstrated protective action of astaxanthin and canthaxanthin on plasma, isolated lymphocytes, neutrophils, LDL and microsomal fractions from various tissues (Carpenter

et al. 1997; Panasenko *et al.* 2000). There are several mechanisms by which a carotenoid can function in cancer prevention. As a provitamin-A, carotenoids have an effect on cellular differentiation and proliferation (Giovannucci *et al.* 1995). It has been reported that retinoids could increase gap junction communication between cells (Mehta *et al.* 1989, Giovannucci *et al.* 1995). The antioxidant function of carotenoids could prevent free radical-induced damage to cellular DNA and other molecules (Burton 1989), immunomodulatory effects could enhance immune surveillance in tumorigenesis (Bendich 1989) and enhanced cell–cell communication would restrict clonal expansion of initiated cells (Zhang and Bertram 1994).

CULTURAL CONDITIONS AFFECTING METABOLISM IN FUNGI

Environmental factors regulate secondary metabolism in filamentous fungi. These include extracellular pH, calcium, inoculum size, light, temperature, carbon and nitrogen sources. In *Aspergillus* species pH regulates production of some of the extracellular enzymes involved in plant cell wall degradation. The major factor responsible for the regulation of pH dependent genes is Pac-C, a DNA binding protein that regulates the expression of cellulases, endopolygalacturonases, arabinofuranosidases and endoxylanases (de Vries 2003). In secondary metabolism of *A. nidulans*, pac-C regulates penicillin biosynthesis and aflatoxin production through transcriptional activation of respective genes (Brakhage 1998; Penalva and Arst Jr 2002).

Metal ions are known as micronutrients and modulators of enzyme action, which are known to promote fungal growth and secondary metabolite production (Dutton 1988). Ca^{2+} ions are essential for growth of a few fungi for growth, asexual spore production and

fruiting body formation (Bollard and Butler 1966). Ca^{2+} deficiency reduces oospore production and maturation in *Phytophthora cactorum* and influences the pattern of hyphal branching (Elliot 1972). Ca^{2+} also controls sexual reproduction and growth in *Pythium graminicola* (Lenny and Klemmer 1966). Calcium is intimately involved in uptake phenomena and protects against injurious effects of other cations like, H^+, Na^+ and K^+ and influences the shape of the pH growth curve (Maggon *et al.* 1977).

Among the factors that determine the morphology of fungal fermentations, the amount, type (spore or vegetative) and age of the inoculum are of prime importance. In *Peniecillum chrysogenum* the influence of initial spore concentration and agitation rate on agglomeration leading to pellet formation is well studied. At low concentrations, agglomeration of hyphal elements was limited and small pellets were formed whereas at higher spore concentrations, agglomeration increased and large pellets were formed (Nielsen *et al.* 1995). The same effect of inoculum size was reported for the steroid transforming filamentous fungus *Rhizopus nigricans* by Matos *et al.* (2000).

Light regulates various metabolic and developmental processes in a wide range of organisms including filamentous fungi such as *Neurospora crassa*, *Phycomyces blakesleanus*, *Mucor circinelloides* etc (Quiles-Rosillo *et al.* 2005). *N. crassa* has photo adoption mechanism, wherein blue light regulates morphological differentiation, developmental processes and metabolism including carotenoid biosynthesis. In asexual life cycle, light induces mycelial carotenoid biosynthesis, conidial formation, change in membrane potential and input resistance and suppresses circadian rhythm of conidiation. In sexual life cycle, it induces accumulation of carotenoids in perithecial walls and phototropism of perithecial beaks (Linden *et al.* 1997).

Temperature is another major factor affecting the fungal metabolism. Nutritional and pH requirement for the growth of fungus is influenced by temperature; similarly dissolved oxygen tension is also temperature dependent and varies inversely with increasing temperature. The rate of medium evaporation is greatly affected by temperature, especially in continuous culture (King *et al.* 1972). Temperature sensitivity of the protoplasmic membrane in the presence of metabolizable carbon substrates is an important determinant of the maximum growth (Madigan *et al.* 2004). Growth at higher temperature reduces the chitin content in the cell wall of *Aspergillus nidulans* (Cohen *et al.* 1969). In submerged fermentation pellet formation was affected by temperature. In *A. awamori* the highest cell volume was obtained at 25^{o}C where, only pellets were observed. At 30^{o}C the pellets initially formed decomposed to filamentous mycelium and at 35^{o}C mainly filamentous mycelium with low amount of pellets was formed (Schu¨gerl 1998). Anderson and Smith (1971) have demonstrated that elevated temperature produced marked morphological changes in the conidia and newly formed hyphae of *A. niger*.

THE PROBLEM

The physiology of *Aspergillus carbonarius* in relation to polygalacturonase secretion has been the intensive area of research, in this laboratory, for over a decade. A mutant strain of the fungus over secreted copious quantities of the enzyme when grown in shake flasks. Studies showed that the enzyme production was induced when pH of the culture broth dropped to 3.0. Intracellular pH homeostasis in the fungus was maintained during growth at pH 3.0 by the incorporation of xanthins in the membrane. One of the xanthins characterized in this study was partially saturated astaxanthin. The details are described in the **First chapter** of the thesis.

Since the mutant *A. carbonarius* accumulated a mixture of xanthins in the membranes and the pigment had industrial applications, studies were carried out for optimizing their production. The results of the study are described in the **Second chapter** of the thesis.

Being a pigment of microbial origin, the fungus containing the xanthin was analyzed for safety. Acute and sub-acute toxicity studies were carried out in albino rats and the results are described in the **Third chapter** of the thesis.

MATERIALS AND METHODS

2.1 MICROBIOLOGICAL METHODS:

All media preparation and culturing of organism were carried out as routine microbiological methods. Culture media were sterilized at 121 ^{o}C for 20 min (15 lbs pressure) and glass wares were sterilized at 180 ^{o}C for 2 h. Inoculation of cultures was performed in laminar air hood and all other aseptic methods described for microbiological work were generally followed. All media preparations were carried out using glass distilled water. Ultra pure water obtained from Milli Q filtration unit (Millipore) was used for buffer preparations for enzyme and antioxidant assay.

2.2 CHEMICALS

All microbiological media were prepared using laboratory grade chemicals. For biochemical studies analytical or pure chemicals were used. Chemicals were obtained from Hi-Media, India; Ranbaxy Laboratories Ltd, India; SRL chemicals, India; s.d.fiNE-CHEM Ltd., India and Sigma Chemicals, St. Louis, USA.

Solvents used for analytical works like, spectrophotometric studies, high performance liquid chromatography, gas chromatography and thin layer chromatography were of analytical grade and HPLC grades, obtained from Merck India Ltd., India. Absolute ethanol (100%) was procured from Hayman Ltd, UK. 1,1-Diphenlyl – 2-picrylhydrazyl (DPPH) and Butylated hydroxy anisole (BHA) were obtained from Sigma Chemicals, USA. Thiobarbituric acid, trichloro acetic acid and dimethyl sulfoxide (DMSO) were obtained from Qualigens fine chemicals (India).

Inhibitors of carotenoid biosynthetic pathway like, lovastatin, nicotine, squalene, piperonyl butoxide and diphenylamine were obtained form Sigma Chemicals St. Louis, USA. Serum enzyme assay kits for acetate dehydrogenase, alkaline phosphatase, asparate amino transferase and alanine amino transferase were obtained form Monozyme India. LTD., India. Ergosterol, squalene, cholesterol and fatty acids standards were obtained form Sigma Chemicals St. Louis, USA.

2.3 THE ORGANISM

Aspergillus carbonarius (Wild type) isolated in this laboratory from grapes (Devi and Rao 1996; Sreekantaiah *et al*. 1975) was maintained at the culture collection of food microbiology department, Central Food Technological Research Institute (CFTRI), Mysore, India under accession number CFTRI 1047. The mutant fungus used was isolated by Venkatesh (2004) and deposited at the culture collection of Food Microbiology Department, CFTRI, under accession number UV-10046.

2.4 MAINTENANCE OF *A. CARBONARIUS*

A. carbonarius was maintained on **Y**east **E**xtract **P**eptone **D**extrose (YEPD) agar slants at 4 ^{o}C. The slants were sub-cultured every 2 months.

2.5 CULTURE MEDIA

1. Yeast extract peptone dextrose gar

	g L^{-1}
Yeast extract	10.0
Peptone	20.0
Dextrose	20.0
Agar	20.0
pH	5.5

2. Pectin agar

	g L^{-1}
Ammonium dihydrogen orthophosphate	0.9
Di-ammonium hydrogen phosphate	2.0
Magnesium sulphate heptahydrate	0.1
Potassium chloride	0.5
Citrus pectin (Sigma)	10.0
Agar- Agar	20.0
pH	5.5

2.6 SHAKE-FLASK CULTURE OF *A. CARBONARIUS*

Inoculum

The inoculum of the mutant *A. carbonarius* was developed in the medium containing the following components.

	g L^{-1}
Corn flour	40.0
Peptone	10.0
Yeast extract	6.0
pH	5.5

A loop full of spores from 3 days old YEPD slants was transferred to 10 mL medium and incubated at 30±1^{o}C in an orbital shaker for 48 h (200 rpm; Kűhner, Switzerland).

Biomass production

After 48 h growth, inoculum was transferred to the growth medium containing the following components.

	g L^{-1}
Corn flour	40.0
$(NH_4)_2$ HPO_4	5.0
NH_4 H_2PO_4	5.0
K_2HPO_4	43.20
pH	3.0 (Adjusted with citric acid)

Glucose medium for physiological experiments

	g L^{-1}
Glucose	30.0 (Sterilized separately and added)
$(NH_4)_2$ HPO_4	5.0
NH_4 H_2PO_4	5.0
K_2HPO_4	42.20
pH	3.0 (Adjusted with citric acid).

The organism was grown at 30±2 ^{0}C on rotary shaker (200 rpm) for 48 h in 500 ml baffled flasks.

2.7 XANTHIN EXTRACTION AND PURIFICATION

Shimadzu UV-Visible spectrophotometer (UV-160A) was used for all spectrophotometric analysis.

The pigmented biomass after 48 h growth was separated from culture broth by filtration using cheese cloth. The biomass was washed with distilled water to remove adhering media components and dried at 50 ^{o}C. The xanthin was extracted using absolute alcohol (30 mL/g) on a platform shaker (200 rpm) at room temperature. The extraction was repeated and the fractions were pooled. The xanthin extract was concentrated by evaporation of ethanol at 40 ^{o}C under 150 mbar pressure using rotary evaporator (Buchi Rotovapor R-205, Switzerland).

2.8 PURIFICATION OF XANTHIN USING THIN LAYER CHROMATOGRAPHY (TLC)

Concentrated xanthin was suspended in minimum quantity of ethanol (5-10 ml) and spotted over analytical TLC plates (Merck) and developed in a TLC chamber saturated with solvent system, isooctane: acetone: diethylether in the ratio of 6:2:2. Since the pigment was coloured, R_f was directly calculated after drying the plates in fume cupboard at room temperature. The yellow pigment was purified repeatedly using preparative TLC plates (Merck). The pigment from silica plates was eluted into absolute ethanol. Silica was removed by centrifugation and the pigment was concentrated by flash evaporation.

2.9 SPECTROPHOTOMETRY

After repeated purification, xanthin was dissolved in absolute ethanol and the absorption spectrum was recorded in UV-Visible spectrophotometer (Shimadzu UV 160) in a wavelength range of 200-600 nm.

2.10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Both the crude and purified xanthin were dissolved in methanol (HPLC grade) and resolved in C18 HPLC column in Shimadzu, LC 10AT model with CR-1 integrator and photo diode array, using mobile phase methanol and water in the ratio 95:5 (run type - isocratic; running time- 40 min; flow rate- 1 mL/min; volume of sample injected- 20 µl). Resolved fractions were recorded in a UV-diode array in a wave length range 400 - 500 nm.

2.11 LIQUID CHROMATOGRAPHY-MASS SPECTROSCOPY (LC-MS)

Xanthin dissolved in HPLC grade methanol was resolved within C18 column (Waters) at a flow rate of 200 µl min^{-1} in HPLC (Waters) with methanol: water in the ratio of 90:10 as solvent system. Mass spectroscopy was carried out in Waters TOF mass spectrometer.

2.12 NUCLEAR MAGNETIC RESONANCE (NMR) SPCETROSCOPY

For all the NMR analysis, the purified pigment was dissolved in heavy chloroform ($CdCl_3$) with Tetra Methyl Silane (TMS) as internal reference.

Carbon 13 NMR (^{13}C NMR)

Carbon NMR was carried out with Gemini-300 NMR spectrophotometer at 75MHz radiofrequency with TMS as internal reference. The pulse angle was 90 ^oC with relaxation delay of 5.0 s and acquisition time of 1.706 s with a total of 3692 repetitions.

Proton NMR (^1H NMR)

Proton NMR was carried out with Inova-500 NMR spectrometer at 500 MHz radio frequency with TMS as internal standard. The pulse angle was 34.6 ^oC with a relaxation time of 20.0 s and acquisition time of 1.891 s with a total of 16 repetitions.

Distortionless Enhancement of Ploarization Transfer (DEPT)

Distortionless enhancement of polarization transfer was recorded with Gimini-300 at 100MHz radio frequency with acquisition time of 3.57 ms. For the pulse angles of 45^o and 135^o, 32 repetitions and for the pulse angle of 90^o, 64 repetitions were recorded respectively.

Heteronuclear Single Quantum Coherence (HSQC)

Heteronulcar Single Quantum Coherence was carried out in Inova-500 spectrometer at 500 MHz frequency with a relaxation delay of 1.1 s, acquisition time of 0.171 s, 128 repetitions and 2 x 64 increments.

Correlation Spectroscopy (COSY)

Correlation Spectrum was performed with Inova-500 spectrometer at 500 MHz frequency with a relaxation delay of 1.1 s, acquisition time of 0.171 s, 8 repetitions and 128 increments.

Rotational Overhauser-effect Enhancement Spectroscopy (ROESY)

Rotational Overhauser-effect Enhancement spectroscopy was carried out in Inova-500 spectrometer with relaxation delay of 1.10 s, mixing time of 0.200 s, acquisition time 0.171 s, 32 repetitions and 2x 200 increments.

2.13 ESTIMATION OF PARTIALLY SATURATED ASTAXANTHIN

Partially saturated astaxanthin was estimated using UV-visible spectrophotometer (Shimadzu, UV-160). Partially saturated astaxanthin extracted from mutant *A. carbonarius* biomass using rectified sprit and purified by preparative TLC was quantified based on optical density (444 nm). Molecular mass and molar extinction coefficient (as E_M) of partially saturated astaxanthin were used for quantification of xanthin using the formula

$$\text{Partially saturated astaxanthin } \mu g\ g^{-1} \text{ dry biomass} = \frac{OD \times 590 \times V_1}{116731.7 \times 1000 \times \text{Dry biomass (g)}}$$

Where in;

OD = Optical density at 444 nm

116731.7 = Molar extinction coefficient of partially saturated astaxanthin

590 = Molecular mass of partially saturated astxanthin

V_1 = Total volume of ethanol extract.

2.14 ESTIMATION OF TOTAL XANTHINS

The total xanthins extracted from mutant *A. carbonarius* biomass using rectified sprit and purified by preparative TLC was quantified based on optical density (414 nm) (Kumarsean 2004). Molecular mass and molar extinction coefficient (as E_M) of partially saturated canthaxantin were used for quantification of xanthin using the formula

$$\text{Xanthin } \mu g\ g^{-1} \text{ dry biomass} = \frac{OD \times 590 \times V_1}{51158.7 \times 1000 \times \text{Dry biomass (g)}}$$

Where in;

OD = Optical density at 444 nm

51158.7 = Molar extinction coefficient of partially saturated canthaxanthin.

574 = Molecular mass of partially saturated canthaxanthin.

V_1 = Total volume of ethanol extract.

2.15 MEMEBRANE STEROL ANALYSIS OF MUTANT *A. CARBONARIUS*

Freeze dried mutant *A. carbonarius* pigmented and nonpigmented biomass were used for the extraction of sterols. Total lipid was extracted using chloroform and methanol (2:1, v/v) from crushed biomass as described by Folch *et al.* (1957). Chloroform : Methanol extract of the biomass was mixed with water (0.2% V/V) and NaCl (10% V/V). In a biphasic system obtained, upper phase contained all non-lipid substances and the lower phase containing essentially all the lipids which was separated using separator flask. Total lipids were further saponified with 10% KOH and extracted with diethyl ether. The ethereal phase was evaporated to obtain total sterols. Sterols analysis was performed using Gas chromatography (Hewlett-Packed Series II 5890). GC separations were carried out using BP-1 fused silica capillary column coated with 5% PMS (30 m long, 0.2 mm ID, Supelco). The conditions used for GC analysis: carrier gas He - 1ml min^{-1}; Injection temperature 250 ^{O}C; Detection temperature 300 ^{0}C; temperature programme; 3 min 105 ^{O}C, 20 ^{O}C min^{-1} 105-300 ^{O}C and followed by 15 min 300 ^{O}C.

2.16 INHIBITION OF XANTHIN BIOSYNTHESIS IN MUTANT *A. CARBONARIUS* BY CAROTENOID BIOSYNTHETIC PATHWAY SPECIFIC INHIBITORS

Carotenoid biosynthetic pathway specific inhibitors like, nicotine, diphenylamine, lovastatin, piperonylbutoxide were used to study the possible biosynthetic pathway of xanthin production by mutant *A. carbonarius.* Three days old spores of fungus were inoculated in 10 ml glucose media and grown at 30 ^{o}C for 48 h on an orbital shaker (200 rpm). The inoculum was transferred to 100 ml glucose media in 500 ml baffled flasks.

Different concentrations of inhibitors (20 μM to 100 μM) dissolved in minimum quantity of DMSO / ethanol were added to glucose medium. The flasks were incubated at 30 ^{o}C for 48 h on orbital shaker (200 rev/min). After 48 h of fermentation biomass was harvested by filtration and washed with distilled water and dried at 50 ^{o}C. Xanthin was extracted from the biomass and quantified as described in section 2.14 of Meterials and Methods.

2.17 INHIBITORY ACTIVITY OF SQUALENE ON SYNTHESIS OF XANTHIN BY MUTANT *A. CARBONARIUS*

The effect of squalene on inhibition of xanthin production from mutant *A. carbonarius* was studied by transferring 48 h old inoculum to glucose medium and incorporating the medium with different concentration of squalene (20 μM to 100 μM). The flasks were incubated at 30 ^{o}C for 48 h on a orbital shaker (200 rev/min), biomass was harvested and xanthin were estimated as described in section 2.14 of Meterials and Methods.

2.18 OPTIMIZATION OF FERMENTATION CONDITIONS FOR XANTHIN PRODUCTION BY MUTANT *A. CARBONARIUS*

RESPONSE SURFACE METHODOLOGY

Three Level Box-Behnken (Box and Behnken 1960) design was used in order to optimize the culture conditions for pigment production by mutant *A. carbonarius*.

Response Surface Methodology (RSM) design

Three level Box Behnken design was performed with the aim of optimizing the culture conditions for xanthin production from mutant *A. carbonarius* UV10046. The

dependent variables selected for this study were xanthin and biomass yield. The independent variables chosen were pH of growth medium, fermentation time and temperature. A second-order polynomial equation was regressed to study the effects of the variables on the yield of xanthin and biomass. The equation indicates the effect of variables in terms of linear, quadratic and cross-product terms.

The equation is of the general form:

$$Y = A_0 + \sum_{i=1}^{N} A_i X_i + \sum_{i=1}^{N} A_{ii} X_i X_i + \sum_{i=1}^{(N-1)} \sum_{j=i+1}^{N} A_{ij} X_i X_j$$

Where Y is yield of pigment or biomass, X_i is independent variable, A_0 is a constant term, A_i is the coefficient of the linear terms, A_{ii} is the coefficient of the quadratic terms, A_{ij} is the coefficient of the cross-product terms and N is the number of variables. The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by F-test. The significance of the regression coefficients was tested by t-test.

Shake flask cultivation

Optimization experiments were carried out in 500ml baffled flasks containing 100 ml of growth medium. Shake flasks were seeded with 10% inoculm and the cultures were maintained at required temperature (25, 30 and 35 ^{o}C) at 200 rpm for 32, 44 and 56 h, according to the experimental design. The mycelium was harvested at the end of fermentation by filtration using cheesecloth. Cells were washed with distilled water to remove the adhering salts. The biomass was dried to constant weight at 50 ^{o}C in

laboratory oven and weights of the mycelia obtained. Xanthin was extracted from the biomass and quantified as described in section 2.14 of Meterials and Methods.

Statistical analysis

The coefficients of the equation were determined by employing Microsoft Excel software version 5.0. Analysis of variance (ANOVA) for the final predictive equation was also done using Microsoft Excel software. The response surface equation was optimized for maximum yield in the range of process variables using the Microsoft Excel solver function. The response surface and contour plot analysis were made keeping one independent variable at middle level, changing other two independent variables.

2.19 CARBON SOURCE OPTIMIZATION

Effect of different carbon sources (3%) on the yield of xanthin was carried out using corn flour, corn starch, sorghum starch, glucose, wheat flour, galactose, xylose, mannose and pectin. Fermentation was carried out at 30 ^{o}C for 48 h. After fermentation biomass and culture filtrate were separated using cheesecloth. Xanthin yield was determined as described in section 2.14 of Meterials and Metods.

2.20 NITROGEN SOURCE OPTIMIZATION

Effect of different nitrogen sources like, ammonium salts, yeast extract, peptone, sodium dihydrogen phosphate, ammonium nitrate, urea, corn steep liquor and ammonium nitrate on xanthin yield from mutant *A. carbonarius* was determined by incorporating nitrogen sources in the growth medium. Fermentation was carried out for 48 h at 30 ^{o}C (200 rpm) and biomass was separated from the culture filtrate and xanthin yield was determined as described in Section 2.14 of Meterials and Metods.

2.21 OPTIMIZATION OF CARBON: NITROGEN (C/N) RATIO

Effect of carbon and nitrogen ratio on xanthin yield was carried out using defined media (carbon source - glucose and nitrogen source - ammonium salts). The growth medium was prepared with different C:N molar ratio (1:1 to 1:12) by varying nitrogen concentration and keeping carbon concentration constant. Fermentation was carried out for 48 h at 30 ^{o}C (200 rpm) and the biomass was separated from the culture filtrate. Xanthin yield was determined using spectrophotometric method. The optimum period of fermentation for the yield of xanthin was determined by incubating the mutant *A. carbonarius* in corn flour medium for a period of 72 h and harvesting the biomass. Xanthin yield was determined as described in section 2.14 of Meterials and Methods.

2.22 ANTIOXIDANT ACTIVITY OF XANTHIN EXTRACTED FROM MUTANT *A. CARBONARIUS*

The antioxidant activity of the xanthin was determined by DPPH free radical scavenging activity, lipid peroxidation inhibition assay and metal chelating activity. The xanthin was dissolved in ethanol (1mg/ml) and the assays were carried out in triplicate.

DPPH Radical scavenging activity

DPPH scavenging activity of the pigment was carried out according to the method of Moon and Terao (1998). 0.2 ml of ethanolic solution of pigment samples at different concentration (20- 100µg ml^{-1}) was mixed with 0.8 ml of Tris HCl buffer (100Mm, pH 7.4) and to this one ml DPPH (500µM in ethanol) solution was added. The mixture was shaken vigorously and incubated for 30 min at room temperature. Absorbance of the resulting solution was measured at 517 nm using UV-Visible Spectrophotometer (UV Visible 160A Shimadzu). BHA was used as positive control and 0.2 ml of ethanol

without xanthin was used as negative control. IC_{50} values of the samples were determined and percentage of antioxidant activity was calculated using the following formula.

$$\text{Antioxidant activity (\%)} = 1 - \left(\frac{A_{\text{sample (517 nm)}}}{A_{\text{control (517 nm)}}} \text{ X } 100 \right)$$

Lipid peroxidation inhibitory studies

Egg lecithin was extracted using egg yolk, homogenized in cold acetone for 10 min. Acetone was decanted and the yolk was re-dissolved in cold acetone till the yellow colour disappeared. Acetone was removed by air drying and egg lecithin was stored in vacuum sealed pack for future use.

Lipid peroxidation inhibitory activity of methanol and ethanol extract of xanthin was measured according to the method of Duh and Yen (1997). Egg lecithin (5mg ml^{-1} in phosphate buffer, pH 7.4) was sonicated in Hielscher GmbH, UP 50H ultraschallprozessor for 30 min to obtain small membrane liposome vesicles. Different concentration of pigment (30-100 μg ml^{-1}) was added to 1.0 ml of liposome mixture and control was prepared without test sample. Lipid peroxidation was induced by adding 10μl of L-Ascorbic acid (200 mM) and $FeCl_3$ (400 mM) the mixture 37 ^oC for one hour. The reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% trichloro acetic acid (TCA) and 0.375% thiobarbuteric acid (TBA). The reaction mixture was boiled for 15 min, cooled and centrifuged at 10,000 rpm for 5 min. Absorbance of the supernatant was measured at 532 nm and percentage of lipid peroxidation inhibition was determined using the formula.

$$\text{Lipid peroxidation inhibitory activity (\%)} = 1 - \left(\frac{A_{\text{sample (532 nm)}}}{A_{\text{control (532 nm)}}} X\ 100 \right)$$

Metal chelating activity

The metal chelating activity was determined by the method of Dinis *et al*. (1994). Xanthin samples at different concentrations (10-100 µg ml^{-1}) were mixed with 2 mM $FeCl_2\ 4H_2O$ and 5 mM ferrozine in a ratio of 10:1:2. The mixture was shaken vigorously for 10 min and absorbance was measured at 562 nm. The percentage of metal chelating activity was measured using the following formula.

$$\text{Metal chelating activity (\%)} = 1 - \left(\frac{A_{\text{sample (532 nm)}}}{A_{\text{control (532nm)}}} X\ 100 \right)$$

Inhibition of auto oxidation of oils

The effect of xanthin from mutant *A. carbonarius* on inhibition of oil auto oxidation was studied with three different oils (Soybean, Peanut and Palm oil). 20g of oil samples were placed in 50 ml beakers and known amount of xanthin extract (0.2% of w/w of oil) in DMSO was mixed with a magnetic stirrer. Synthetic antioxidant BHT was used as standard antioxidant. The autooxidation of samples was studied by Schall oven test as described by Economou and Oreopoulou (1991). Blank samples were prepared under the same condition without xanthin. The rate of auto oxidation of oils was estimated according to increase in peroxide value as described by Bandoniene *et al.* (2000).

2.23 ANTIBACTERIAL ACTIVITY OF XANTHIN EXTRACTED FROM MUTANT *A. CARBONARIUS*

Ten species of food borne pathogens were used to study the antibacterial activity of xanthin extracted from *A. carbonarius.* Pure bacterial cultures of *Bacillus cereus, Pseudomonas aeruginosa, Yersinia enterocolitica, Proteus vulgaris, Listeria monocytogenes, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei* were obtained from the stock culture of Food Microbiology Department, Central Food Technological Research Institute, Mysore and maintained on nutrient agar.

Disc diffusion assay

Disc diffusion assay was carried out according to the method of Murray *et al*. (1998) with some modification. A loop-full of organisms was pre-cultured in 10 ml nutrient broth for 12 h. The turbidity of the culture was adjusted to 0.5 McFarland optical densities. 0.1ml of bacterial suspension was used to seed Mueller Hinton agar plates. Sterilized Whatman No 42 filter paper discs (6mm diameter) impregnated with 10 µl of xanthin extracts (both pure xanthin and crude xanthin in DMSO) of different concentrations (10, 20, 30 µg/disc). The disks containing pure and crude xanthin were placed on the agar plates and inhibitory zones were measured after 24 h of incubation at 37 ^{O}C. Oxacillin (1µg/disk) and norfloxacin (10 µg/disk) were used as positive controls for gram positive and gram-negative bacteria respectively.

Minimum inhibitory concentration determination

Minimum inhibitiory concentration of the xanthin against food borne pathogens was determined according to the method of Vairappan (2003) with slight modification. The xanthin was dissolved in 200 µl DMSO to obtain different concentrations (25 to 500

μg/mL) and was added to bacterial culture suspension (10^4 CFU in 9.8 ml of nutrient broth). 200 μl of DMSO was used as control. Bacterial growth was monitored at 4, 8, 12, 16, 20 and 24 h and viability was determined using plate count agar.

2.24 SAFTEY EVALUATION STUDIES ON FREEZE DRIED BIOMASS OF MUTANT *A. CARBONARIUS*

Biomass harvesting and proximate analysis

The mutant *A. carbonarius* was grown in corn flour medium and biomass was harvested after 48h of fermentation using cheesecloth. The biomass was washed with distilled water and freeze-dried. Proximate analysis of fungal biomass carried out using following methods. Moisture content was measured by gravimetric method (AOAC 925.09, 1990). Crude protein was estimated by Micro-Kjeldahl method (AOAC 955.04, 1990). Lipid content was determined by the soxhlet extraction method (AOAC 945.39,1990). Ash content was estimated according to the AOAC method (AOAC 924.05, 1990). The total carbohydrate (%) was calculated from the above values.

Animals and maintenance

Adult male rats (CFT – Wistar strain- 8 week old, 230-250 g) were employed for the acute toxicity study and weanling male and female rats (CFT – Wistar strain, 35-40 g) were employed for the sub-acute toxicity studies. The animals were obtained from the stock colony of the animal house facility, Central Food Technological Research Institute, Mysore, India, and were caged in individual stainless steel cage with screen bottom. The rats were housed in a room maintained at 25± 2 ^{o}C with a relative humidity of 60-70%.

Rats were fed with commercial diet (M/S Gold Mohar, Lipton India Ltd.), for acute toxicity studies freeze dried *A. carbonarius* biomass was incorporated into the diet

based on body weight and for sub-acute toxicity studies the biomass was fed with different dietary levels (0.5-2.0 %).

Acute Toxicity study

Freeze dried fungal biomass of *A. carbonarius* was fed through diet on day one, at different concentrations like, 0.0, 0.5, 1.0, 2.5 and 5.0 g/kg body weight. Prior to dosing, rats were fasted overnight. Rats were observed thoroughly for onset of any immediate toxic signs and also delayed toxic effects during the observation period of one week. Fecal matter was collected at regular intervals of 24, 48, and 72 h for viability studies. All the animals were scarified after seven days and selected vital organs were excised, blotted, weighed and processed for routine histological examination. Blood for hematological studies was collected by heart puncture.

Sub acute toxicity study

Weanling rats of both sexes were randomly assigned to control and test groups. The rats were fed diets containing 0.0, 0.25, 0.50, 1.0 and 2.0% freeze dried fungal biomass to assess the cumulative effect of low doses of xanthin containing fungal biomass. Diet and water were given *ad libitum* for a continuous period of 14 weeks. Daily feed intake and weekly body weight gain of rats were monitored. The animals were observed thoroughly for the onset of any sign of toxicity. At the end of experimental period (14 weeks) all the animals were sacrificed by light ether anesthesia.

Organ weight and histopathological studies

The following vital organs of each rat like, liver, lungs, kidney, spleen, brain, adrenals, gonads, and heart were excised, blotted and weighed and the organ/body weight ratio were calculated. Organ samples fixed in Bouin's fixative were embedded in

paraffin wax to obtain 5 µm section. After staining with haematoxylin and eosin a detailed microscopic examination was carried out.

Hematological Study

Blood samples were collected from 5 rats of each group and were examined for hemoglobin concentration, packed cell volume, erythrocytes, leucocytes and differential counts. Mean corpuscular hemoglobin (MCH) was calculated from hemoglobin and erythrocytes counts. Mean corpuscular hemoglobin concentration (MCHC) was determined from hemoglobin concentration and packed cell volume (Bharoucha 1976).

Serum enzymes assay

The whole blood taken from each group was allowed to clot and the serum separated and analyzed for, acetate dehydrogenase (Zimmerman 1956), alkaline phosphatase, asparate amino transferase (ASAT) and alanine amino transferase (ALAT) (Bergmeyer 1976) using commercially available enzyme kits (Monzyme, India).

Viability test

Viability of Freeze dried fungal biomass and presence of live fungal spores in fecal matter were determined by standard plate count method. One gram of fecal pellet collected under aseptic condition was suspended in 100 ml of saline. Serial dilution of suspended samples were carried out and plated on pectin agar. The plates were incubated at 28 ^{o}C for 72 h. The number of colonies formed were counted and expressed as colonies forming units per gram (CFU/g).

MICROBIAL PIGMENTS AS METABOLITES

Metabolites produced by microorganisms are generally classified as primary or secondary. Primary metabolites secreted during early active growth phase are important for survival unlike secondary metabolites that originate during late log or stationary phase of growth (Adams and Yu 1998). Secondary metabolites of filamentous fungi have wide application as drugs and as feed. Secondary metabolites like statins, naphthoquinones and carotenoids characterized from filamentous fungi have pharmaceutical applications and possess antimicrobial, antioxidant and anticancer activities (Azuine *et al.* 1992; Huang *et al.* 1992; Bennedsen 1999; Dhale Mohan *et al.* 2006; Kumaresan *et al.* 2008). A few such as aflatoxins, ochrotoxin and zearalinone are harmful (Abarca *et al.* 2004). The carotenoid pigments produced by fungi have important applications in the nutraceutical, cosmetics, food and feed industries (Martin *et al.* 2003).

Fungal secondary metabolites are classified into three broad categories. Those that activate sporulation like linoleic-acid derived *psi* factor, zearalenone and butyrolactone-I required for melanins and spore pigment synthesis and finally the toxic metabolites secreted by growing colonies at the approximate time of sporulation. Melanin pigments, 1,8-dihydroxynaphthalene (DHN melanin), L-3,4-dihydroxyphenylalanine (DOPA-melanin) produced in fungi have been implicated to pathogenesis (Hamilton and Gomez 2002). Some of these induce morphological differentiation during asexual and sexual sporulation. It appears the phenomenon is common across species and genera (Linden *et al.* 1997; Adams and Yu 1998).

Numerous observations have supported the hypothesis that microbial secondary metabolite production and sporulation are intimately associated (Bu'Lock 1961; Paul *et*

al. 1995; McIntyre *et al.* 2002; Hamilton and Gomez 2002). Media with nutrient limitations increase sporulation in submerged cultures wherein, sporulation is induced by stress factors like availability of carbon, nitrogen and pH (Adams *et al.* 1998). The nature and concentration of a carbon source can also regulate secondary metabolism through catabolic repression. In *Aspergillus terreus,* lovastatin biosynthesis is affected by carbon-nitrogen sources, strain used, composition and constituents of the culture media (Manzoni 1999). Glucose, fructose, sucrose, and sorbitol were shown to induce high growth, sporulation and aflatoxin production in *Aspergillus flavus*. Similarly, ammonium based media increased and nitrate decreased aflatoxin production. Aflatoxin production in *A. flavus* suggested oxidative stress as a prerequisite for toxin synthesis as toxigenic strains of *Aspergillus* exhibit higher enzymatic activities related to free radical and lipid peroxidation metabolism (Wallace *et al.* 1996).

In *Aspergillus*, the processes of asexual and sexual sporulation usually culminate in the production of yellow, green, red or black pigmented conidia (Rapper and Fennell 1965). Characterization of pigments in *A. nidulans* that produce asexual green spores and sexually generated red spores revealed that they were structurally related to anthroquinone. Most of the fungal pigments are the dark brown melanin pigments formed by oxidative polymerization of phenolic compounds associated with sporulation structures and deposited in the cell wall. Melanin biosynthesis in pathogenic fungi is well studied. The pigment serves to protect spores against UV damage and also act as an important virulence factor. The melanin pigment produced by *A. nidulans* has been shown as a potential antioxidant since it quenched free radical generated by HOCl and H_2O_2 (Babitskaya *et al.* 1999).

Biosynthesis of secondary metabolites begins with acetyl-CoA, the precursor for different pathways. Isoprenoid pathway is characterized by isomerization and rearrangement of acetyl-CoA that result in the production of isoprene units. In polyketide biosynthesis, monomers condensed to form polymers such as melanin, mycotoxins and macrolide antibiotics or terpenoids like carotenoids and phytohormones (Liu and Reynolds 2001).

Carotenoids are amongst the most widely distributed pigments in biological systems. Carotenoid pigments are produced by all green plants and by a wide range of microorganisms. Eubacteria, yeasts and fungi are of special interest in carotenoid research because they grow easily and can be manipulated in the laboratory. The carotenoids, notably astaxanthin, have commercial value for the aquaculture of salmonid fish. They also possess nutraceutical properties (Moller *et al.* 2000). To date, over 750 carotenoids have been identified in nature but only 24 of them have been detected in human tissues. The common chemical feature of carotenoids is the polyene chain, a long conjugated double bond system forming the backbone of the molecule. This chain may be terminated by cyclic end groups that contain oxygen bearing substitutes. The electron rich conjugated system of the polyene is responsible for the antioxidant activities of the carotenoids, since they can quench singlet oxygen and scavenge free radicals to terminate chain reactions (Lauver *et al.* 2005). Biological benefits of certain carotenoids are due to their specific physicochemical interactions with the membranes (Britton 1995).

Carotenoids play an important role in the protection of cell functions and much of their role is against photo-oxidative and free radical induced damages. Carotenoids absorb excess light energy in order to dissipate it, likely as heat (Demming and Adams

1996). In *Neurospora crassa,* blue light regulates reproduction and induces carotenoid biosynthesis. During asexual life cycle, light induced mycelial carotenoid biosynthesis occurred in *Neurospora crassa* which was accumulated in the perithecial walls (Linden *et al.* 1997). Environmental and nutritional factors also regulate carotenogenesis. In *Xanthophyllomyces dendrorhous*, acidic pH induced and glucose repressed carotenogenesis (Garbayo *et al.* 2003). *Mucorales* (*Mucor* and *Phycomyces*) generally accumulated carotenoids as a positive response to light while biosynthesis and accumulation of β-carotene in *Blakeslea trispora* is inhibited by continuous light exposure and appeared to be regulated by sexual interaction with opposite mating type (Quiles- Rosillo *et al* 2005).

In this laboratory (Venkatesh 2004; Kumaresan 2007), a mutant *A. carbonarius* isolated for polygalactruronase over production in shake flasks was shown to secrete copious quantities of the enzyme when the culture medium was acidic. Growth in such an extreme environment caused the mutant to produce and incorporate xanthin in the cell membrane to maintain intracellular pH homeostasis by preventing H^+ ion entry. The pigment, identified as partially saturated canthaxanthin by mass spectrometry and nuclear magnetic resonance spectroscopy, induced apoptosis of retinoic acid receptor - β expressing prostate cancer cell line (Kumaresan *et al.* 2008). The pharmaceutical importance of the pigment suggested studies on the fungal physiology in relation to the pigment production.

The natural product chemistry of *Aspergillus* metabolites has been extensively studied. A new chromone derivative from *A. versicolor,* isolated from the sponge *Xestospongia exigua*, has been characterized (Lin *et al.* 2003), so also the drimane

lactone, an unusual nitrobenzoyloxy substituent of *A. versicolor,* isolated form marine green alga *Penicillus capitatus* (Belofsky *et al.* 1998). Babitskaya *et al.* (1999) reported production of pigments erytroglaucin (red), auroglaucin (orange) and flavoglaucin (yellow) by *Aspergillus glaucus*. Other pigmented members of *Aspergillus* and *Penicillium* secrete the naphthoquinone mycotoxins (Hawksworth *et al.* 1999). A pigment of commercial importance, anthraquinone, was reported from *Aspergillus* and *Curvularia* strains (Gill 1999). Other pigments of *Aspergillus* are xanthomegnin (yellow) and viomellein (yellow) of *Aspergillus tawarii* and *Aspergillus sydowii*, derivatives of fusarubin (red) from *Aspergillus sydowii* (Nelson *et al.* 2002) and derivatives of melanin associated with developmental structures of *Aspergillus* species. True mealnins of dihydronaphthalene group were reportedly synthesized by micromycetes *Paecilomyces variotii* and *Aspergillus carbonarius* (Babitskaia *et al.* 1999). A β-carotene group of six carotenoids synthesized by *A. giganteus* was reported by Van *et al.* (2004). Since *A. carbonarius* mutant produced a mixture of pigments and one of these was characterized as partially saturated canthaxanthin (Kumaresan 2007), a study characterizing another pigment is described in this chapter.

3.1 PIGMENTS OF MUTANT *ASPERGILLUS CARBONARIUS*

Preliminary experiments were carried out for separation of pigments extracted, from *A. carbonarius* biomass in rectified spirit. Thin layer chromatography using silica in a solvent system made of isooctane: acetone: diethylether (6:2:2) was used to separate the pigments.

Silica gel thin layer chromatography showed the presence of another yellow pigment, apart from partially saturated canthaxanthin in alcohol extract of *A. carbonarius* biomass **(Figure-4)**.

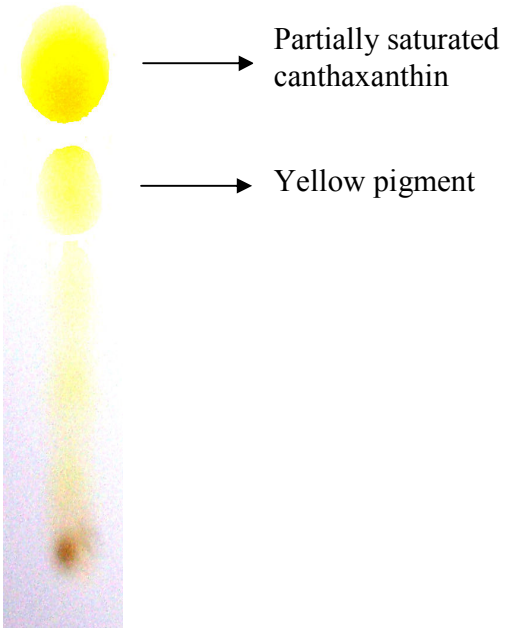


Figure: 4. Silica gel thin layer chromatography of crude pigment extracted from *A. carbonarius* biomass in rectified spirit

3.2 EXTRACTION OF THE YELLOW PIGMENT FROM MUTANT *A. CARBONARIUS* BIOMASS

A wide range of natural pigments like, carotenoids, anthocyanins, flavonoids and anthroquinone are used as color additives and different methods have been described to analyze these for use in foods. Among these, thin layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) with a visible light absorbance detector or a photodiode array detector and HPLC–mass spectrometry (LC–MS) are the most powerful analytical separation methods. In the recent years, capillary electrophoresis (CE) for analysis of food colorants has also been described (Toshiro and Shigeru 2000).

In the separation procedures, appropriate solvent system is an important criterion for properly resolving the compound of interest. In a HPLC method, developed by Orosa *et al.* (2005), chlorophylls a & b, carotenes and xanthophylls were resolved in a short-period of time from the total pigments of *Haematococcus pluvialis* extracted using dimethyl sulfoxide (DMSO). Different solvents such as DMSO, acetone, ethanol etc., have been used to extract carotenoids from yeasts, like *Rhodotorula glutinis, Dioszegia takashimae*, *Sporobolomyces coprosmae, Sporobolomyces roseus* and *Xanthophyllomyces dendrorhous* (Roland *et al.* 2007). Namthip *et al.* (1997) used acetone for extraction and analysis of astaxanthin from *Phaffia rhodozyma*.

The bright yellow pigment **(Figure-5)** from the pigmented biomass of *A. carbonarius* that was separated by TLC (R_f - 0.359) in a solvent system made of isooctane: acetone: diethylether (6:2:2) showed an absorption maxima at 444 nm. The concentrated pigment in the solvent appeared reddish orange in colour **(Figure-6)** and

spectrum of pure pigment showed double shouldered peak characteristic of carotenoid nature **(Figure- 7)**.

High Performance Liquid Chromatography (HPLC) was performed for purity check. For HPLC, the pigment was separated and detected using 30mm x 5mm C-18 (supelco) column of 5 μm particle sizes and UV-Visible detector respectively. The pigment was eluted after retention time of 3.213 min **(Figure-8)**. It was further purified by TLC for characterization by mass spcetrophotometry and nuclear magnetic resonance spcetrophotometry.

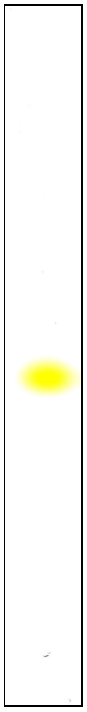


Figure: 5. Thin layer chromatography of pure pigment

Figure: 6. Rectified spirit extract of pigment from 48 h old *A. carbonarius* mutant biomass

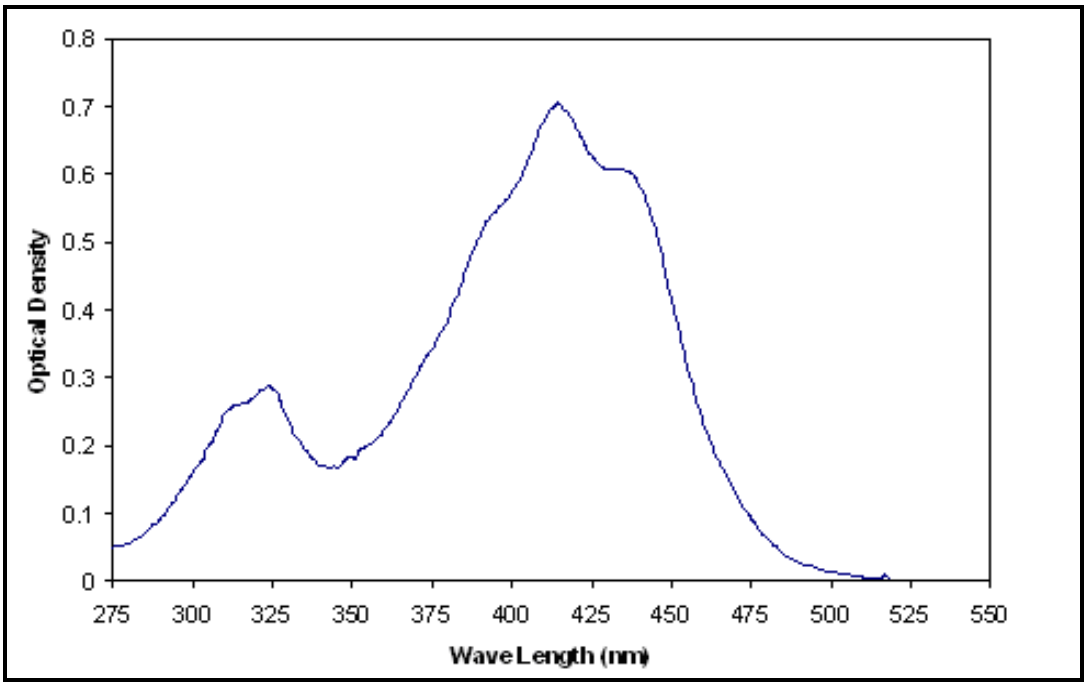


Figure: 7. UV-Visible spectrum of pure pigment

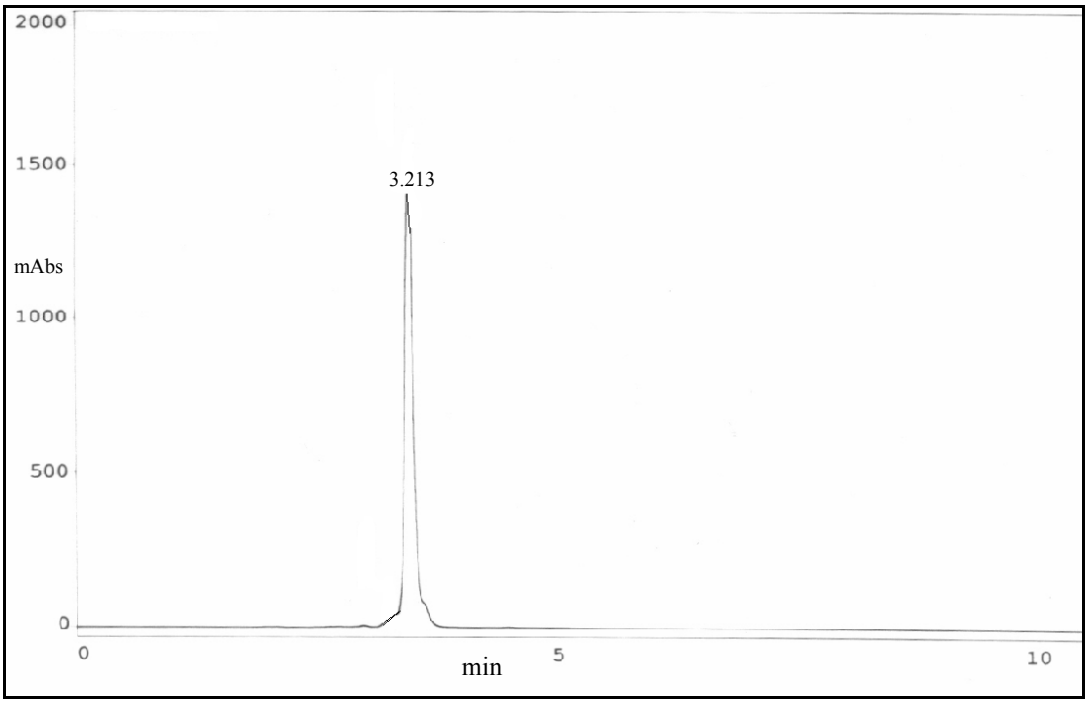


Figure: 8. HPLC profile of pure pigment

3.3. MOLECULAR MASS DETERMINATION

Spectroscopic determination of the mass of a compound relies on bombarding molecules with high energy electrons and converting them to molecular ions. The ions accelerated in an electric field are separated based on their mass to charge ratio (*m/e*) by a spectrometer for estimation of molecular mass of the compound. Liquid Chromatography Mass Spectrometer (LCMS) resolves individual compounds in a mixture and determines the mass of each molecule.

For an insight on the structural details of the yellow pigment purified, mass was estimated by LCMS.

The pigment extracted in rectified sprit and purified by repeated TLC was used for LCMS (Materials and Methods, Secton-2.8).

Though it appeared pure, LCMS resolved a number of fractions **(Figure-9)**. The molecular mass of the major compound was 590. A major fragment spectrum at 309 and 311 corresponded to the hydrocarbon chain linking cyclohexane moieties. In addition, loss of $-H_2O$, $-CH_2$, $-CH_3$ moieties were identified by peaks with molecular weight difference of 18, 14 and 15 respectively.

Mass spectrum data supported the identification of major compound as partially saturated astaxanthin in the mixture.

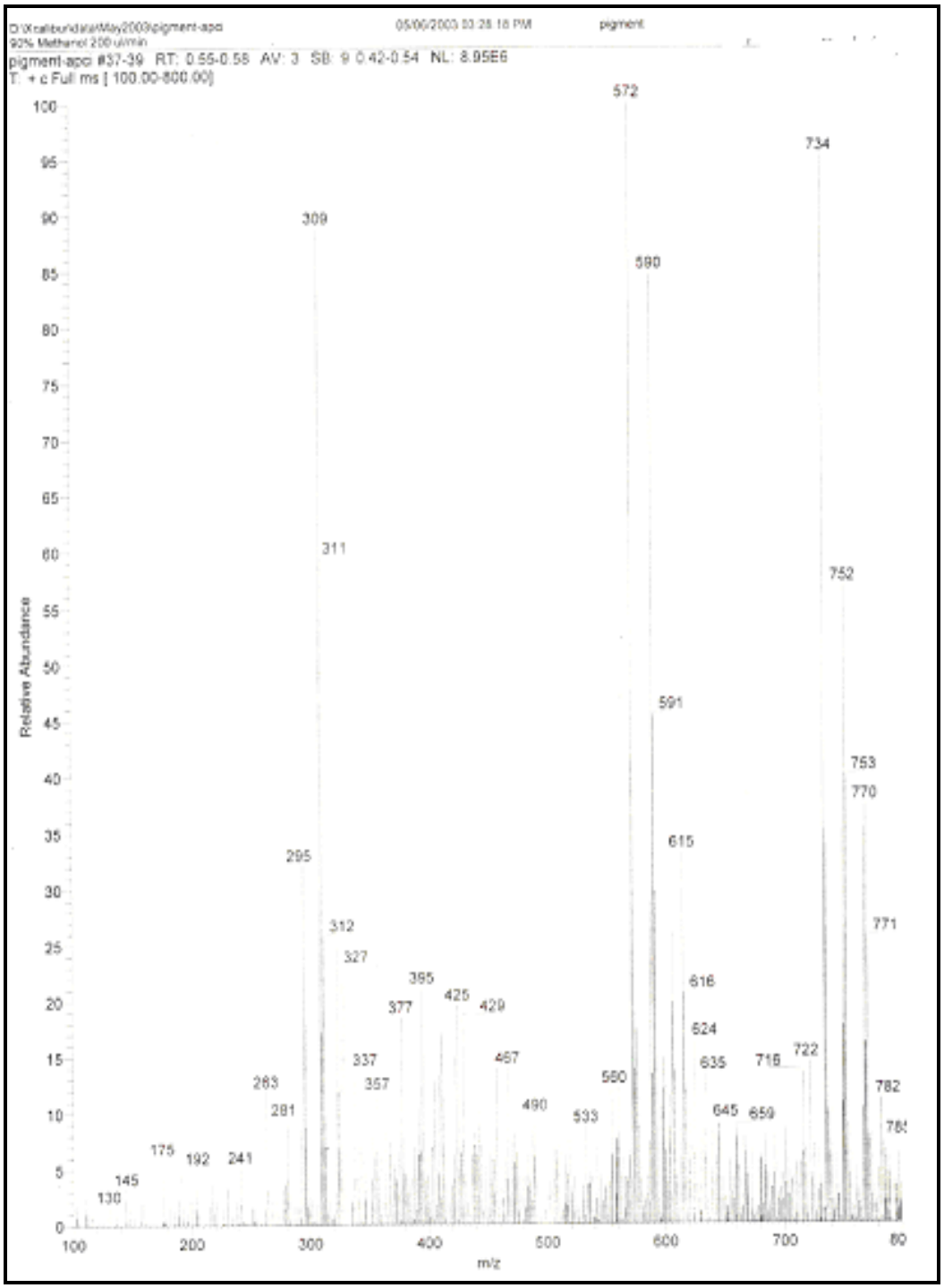


Figure: 9. Mass spectrum of purified pigment

3.4 FURTHER CHARACTERIZATION OF PARTIALLY SATURATED ASTAXANTHIN BY NUCLER MAGNETIC RESONANCE (NMR) SPECTROSCOPY

^{1}H and ^{13}C NMR spectra were recorded for the purified pigment used for LCMS.

^{1}H NMR spectrum was assigned to the hydrogen atoms in the molecules. The assignment strategy involved simultaneous analysis of 1D – ^{1}H NMR **(Figure-10)** and ^{13}C-NMR spectrum **(Figure-11)** of the compound along with the 2D – correlated spectrum (COSY) **(Figure-12)**. Peaks of the 1D spectrum were assigned with the aid of COSY spectrum and verified to match the integrated intensities of 1D spectrum. The assignment of ^{1}H signals is shown in **Table-1**. Similarly, the data from ^{13}C-^{1}H HSQC (Heteronulear Single Quantum Coherence) **(Figure-13)** and ^{13}C–DEPT (Distortionless Enhancement by Polarization Transfer) **(Figure-14)** spectra were analyzed simultaneously for assigning the individual carbons in the molecule **(Table-1)**.

NMR spectra indicated that the compound contained aliphatic chains evidenced by the number of ^{13}C peaks in the range 34.6-14.2 ppm and corresponding proton peaks in the range of 0.95 – 2.04 ppm. The most downfield ^{13}C peak at 180.4 ppm without any proton cross peaks, identified as a quaternary carbon in DEPT spectrum, indicated the presence of ketone group. The number of ^{13}C peaks between 128.4 – 131.0 ppm and corresponding proton cross peak in the range 5.98 – 6.5 ppm showed the presence of olefinic group, probably in conjugation. Similarly, a bunch of ^{13}C peaks between 34.6 – 14.2 ppm range and the corresponding proton peaks in the 0.95 – 2.04 ppm range indicated the presence of saturated hydrocarbons. The ^{13}C peak at 180.2 ppm for carbonyl group C1 and C1′ suggested a symmetrical structure for the compound. The ^{13}C at 32.5 and the corresponding proton peak in the 2.39 ppm (triplet, 2H) indicated the

presence of C2 and C2′ (-CH2-). Similarly, C3 and C3′ were confirmed by ^{13}C peak at 35.0 ppm. The quaternary carbons C4 and C4′ were confirmed by the ^{13}C peaks at 33.6 ppm. Likewise, other two sets of quaternary carbon C5, C5′ and C6, C6′ connected through double bonds were confirmed by ^{13}C peaks at 128.1 and 128.6 ppm respectively. The olefinic groups C7, C8 and C10 and corresponding C7′, C8′ and C10′ were identified with the ^{13}C peaks at 131.0, 128.4 and 130.4 ppm and the corresponding protons at 6.5 (doublet, H), 6.3 (doublet, H), 5.98 (multiple, H) respectively. Saturated carbon chains were confirmed by ^{13}C peaks at 26.0 ppm (^{1}H at 2.04) for C11 and C11′, 34.6 (^{1}H at 1.32 ppm) for C12 and C12′, 30.2 ppm (^{1}H at 1.36 ppm) for C13 and C13′, 32.8 PPM (^{1}H at 1.10 ppm) for C15 and C15′ and all the proton peaks are multiplets, which further supports the saturated carbon chain. Methyl groups of C16, C17, C18 and C19 (C16′ or C17′ C18′ and C19′) showed cross peaks at 27.8, 18.0 and 14.2 and the corresponding proton peaks at 1.32, 2.00 and 1.64 ppm, which are singlet indicated that they have been attached to quaternary carbons. Similarly, the –CH3 at C20 and C20′ were confirmed by ^{13}C peaks at 23.0 (^{1}H at 0.95 ppm). The assignment thus obtained from the carbon and hydrogen atoms in the molecules are shown in **Table-1** and the representative structure identified is provided in **Figure-16**.

Table: 1. ^{1}H and ^{13}C assignment of partially saturated astaxanthin

Assignment	^{1}H δ (ppm)	Assignment	^{13}C δ (ppm)
-	-	C1, C1′	180.2
H2,H2′	2.5 (2H,t)	C2, C2′	32.5
H3,H3′	2.39	C3, C3′	35.0
-	-	C4, C4′	33.6
-	-	C5, C5′	128.1
H7,H7′	6.5(H, d)	C7, C7′	-
H8, H8′	6.3 (H, d)	C8, C8′	131.0
-	-	C9, C9′	128.4
H10, H10′	5.98 (H)	C10, C10′	130.4
H11, H11′	2.04 (2H)	C11, C11′	26.0
H12, H12′	1.32 (2H, m)	C12, C12′	34.6
H13, H13′	1.36 (H, m)	C13, C13′	30.2
H14, H14′	1.10 (2H, m)	C14, C14′	32.8
H15, H15′	1.10 (2H, m)	C15, C15′	29.2
H16, H16′	1.32 (3H)	C16, C16′	27.8
H17, H17′	1.32 (3H)	C17, C17′	27.8
H18, H18′	2.00 (3H)	C18, C18′	18.0
H19, H19′	1.64 (3H)	C19, C19′	14.2
H20, H20′	0.95 (3H, d)	C20, C20′	23.0

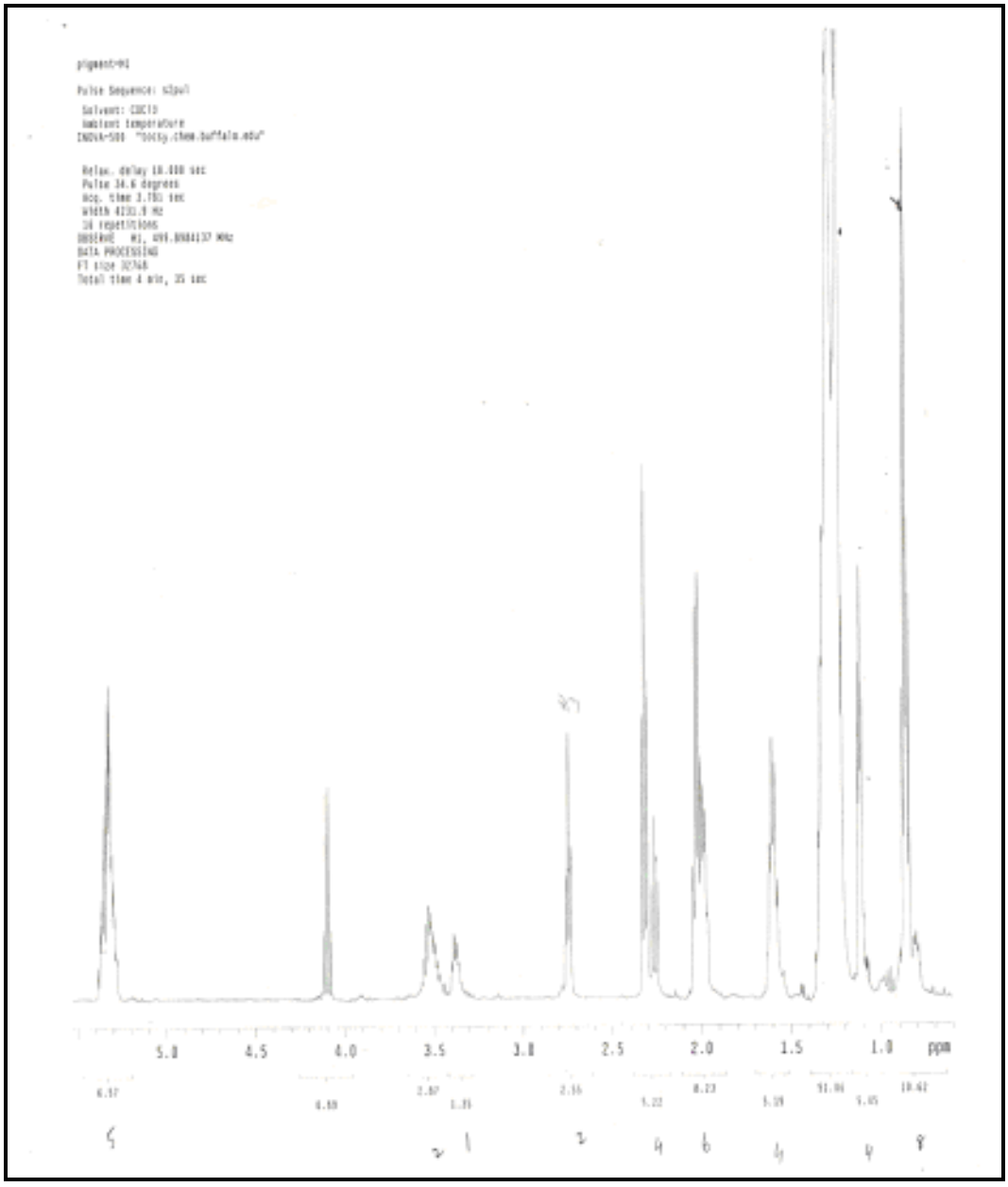


Figure: 10. ^{1}H NMR of purified pigment

A ROESY spectrum was also collected for the pigment. The ROESY peaks showed only the interactions expected from nearest neighbors as identified through COSY assignments. This indicated that the molecule does not adopt any preferred conformation in solution. The confirmation is probably a distribution of allowed conformation in solution. Also, due to overlap in the aliphatic region of the ^{1}H spectrum, it was not possible to identify of diagnostic coupling constants to fix the geometry and the bonds in the hydrocarbon chain connecting the two ends of the molecule.

With evidence gathered from the NMR spectra (^{1}H, ^{13}C, 2D-COSY, 2D-HNQC and ^{13}C-DEPT) and mass spectrophotmetric data we postulate the major compound present in the pigment as two isomers of partially saturated astaxanthin. The simulated NMR spectra agreed reasonably with the mass spectra. The structure of the partially saturated astaxanthin is shown in **Figure-16**.

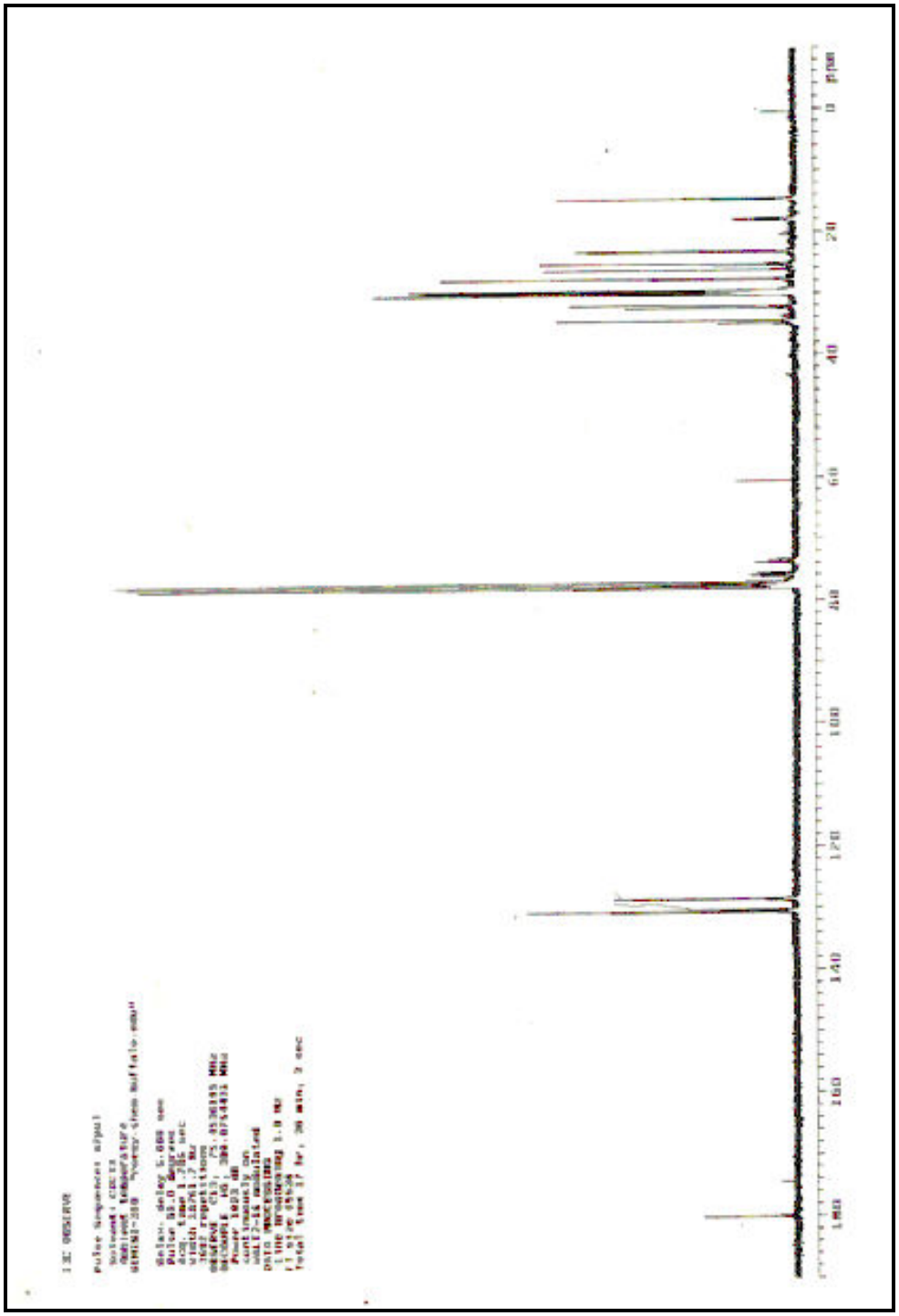


Figure. 11. ^{13}C NMR Spectrum of purified partially saturated astaxanthin

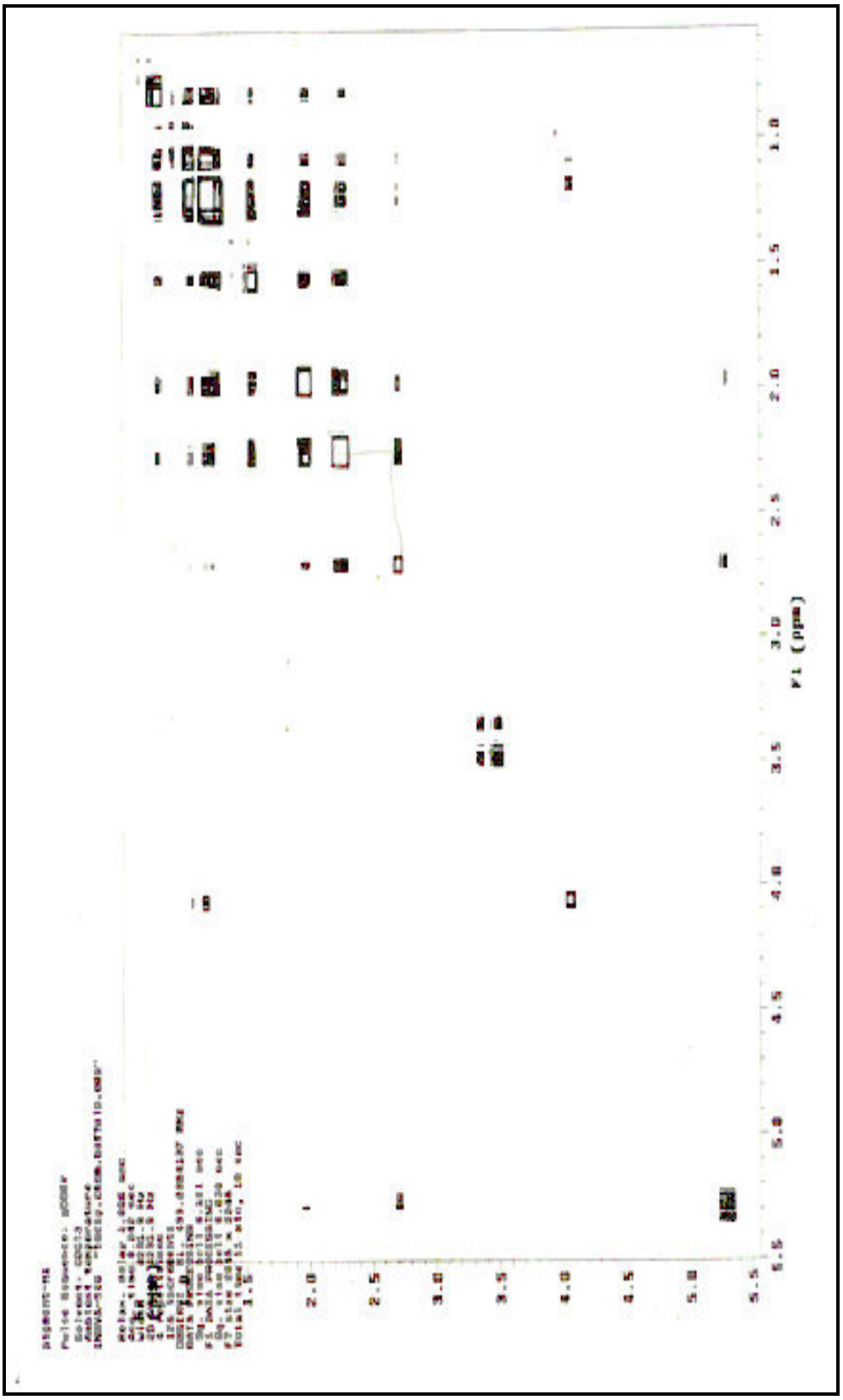


Figure: 12. Correlation spectroscopy of purified partially saturated astaxanthin

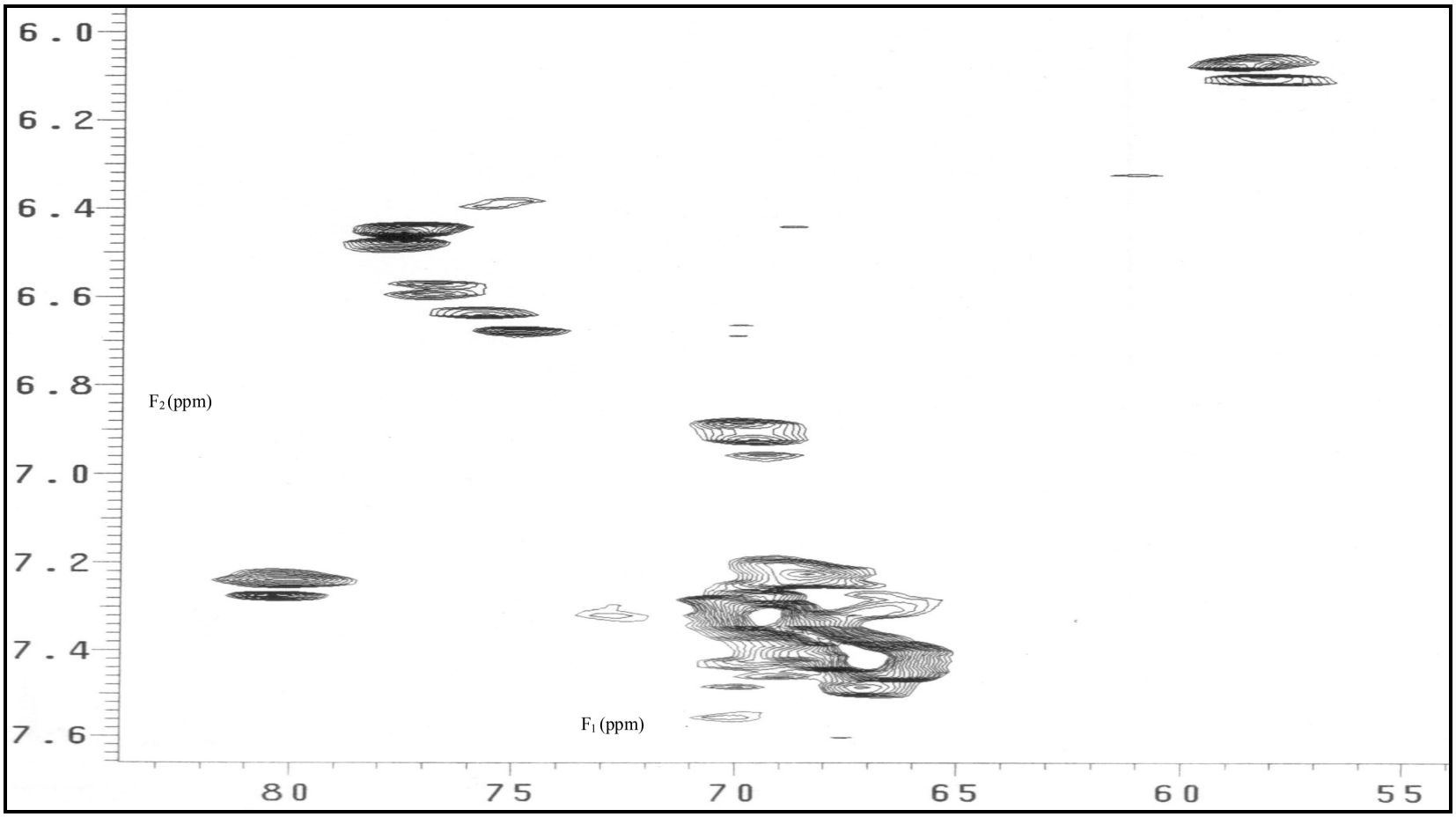


Figure: 13. Gradient HSQC spectrum of purified partially saturated astaxanthin

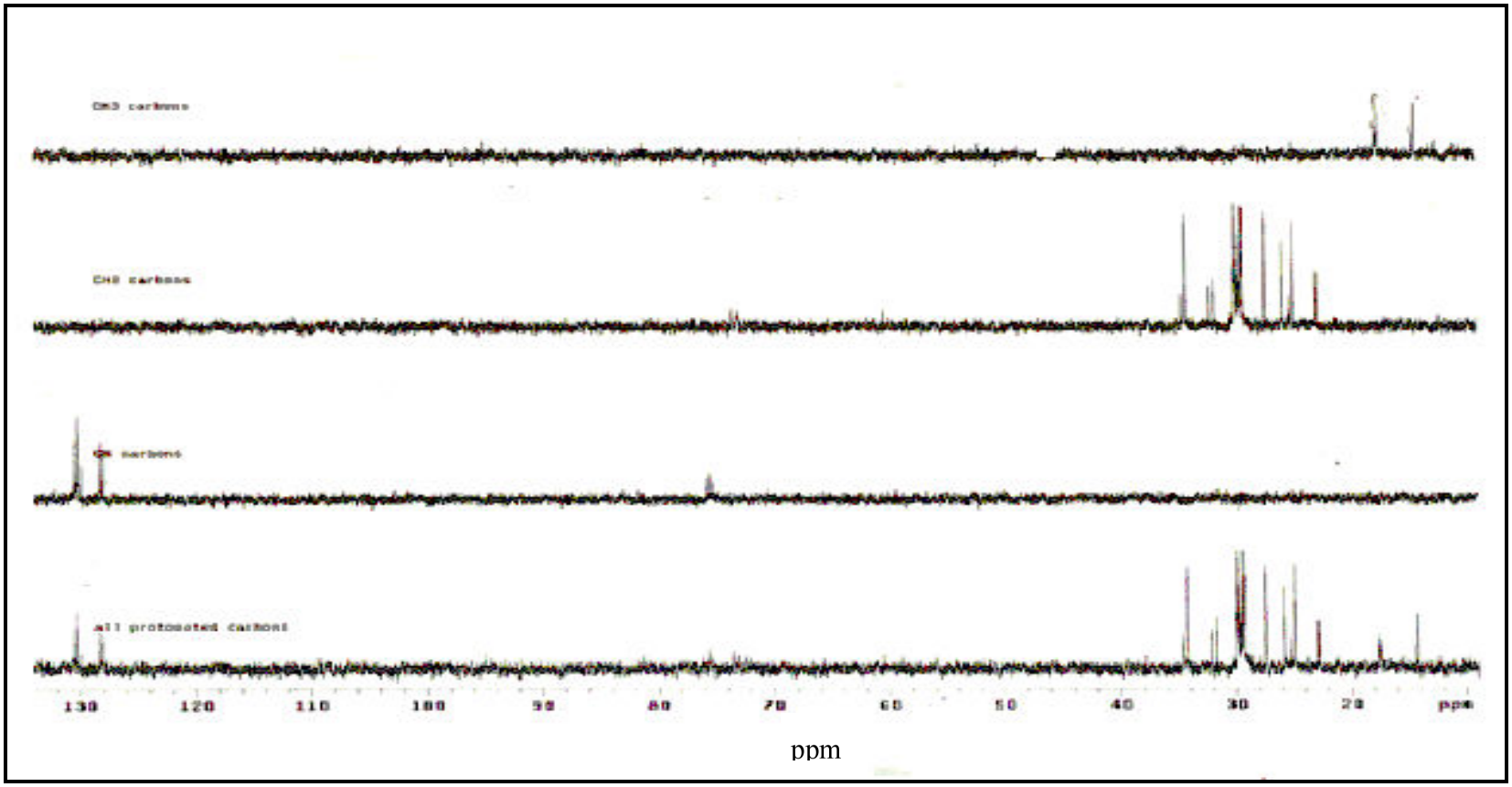


Figure: 14. DEPT Spectrum of Partially saturated astaxanthin

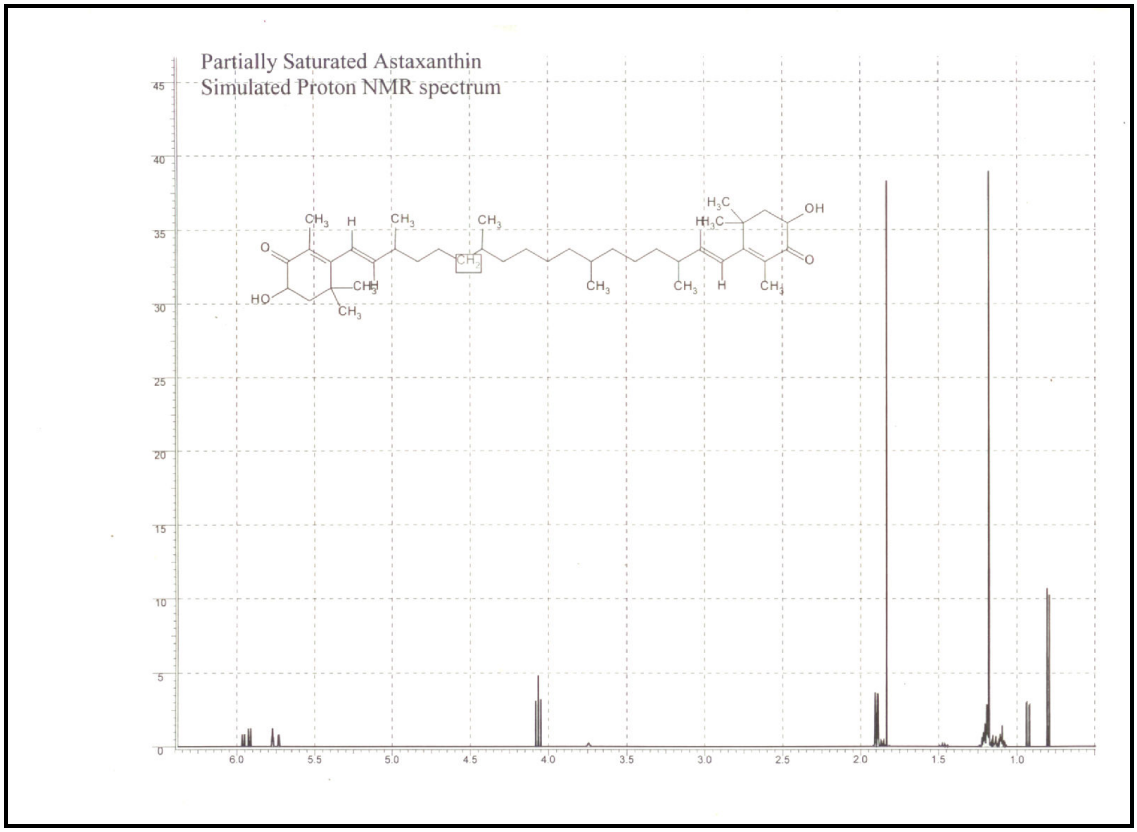


Figure: 15. Simulated proton NMR of partially saturated astaxanthin

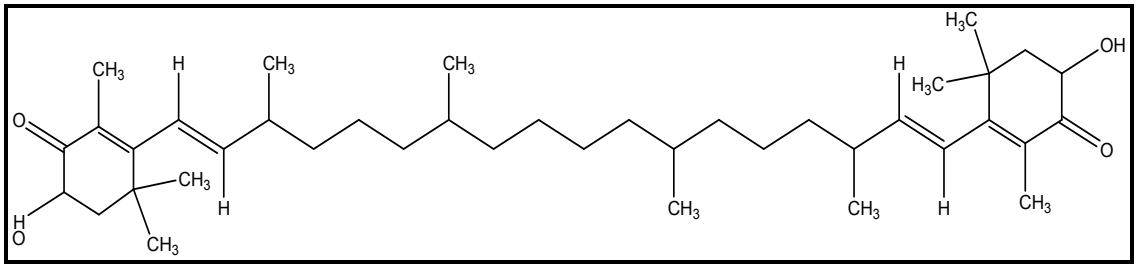


Figure: 16. Structure of partially saturated astaxanthin

3.5 MEMBRANE STEROLS OF MUTANT *A. CARBONARIUS*

Carotenoids like zeaxanthin, astaxanthin and canthaxanthin incorporate into cell membranes and change fluidity and integrity in order to strongly decrease water and small molecules permeability (Tamara *et al.* 1997). Free ion diffusion is also decreased due to change in structural and mechanical properties of membrane brought about by the polar end groups and hydrophobic middle chains of carotenoids (Gabrielska and Gruszecki 1996). Some microorganisms especially *Aspergilli*, reduce the pH of culture media to extreme acidity during growth. The internal pH homeostasis for growth sustenance under such an extreme condition is maintained either through the cytoplasmic-membrane P-ATPases which expel free protons (Serrano 1988; Hesse *et al.* 2002) or by incorporating sterols, squalene, polyisoprenes (dolichol, ubiquitin), saturated fatty acids and unusual lipids into the lipid bilayers (Lazrak *et al.* 1988; Albers *et al.* 2001). The plasma membrane P-ATPase usually controls the process of free proton removal at the expense of ATP hydrolysis to maintain the pH and electrochemical potential differences across the membrane (Blatt and Slayman 1987; Serrano 1998). Thus response of intracellular pH to different conditions, especially variation in extracellular pH levels clues to mechanism of pH regulation in fungi (Kugel *et al.* 1987). In eukaryotes, sterols function as a component of biological membranes by modulating their properties, especially their fluidity. In certain prokaryotes tolerance to extreme extracelluar acidity has been related to the lipid composition (Davoli *et al.* 2002). Selective plasma membrane permeabilisation in *Penicillium cyclopium* was due to the advantage of the pore complex formation between nystatin and ergosterol. Selectivity towards the plasma membrane is based on the significantly higher sterol (mainly

ergosterol) content of the fungal plasma membrane as compared to the fungal tonoplast (Albers *et al.* 2001).

Effect of pH on mutant *A. carbonarius* growth in phosphate citrate buffer media (250 mM) showed that the optimum pH for xanthin (partially saturated canthaxanthin and astaxanthin) production was 3.0. Accumulation of pigment within the growing mycelia occurred when fungus was grown in a medium pH, 3.0 or 4.0. Thus it appeared that xanthin had a role in protecting the cells from acidic pH stress and its synthesis occurred at the expense of membrane sterols. In order to relate proton impermeability to change in membrane lipids during growth at acidic pH, concentration of sterols in the pigmented and non pigmented mutant *A. carbonarius* biomass was estimated by gas chromatography.

In this experiment from crushed biomass (1g) total lipids were extracted with chloroform and methanol. For sterols quantification, they were extracted into petroleum ether (Materials and Methods) (Folch *et al.* 1956).

Estimation of total sterol content in pigmented **(Figure-17)** and non-pigmented **(Figure 18)** biomass showed a reduced sterol content in pigmented biomass. GC analysis showed absence of ergosterol peak in the sample extracted from the pigmented biomass.

The reduction in the concentration of membrane sterols, in general and absence of synthesis of ergosterol in particular within the pigmented biomass of mutant *A. carbonarius* suggested a mutation that diverted sterol precursors for the synthesis of xanthin. The gas chromatogram of standard ergosterol is presented in **Figure-19**. The results are discussed in the last section of thesis.

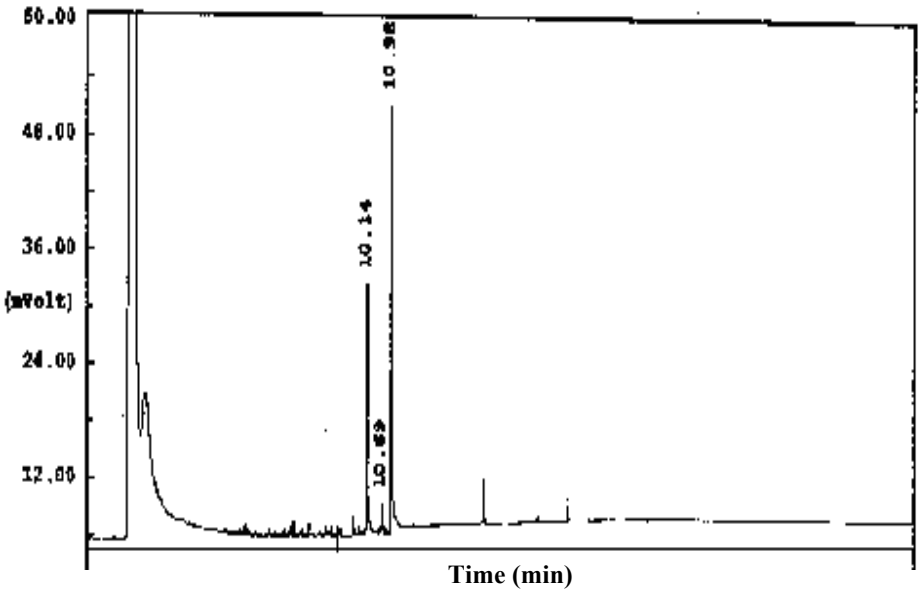


Figure: 17. Gas Chromatogram of sterols extracted from pigmented mutant *A. carbonarius* biomass. The fungus when grown in pH 3.0 incorporated the pigment in biomass

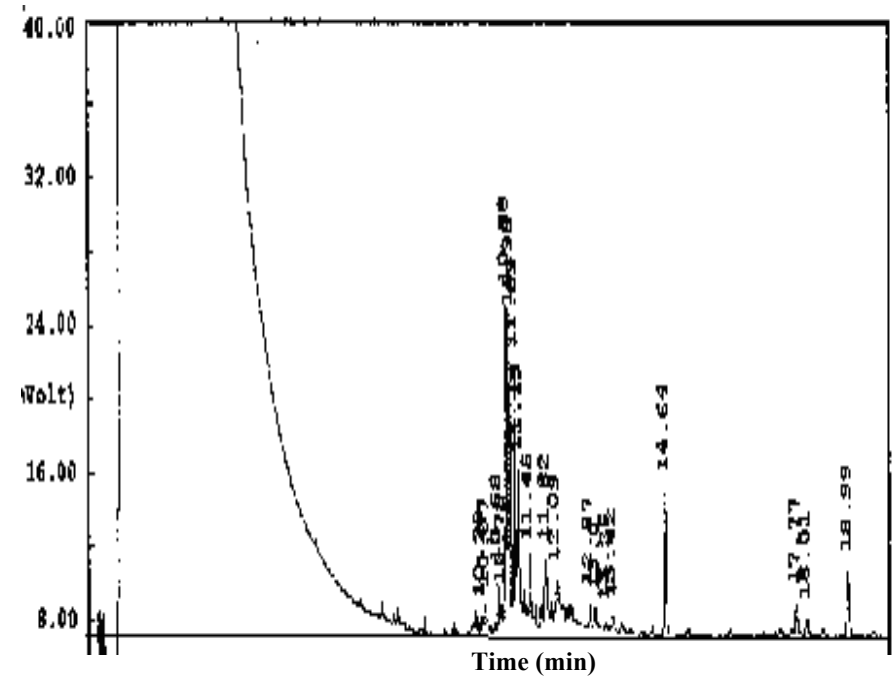


Figure: 18. Gas Chromatogram of sterols extracted from non-pigmented *A. carbonarius* biomass. The fungus when grown in pH 5.5 did not synthesize the pigment

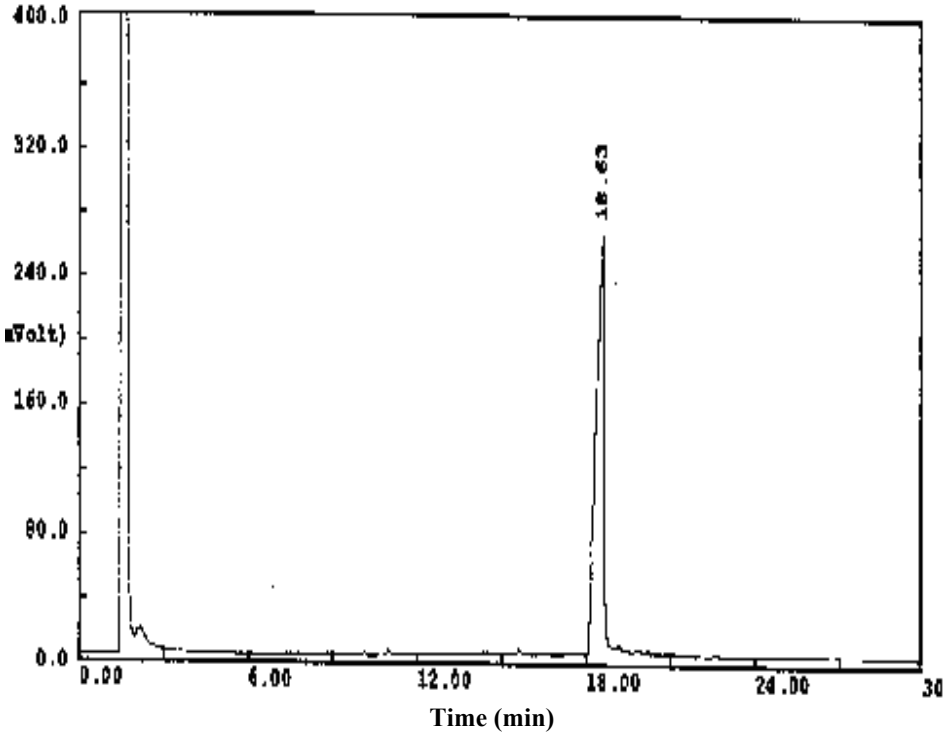


Figure: 19. Gas chromatogram of standard ergosterol

3.6 INHIBITION OF XANTHIN BIOSYNTHESIS IN MUTANT *A. CARBONARIUS* BY PATHWAY SPECIFIC INHIBITORS OF CAROTENOID BIOSYNTHESIS

The biosynthetic pathways involved in carotenoid formation were elucidated in 1950s and 1960s by classical biochemical approaches, using specific inhibitors which blocked the synthesis at certain steps in the pathway. In the early 1970s *in vitro* systems were developed for elucidation of pathways (Bramley 1985).

Most of the enzymes involved in carotenoid biosynthesis are membrane associated. Their sensitivity against detergents used for solublilization and their lower concentration makes the isolation and purification rather difficult (Bramley 1985). A number of carotenoid biosynthesis inhibitors, such as nicotine, norflurazon, diphenylamine, lovastatin, fluridone have been used for pathway elucidation and for isolation of resistant mutants against carotenoid biosynthesis inhibitors (Jin *et al.* 2004).

Carotenoid biosynthesis in fungi occur through isoprenoid pathway. The acetate/mevalonate (MVA) pathways are the biosynthetic routs leading to isopentenyl diphosphate (IPP), the central intermediate of isoprenoid biosynthesis. C40 carotenoids are made starting with the synthase-catalyzed condensation of two molecules of geranyl-geranyl pyrophosphate (C_{20}PP) to form phytoene. Different types and levels of modification of this C_{40} backbone lead to production of majority of known carotenoids (Goodwin 1980). These C_{40} condensation products are dehydrogenated in a stepwise manner by desaturase enzymes, which represent the next important branch points for pathway diversification. In bacteria and fungi, a single desaturase catalyzes the entire sequence of carotenoid desaturation steps. This is followed by cyclization, catalyzed by β- or ε-cyclase which leads to carotenoids with one or two cyclized ends. The 700 known

carotenoids are ketolated, hydroxylated, glycosylated and/or oxidatively cleaved form of the C_{40} carotenoids **(Figure-20)**. Xanthophylls are oxygenated carotenoids that play significant role in the photosynthetic apparatus of higher plants (Britton 1995). The first step in xanthophylls biosynthesis is the hydroxylation of the ε- and β -ionone rings of α-carotene (ε–β-carotene) and β-carotene (β–β-carotene) through the insertion of iron-activated oxygen.

Complete carotenoid biosynthetic pathway of *Thermus thermophilus* was established using the inhibitors diphenylamine and 2-(4-chlorophenylthio)-triethylamine-HCl (CPTA) (Akihiro *et al.* 1996). 2-methylimidazole, triethylamine and piperonyl butoxide were used for elucidation of monocyclic carotenoids pathway in *Phaffia rhodozyma* (An *et al.* 1999). The biosynthetic pathway of astaxanthin in the green algae *Haematococcus pluvialis,* was studied using inhibitor diphenylamine. Cultures of *Haematococcus pluvialis* when induced to produce astaxanthin in the presence of the diphenylamine (30μM), biosynthesis of astaxanthin was specifically inhibited in the conversion of ß-carotene to echinenone and canthaxanthin (Lu *et al.* 1995). In a study on carotenoid formation in a mutant *Mucor circinelloides* using diphenylamine, nicotine and CPTA showed that diphenylamine prevented desaturation, nicotine reduced carotene levels while CPTA caused an increase in total carotenoid content but reduced β-carotene with the accumulation of lycopene (Paul *et al.* 1995).

The finding that xanthin is produced by mutant *A. carbonarius* required analysis for the occurrence of the biosynthetic pathway. For this, carotenoid pathway specific inhibitors were used **(Table-2)** to study their effect on xanthin synthesis.

The mutant *A. carbonarius* was grown in a defined medium buffered to pH 3.0, with glucose and ammonium salts as carbon and nitrogen sources respectively. Inhibitors at a concentration ranging from 20 μM to 100 μM (dissolved in dimethyl sulfoxide (DMSO) or ethanol were added in the growth medium. The control flasks contained the highest concentration of ethanol or DMSO, used for dissolving the inhibitors. Biomass was harvested after 48 h growth and the pigment extracted was quantified (Materials and Methods).

All the inhibitors used in this study showed a concentration dependent inhibition of xanthin synthesis. Lovastatin, diphenylamine and pipernylbutoxide completely inhibited fungal growth at 100 μM concentration. Xanthin yield was inversely proportional to the concentration of inhibitors **(Figures 21 to 26)**. At lower inhibitor concentrations (20 μM) xanthin synthesis was comparable with the control.

Inhibitors of carotenoid biosynthesis affected xanthin synthesis at higher concentration (40-100 μM). It appeared that the enzymes of mevalonate pathway (HMG CoA reductase, phytoene saturase, lycopene β-cylase, β-carotene hydroxylase) were inhibited, resulting in reduced xanthin yield. Use of pathway specific inhibitors of carotenoid biosynthesis showed that mutant *A. carbonarius* possibly utilizing isoprenoid pathway for the synthesis of xanthin.

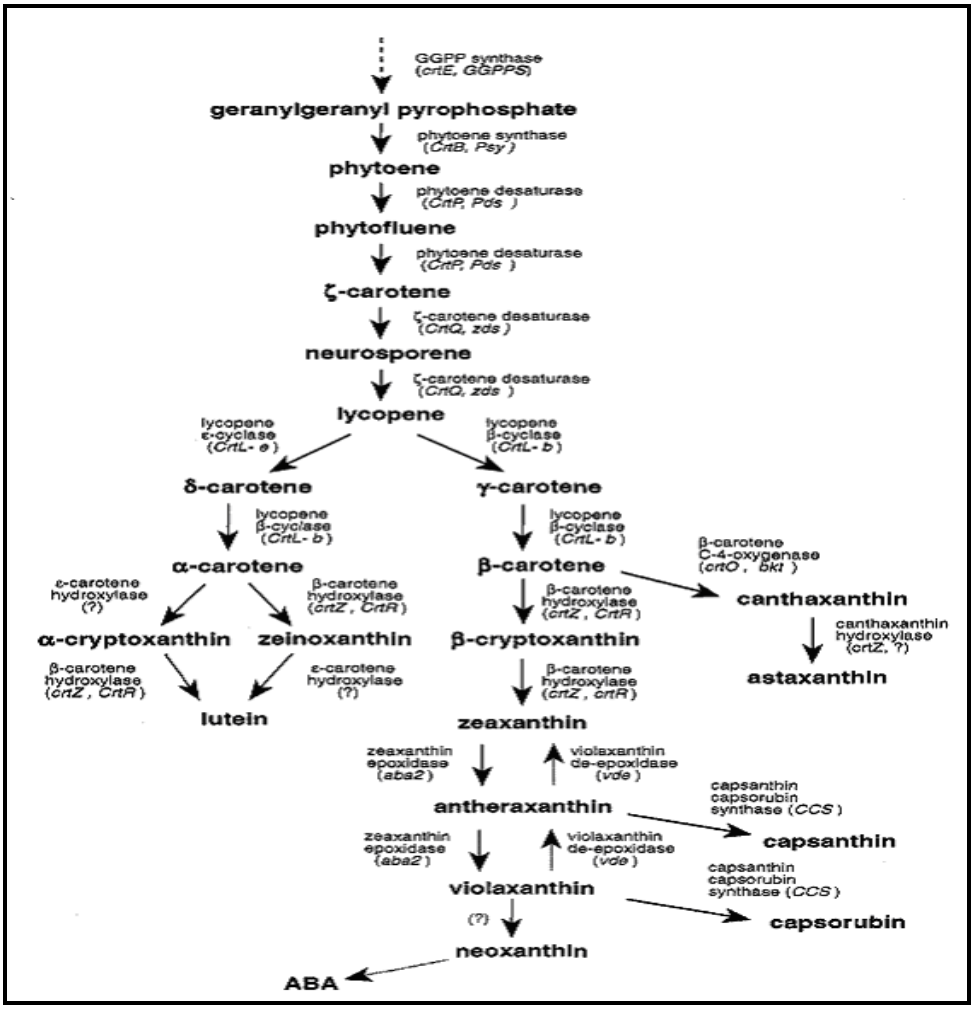


Figure: 20. Isoprenoid pathway of carotenoid biosynthesis (Source: Britton 1995)

Table: 2. Enzymes Inhibitors of carotenoid biosynthetic pathway

Sl. No	Enzymes	Inhibitors
1	HMG-CoA reductase	Lovastatin
2	Phytoene desaturase	Nicotine
3	Lycopene β- cylase	Diphenylamine
4	B-carotene hydroxylase	Pipernylbutoxide
5	HMG-CoA reductase	Squalene

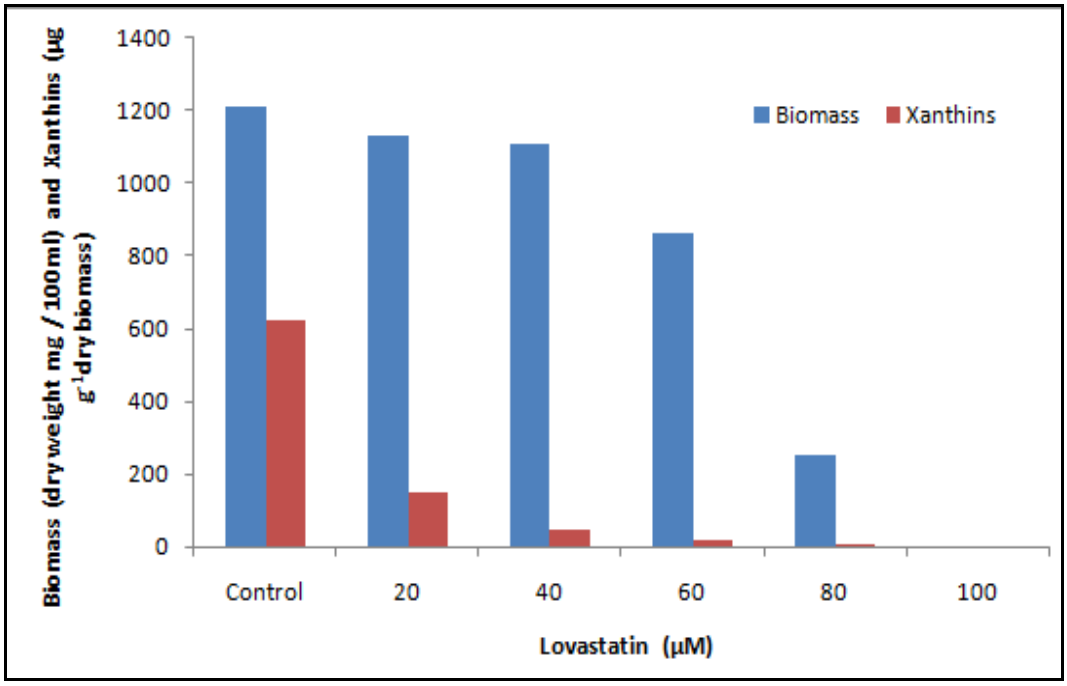


Figure: 21. Effect of lovastatin on xanthins of *A. carbonarius* mutant

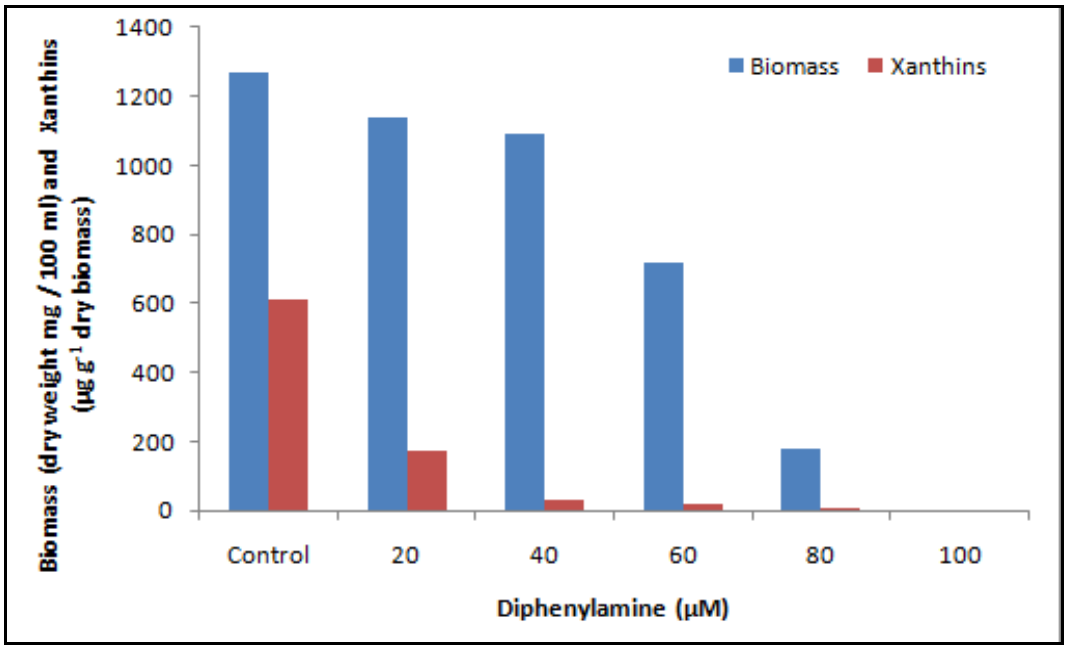


Figure: 22. Effect of diphenylamine on xanthins of *A. carbonarius* mutant

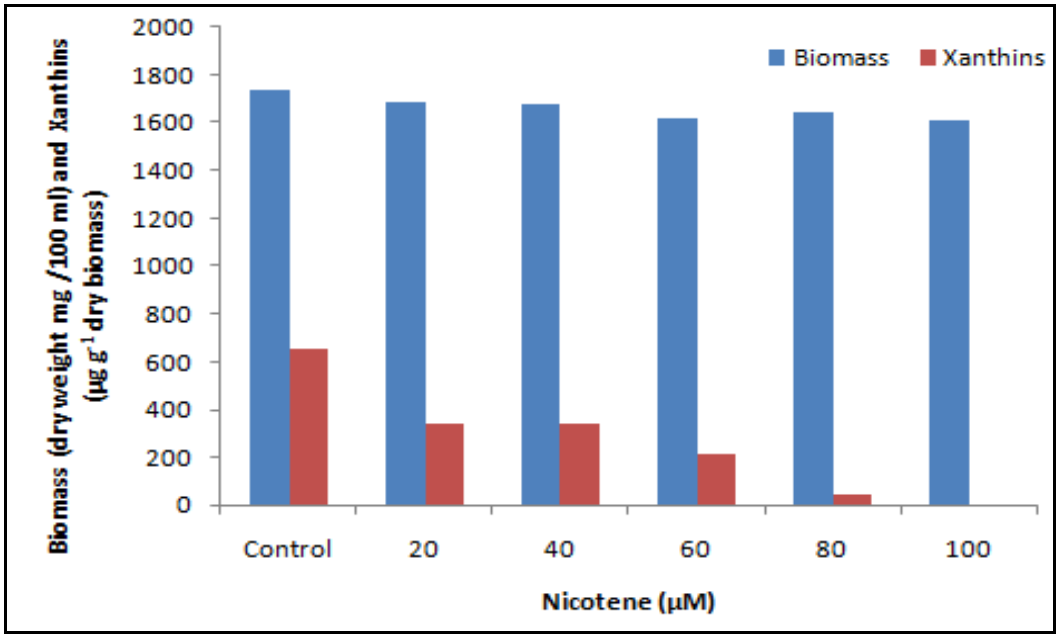


Figure: 23. Effect of nicotine on xanthins of *A. carbonarius* mutant

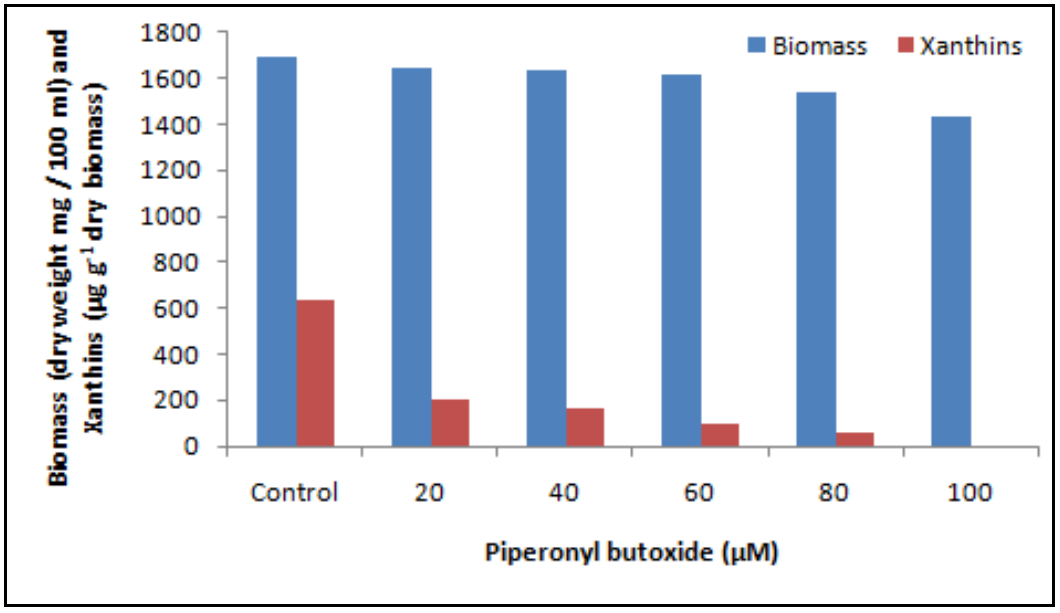


Figure: 24. Effect of piperonylbutoxide on xanthins of *A. carbonarius* mutant

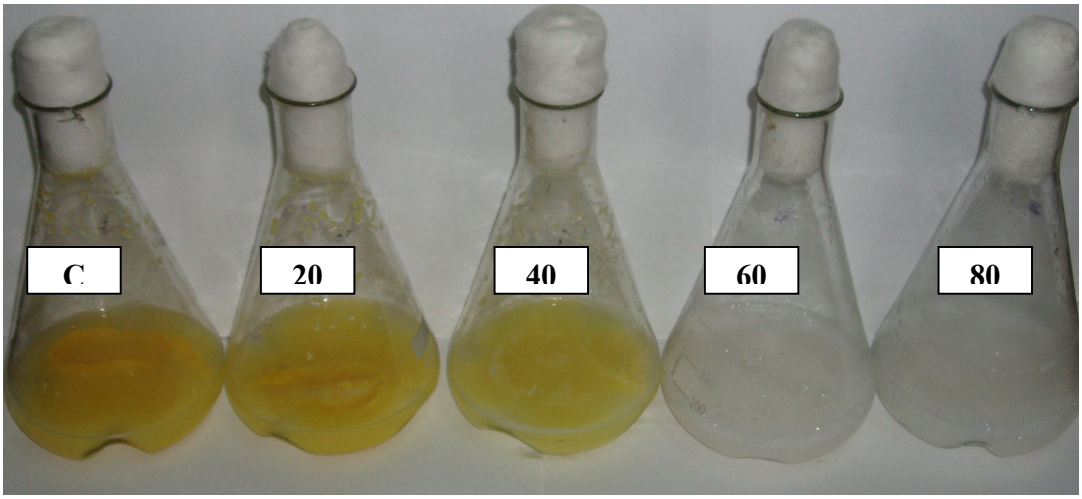


Figure: 25. Concentration dependent (20-100 µM) inhibition of xanthin synthesis by lovastatin

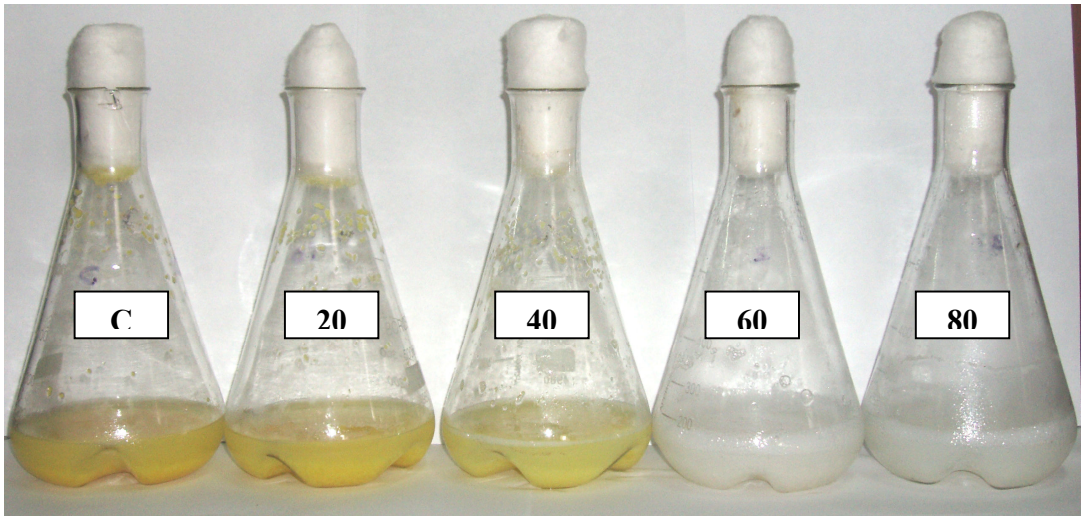


Figure: 26. Concentration dependent (20-100 µM) inhibition of xanthin synthesis by diphenylamine

3.7 EFFECT OF SQUALENE ON XANTHIN SYNTHESIS IN MUTANT *A. CARBONARIUS*

Squalene, a triterpenoid hydrocarbon oil, is a precursor of cholesterol and the steroid hormones. Squalene, formed from farnesol by squalene synthase, is the first metabolite for cholesterol synthesis (Terese *et al.* 2000). Squalene is made up of universal C_5 building block isopentenyl diphosphate (IPP), formed from three molecules of acetyl-CoA via the mevalonate pathway. Squalene stimulates the activity of Acyl Coenzyme A: cholesterol Acyltransferase (ACAT) but strongly inhibits the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of mevalonate pathway for synthesis of sterols and carotenoids (Strandberg *et al.* 1989; Britton 1995).

In the earlier experiment, squalene when added to the growth medium inhibited xanthin production by mutant *A. carbonarius*. Hence concentration effect of squalene (20-100μM) on pigment synthesis was studied.

Squalene was incorporated into the growth medium of mutant *A. carbonarius* and incubated for 48 h. Mycelium was harvested and the pigment extracted was quantified (Materials and Methods).

Squalene inhibited the synthesis of pigment in mutant *A. carbonarius*. There was a significant decrease in pigment yield with increasing concentration of squalene **(Figures 27 & 28*)***. However there was no effect of squalene on the yield of biomass. It appeared that pigment synthesis was decreased due to the inhibition of HMG-CoA reductase, the preliminary step in carotenoid and sterol biosynthesis. Thus it appeared that the mutant *A. carbonarius* followed the mevalonate pathway for pigment production.

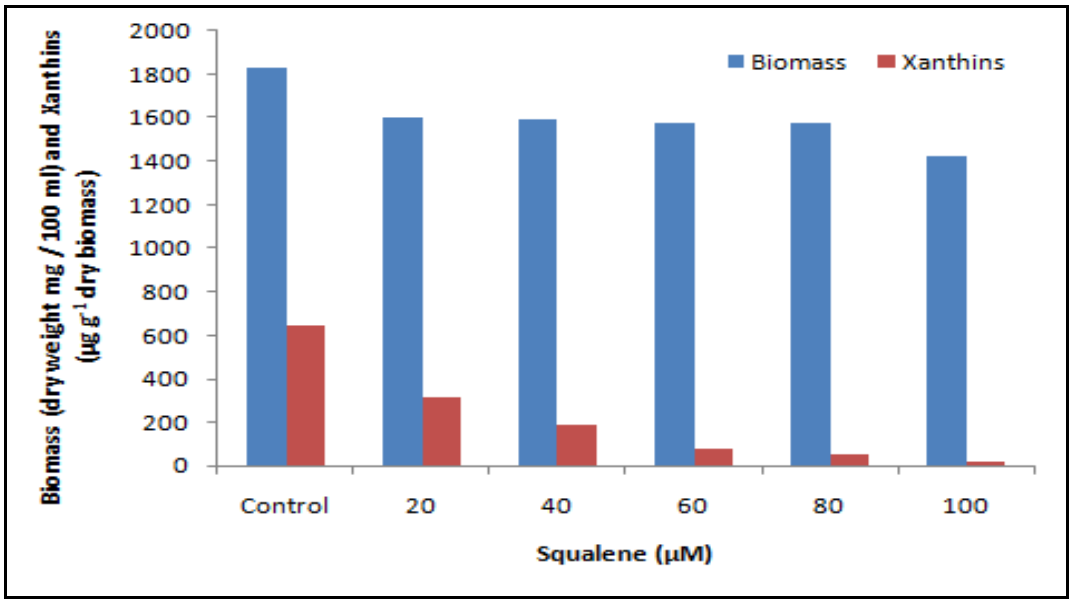


Figure: 27. Effect of squalene on xanthin production by *A. carbonarius* mutant

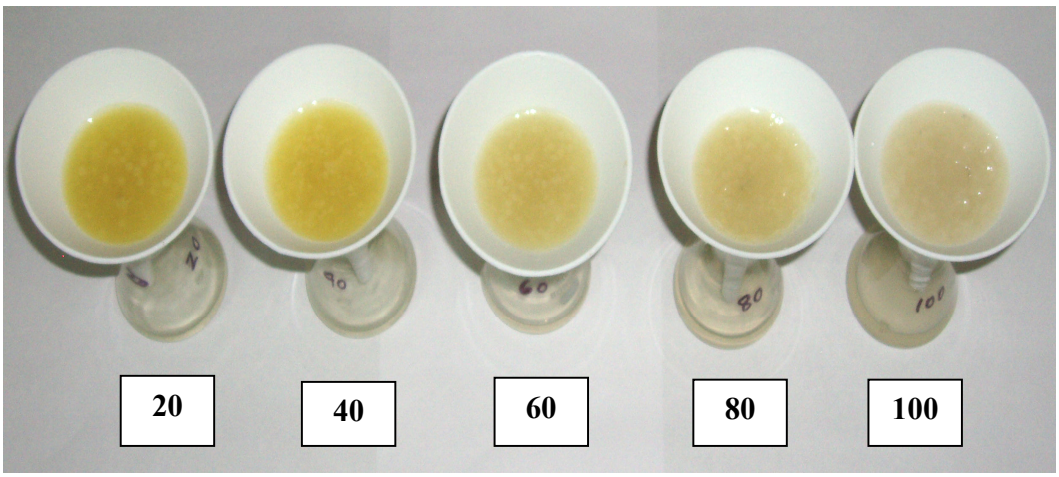


Figure: 28. Production of xanthin by mutant *A. carbonarius* grown in medium containing different concentration (µM) of squalene

OPTIMIZATION OF CULTURAL CONDITIONS FOR XANTHIN PRODUCTION USING MUTANT *A. CAROBONARIUS*

Microbial fermentations are widely explored in industries for the production of a variety of fine and commodity chemicals like alcohols, organic acids, antibiotics, enzymes, vitamins, amino acids, and pharmacologically active agents (Nagasawa and Yamada 1995). In these processes, optimization of cultural parameters is a prerequisite since they are not easily defined. Various parameters that affect microbial physiology and metabolite yield are inoculum density, pH, temperature, agitation and media composition (Wang *et al.* 1999; Castinmiller *et al*. 1999). Hence, there is a requirement to design fermentation conditions for metabolite production by microbes.

Filamentous fungi are heterotrophic and require simple sugars like D-glucose, D-fructose, sucrose etc. While complex organic compounds such as polysaccharides, amino acids, lipids, organic acids, proteins, alcohols and hydrocarbons support growth, their nature affects metabolite yield. Citric acid production is greatly influenced by the nature of carbon source since it exerts a strong effect on enzymes within the TCA cycle (Xu *et al.* 2003). Media composition greatly affects the growth and product yield. In penicillin production by submerged fermentation, addition of precursors to the usual sources of carbon, nitrogen, minerals and buffers increases the yield of the antibiotic (Atkinson and Mavituna 1991). The use of complex media may affect not only the formation but also the degradation rate of the product. Christensen *et al.* (1994) showed that the rate of penicillin-V degradation was significantly higher when media contained corn-steep liquor (CSL) with phosphate. Studies on conventional batch culture for citric acid production

showed that, initial glucose concentration in the fermentation medium affected the morphology of *Aspergillus niger* (Papagianni *et al.* 1999b).

Nitrogen may be supplied as ammonia, nitrate or organic compounds such as amino acids, beet or cane molasses, corn-steep liquor, whey powder, soy flour and yeast extract. Nitrogen limitation induces pellet formation in filamentous fungi (Braun and Vecht-Lifshitz 1991). Study conducted on the growth behavior and glucoamylase production by a mutant *Aspergillus niger* without nitrogen source, showed low mycelial metabolic activity with broken or partially empty hyphal tips (Schrickx *et al*. 1995).

Industrial production of metabolites by filamentous fungi is susceptible to regulation by dissolved oxygen tension of the medium (Kubicek *et al.* 1980). Influence of oxygen in citric acid production by *Aspergillus niger* has been well established (Shu and Johnson 1984). Production process of penicillin by *Penicellium chrysogenum* is highly sensitive to dissolved oxygen tension. The specific penicillin production rates at different dissolved oxygen levels showed that below 30% air saturation, yield decreased sharply (Vardar and Lilly 1982).

The pH of the medium is very important because it can profoundly affect cellular activities. The pH of a solution is a measure of the concentration of H^+ ions present. Conidial fungi can grow over a wide range of pH. Most of them tolerate a pH range from 4 to 9 but grow and sporulate maximum near neutral pH (Braun and Vecht-Lifshitz 1991). The composition of the medium can affect direction of pH drifts during growth of the fungus. Poorly buffered media containing ammonium salts are likely to become more acidic during growth, while media containing nitrate are likely to become alkaline.

Minimizing pH drifts during growth is a desirable objective that is often difficult to achieve.

Study of melanin production by *Aspergillus nidulans* showed that pH 3.0 was the lower limit for growth and metabolism (Rowley and Pirt 1972). Pirt and Callow (1999) reported that increasing the pH of steady-state cultures of *Penicillium chrysogenum,* above 6.0, caused a corresponding decrease in hyphal length. In *Aspergillus awamori,* pH influences α-amylase formation, growth and morphology of fungus (Carlsen *et al.* 1995). In *Aspergillus niger*, pellets formation occurs only at inoculum sizes below 10^8 spores/ml (Van Suijdam *et al.* 1980), unlike in *Penecillium chrysogenum* where in pellets formed at inoculum sizes below 10^4 spores/ml (Calam 1987).

Xanthin production by mutant *A. carbonarius* occurred when the fungus was grown at pH 3.0. Since the fermentation condition required for xanthin synthesis appeared stringent, media optimization studies were carried out and the results of this study are described in this section of thesis.

4.1 EFFECT OF AERATION ON XANTHIN PRODUCTION IN MUTANT *A. CARBONARIUS*

Fungi are morphologically complex organisms and growth conditions influences their morphogenetic behavior. In submerged cultures, a large number of factors affect fungal development and differentiation. These include the type and concentration of carbon, nitrogen, phosphate, trace minerals, dissolved oxygen, carbon dioxide, pH and temperature. Growth and fermentation are also affected by fermentor geometry, agitation systems used, rheology and the culture methods (batch, fed-batch or continuous).

Fungi can grow over wide ranges of oxygen tensions. Industrial production of metabolites by filamentous fungi is susceptible to regulation by the dissolved oxygen tension of the medium. Effect of aeration on citric acid yield has been well studied in *A. niger*. Higher aeration resulted in the production of small compact pellets and interruptions of aeration up to 20 min did not reduce viability but resulted in complete loss of ability to produce citric acid. The cultures of small and compact pellets yielded higher levels of citric acid than the cultures composed of free filaments and loose pellets (Kubicek *et al.* 1980).

In *A. niger* pellet morphology and citric acid yield were associated with stirring conditions in submerged fermentation. At higher stirrer speed (1000 rpm), the small compact pellets formed predominated, while at lower speed (450 rpm) a mixture of free filaments and loose pellets arose (Carlsen *et al.* 1995).

In *Streptosporangium* the dissolved oxygen tension (pO2) is an important parameter that regulates secondary metabolite production during submerged fermentation (Christinson *et al.* 1994). Oxygen concentration of more than 1.2 μM introduced into cultures of *Mucorella racemosus,* caused the organism to grow exclusively in the hyphal

form. Oxygen influenced the morphological development of the dimorphic fungus *Mucor circinelloides* (McIntyre *et al.* 2002). Studies carried out on three morphogenetic transition status of conidial formation in *Neurospora crassa* showed that exposure of the mycelial mat to air induced hyphal adhesion, growth of aerial hyphae and conidia formation (Micha´n *et al.* 2003).

Xanthin was synthesized by the mycelia of mutant *A. carbonarius* when grown in a medium made up of corn flour and ammonium salts in a fermentor. Since the xanthin synthesis occurred only when the fungus was grown in a fermentor and not in shake flasks, it appeared that aeration was a major factor that affected xanthin production.

A. carbonarius mutant was grown in corn flour, ammonium salt medium buffered to pH 3.0. Baffled flask (500 ml) for increased aeration was used for the experiment. Controls consisted of the fungus grown in Erlenmeyer (500 ml) flasks under identical conditions. After 48 h growth the biomass was harvested for xanthin extraction (Materials and Methods).

Xanthin production by *A. carbonarius* in baffled and control flasks is given in **Table- 3**. Though there was no apparent difference in pH of culture filtrate and biomass yield of the fungus, there was increase in the xanthin yield (1248 μg g^{-1} dry biomass) when the organism was grown under baffled conditions. Thus it showed that aeration was important for xanthin production and mutant fungus produced more xanthin compared to the parent.

Table: 3. Effect of aeration on the yield of xanthin from mutant *A. carbonarius*

Organism	**Growth condition**	**Culture pH after 48 h**	**Biomass yield ***	**Xanthin yield [@]**
Mutant	Baffled	2.94	2.41	1248
	Control	2.92	2.50	52
Parent	Baffled	2.94	2.45	25
	Control	2.94	2.48	23

* Dry weight g /100 ml [@] µg g^{-1} dry biomass

4.2 OPTIMIZATION OF XANTHIN PRODUCTION BY MUTANT *A. CARBONARIUS* USING RESPONSE SURFACE METHODOLOGY

Traditionally, optimization of cultural conditions for fungal metabolite production is done by varying each factor one by one. Such an optimization method is tedious since a large number of factors are involved and relation between two interrelated factors becomes difficult to interpret. Statistical methodologies have been applied in biotechnological process to define fermentation process and to deal with a large number of variables (Manuel and Antonio 1997). Of the many statistical methods, Response Surface Methodology (RSM) is a powerful mathematical model that tests multiple process variables with fewer experimental trials (Sreekumar *et al*. 1999).

RSM designs provide a systematic and efficient means of studying several control factors in fermentation process. It is widely used to examine operational variables in designing an experiment by predicting a model based on factors and conditions used for optimization (Buchanan and Philips 1990). In the optimization of medium composition interactions between variables are identified and quantified for designing the model (Box GEP and Wilson 1951; Ergun and Mutlu 2000; Escamilla *et al.* 2000; Li *et al.* 2001; Xu *et al.* 2003; Mao *et al.* 2005).

Zhu *et al.* (1996) used RSM-based experimental designs to optimize enzyme production in solid-state fermentation. Optimization of lignin peroxidase production form white rot fungus, *Phanerochaete chrysosporium,* was carried out using RSM methodology (Manimekalai and Swaminathan 1999). Chang *et al.* (2002) used RSM to optimize culture medium for production of lovastatin by *Monascus ruber*. In this experiment cultural conditions were optimized for the production of xanthin from mutant *A. carbonarius* in submerged fermentation.

Three level Box Behnken design was performed with the aim of optimizing the culture conditions for xanthin production. The variables involved in this study were, pH (X_1), temperature (X_2) and fermentation time (X_3). The upper and lower levels of variables in this design, were chosen based on previous data on xanthin production by *A. carbonarius*. **Table-4** shows the codes and actual levels of independent variables for design of experiment.

Table: 4. Codes and independent variables for designing RSM

Independent variables	**Symbols**	**Coded Levels**		
		-1	0	+1
PH	X_1	2.5	3.5	4.5
Temperature (^{0}C)	X_2	25	30	35
Time (hours)	X_3	32	44	56

The experimental data obtained for the matrix corresponding to factorial design is shown in **Table-5**. To avoid bias, a total of 15 runs were performed in a random order (overall randomization). The coefficients of the equation were determined by employing Microsoft excel software version 5.0. Analysis of variance (ANOVA) for the final predictive equation was also carried out using Microsoft excel software. The quality fit of the first-order model equation was expressed by the coefficient of determination (R^2) and its statistical significance was determined by *f*-test. The significance of the regression coefficients was tested by *t*-test.

The results obtained by observed experimental data were used to develop model. Each dependent variable was obtained as the sum of the contributions of the independent variables through second order equation and interaction terms (Equation-1, in Material and Methods). In this experiment the quantity of xanthin produced by *A. carbonarius* ranged form 54.0 to 851.0 µg/g dry biomass. Runs # 6 and # 13 had the minimum and maximum xanthin yield respectively.

Statistical testing of the model was done using ANOVA to test the significance and adequacy of the model. The results of ANOVA for the quadratic regression model for xanthin yield is presented in **Table-6.**

Table: 5. Three level Box-Behnken design matrix and the experimental responses of the dependent variables on xanthin yield

Run No.	Independent variables code levels			Xanthin yield µg g^{-1} dry biomass			Biomass dry weight g / 100ml culture media		
	pH	Temp (^oC)	Time (h)	A	B	C	A	B	C
1	1	1	0	64.0	29.0	54.1	1.80	1.79	0.3
2	1	-1	0	68.0	86.0	26.0	1.81	1.83	1.2
3	-1	1	0	389.0	371.0	4.6	1.75	1.72	1.3
4	-1	-1	0	584.0	618.0	5.9	1.77	1.77	0.3
5	1	0	1	89.0	127.0	43.6	2.18	2.16	0.8
6	1	0	-1	54.0	32.0	40.7	1.26	1.29	0.07
7	-1	0	1	689.0	710.0	3.2	2.10	2.09	0.06
8	-1	0	-1	362.0	323.0	10.8	1.11	1.15	1.0
9	0	1	1	692.0	687.0	0.6	2.16	2.18	1.1
10	0	1	-1	389.0	445.0	14.5	1.42	1.43	0.2
11	0	-1	1	896.0	839.0	6.3	2.14	2.13	0.2
12	0	-1	-1	594.0	598.0	0.7	1.48	1.45	1.3
13	0	0	0	851.0	844.0	0.6	2.04	2.06	3.3
14	0	0	0	838.0	844.0	0.5	2.00	2.06	2.9
15	0	0	0	843.0	844.0	0.08	2.05	2.06	0.6

[A] Observed, [B] Predicted, [C] Relative deviation, Values average of triplicate experiment.

The Fisher's variance ratio, the *F*-value, is a measure of the variation of the data about the mean. Generally the calculated *F*-value should be several times greater than the tabulated *F*-value if the model is a good prediction of the experimental results and the estimated factor effects are real. ANOVA of the regression model for xanthin yield demonstrated that the model was highly significant, due to calculated *F*-value of 106.5 with low probability (P model >*F* – 0.0005). Analysis of variance (*F*-test) for the model of xanthin yield explained the response of the dependent variable Y and showed that the

coefficient of determination R^2 was 0.995. This indicated that nearly 99.5% of the variations could be explained by the model. Thus the accuracy and general ability of the polynomial model was good and analysis of the response trends using the model was reasonable. It also showed that the experimental yields fitted the second-order polynomial equation, indicated by high R^2 values.

Table: 6. Analysis of variance (ANOVA) for optimization of xanthin production from mutant *A. carbonarius*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Regression	2599339.01	9	288815	106.759	0.0005
Residual	13526.49	5	2705.3	-	-
Total (corr.)	2612865.50	14	-	-	-

R^2= 0.9948, Adjusted R^2= 0.9855, Multiple R= 0.9974, Standard error =52.01

The regression coefficients, along with the corresponding *P*-values, for the pigment production model are described in **Table-7**. The *P*-values were used as a tool to check the significance of each of the coefficients, which in turn indicated the pattern of the interactions between the variable. The smaller the *P*-value, the more significant was the corresponding coefficient (Liu *et al.* 2003). The regression coefficients of all the linear term and all quadratic coefficients of X_1, X_2, X_3, were significant at < 1% level and interaction coefficient of X_1 and X_3 was significant at < 5% level. ANOVA showed the model to be significant at $P<0.01$. The quadratic terms were highly significant compared to linear and interaction coefficients.

Table: 7. Regression analysis of second order polynomial model for optimization of xanthin production from mutant *A. carbonarius*

	Coefficients	**Standard error**	***t*- statistics**	***P*- value**
Intercept	1244	30.029	41.426	0.000**
A_1	-218.575	18.389	-11.886	0.000**
A_2	-76.05	18.389	-4.135	0.009**
A_3	120.875	18.389	6.573	0.001**
A_1A_1	-655.95	27.068	-24.233	0.000**
A_2A_2	-311.7	27.068	-11.515	0.000**
A_3A_3	-289.55	27.068	-10.697	0.000**
A_1A_2	47.65	26.006	1.832	0.126
A_1A_3	-73	26.006	-2.807	0.037*
A_2A_3	0.25	26.006	0.009	0.992

***Significant at P< 5%level, **Significant at P< 1% level.**

From the 3D response surface plots and the corresponding contour plots, the optimal values of the independent variables and the interaction between each independent variables pair were described. The graphical representations of the regression equation, called the response surfaces for xanthin yield obtained using the same software are shown in **Figures-29 to 31**.

Response surface analysis indicated that a local optimum existed in the area experimentally investigated. The optimal levels in coded values for pH and temperature were between 0 and -1 and for fermentation time, 0 and +1. The orientation of the principal axes of the contour plots between the variables pH and temperature, pH and fermentation time indicated that the mutual interactions between these set of variables had a significant effect on the pigment yield. When the third independent variable,

fermentation time, was kept constant at 44 h **(Figure-29)** the interaction between the two variables (temperature and pH) showed that the xanthin yield was sensitive when pH and temperature were subject to small alterations. Under certain condition a maximal contour (xanthin yield, 1283.00 µg/g dry biomass) could be determined, meaning that further change in temperature and pH would not increase the xanthin yield further.

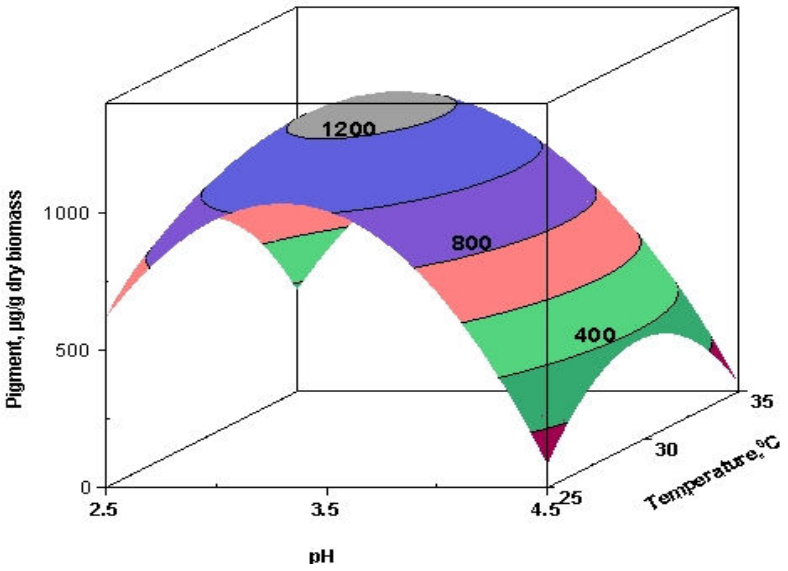


Figure: 29. Three dimensional plots along with contour level showing the effect of pH and temperature on xanthin yield (Fermentation time-44 h)

The other pair of the independent variables, pH and fermentation time showed similar effects while keeping the third independent variable temperature constant at 30^0C **(Figure-30)**. The 3D surface response for temperature and fermentation time on the yield of xanthin **(Figure-31)**, where pH was constant, showed that the mass yields are obtained in the middle level of the process variables. As the values of process variables increased, the yield also increased but only up to the midpoint of variables and decreased thereafter

even though the values of variables increased. The xanthin yield was significantly affected by pH, temperature and fermentation time and pH had greater effect.

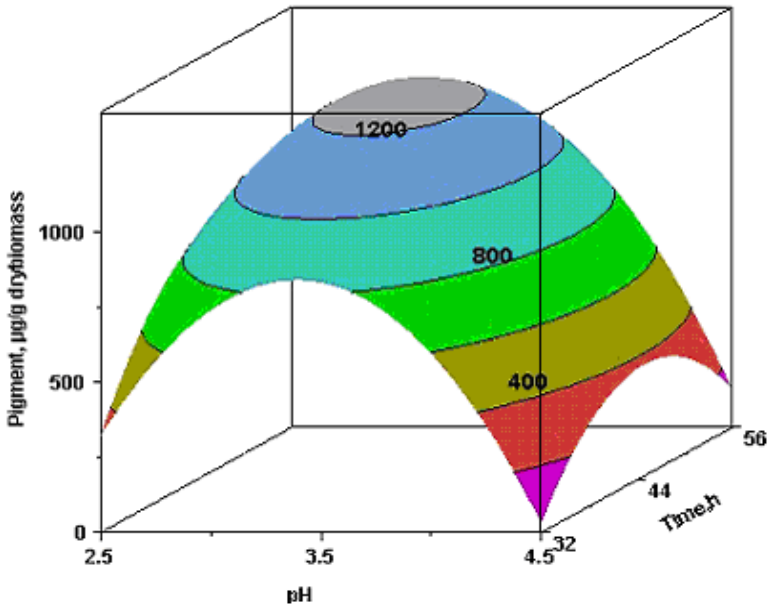


Figure: 30. Three dimensional plot along with contour level showing effect of pH and fermentation time on xanthin yield (temperature 30^{0}C)

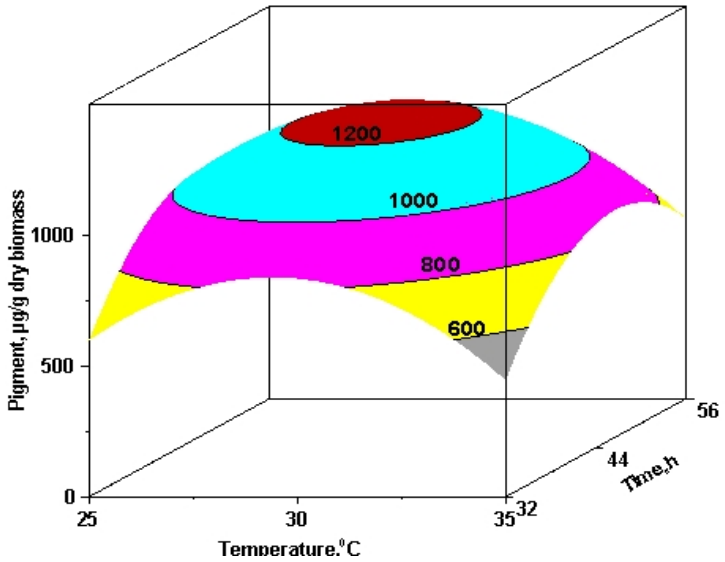


Figure: 31. Three dimensional plot along with contour level showing the effect of temperature and fermentation time on xanthin yield (pH 3.5)

Based on the models, the optimal condition obtained for high xanthin yield was; pH of growth media, 3.3, temperature, 29.3^{0}C and fermentation time 46.7 h. Under these conditions the model predicted a xanthin yield of 1283.00 µg/g dry fungal biomass. The validation experiment conducted on above optimum condition yielded 1298.00 µg xanthin per gram of dry fungal biomass. The average relative deviation (ARD) of the predicted yield from experimental yield for xanthin was found to be 0.4 %.

4.3 OPTIMIZATION OF BIOMASS YIELD BY RESPONSE SURFACE METHODOLOGY

The conditions of Box-Benkhen design used in the earlier experiment were followed for optimizing the *A. carbonarius* mutant biomass yield. The variables involved in this study were pH (X_1), incubation temperature (X_2) and fermentation time (X_3). The upper and lower levels in this design were chosen in reconciliation with the data of our previous work on xanthin production by *A. carbonarius.* **Table-8** shows the codes and actual quantity of independent variables used for experimental design.

Table: 8. Codes and independent variables for designing RSM

Independent variables	Symbols	Coded Levels		
		-1	0	+1
PH	X_1	2.5	3.5	4.5
Temperature (^{0}C)	X_2	25	30	35
Time (hours)	X_3	32	44	56

The conditions used for the optimization were as following: pH, 2.5, 3.5 and 4.5; temperatures, 25, 30 and 35^{o}C and fermentation time, 32, 44 and 56 h. **Table-9** shows results of ANOVA for the biomass production from mutant *A. carbonarius.* ANOVA of the regression model demonstrated that the model was highly significant, as evidenced from the calculated *F*-value 36.96, very low probability value (P model >F - 0:0005) and with high R^2 (98.51%) value.

Table: 9. Analysis of variance (ANOVA) for optimization of biomass production from mutant *A. carbonarius*

Source	Sum of Squares	Degree of Freedom	Mean Square	*F*-value	Significance-*F*
Regression	0.864	9	0.096	36.969	0.0005
Residual	0.012	5	0.002	-	-
Total model	0.877	14	-	-	-

R^2= 0.985, Adjusted R^2= 0.958, Multiple R= 0.992, Standard error =0.509

The regression coefficients along with the corresponding *P*-values for the model of mycelial production by *A. carbonarius* is presented in **Table-10**. The regression coefficients of the linear term X_3 and quadratic coefficients of X_1&X_2 were significant at <1% level. The quadratic term of X_3 and interaction term of X_1 and X_3 were significant at <10% level. The response analysis reveled that maximum yield of biomass (2.443g dry biomass/ 100ml media) could be obtained when the organism was grown for 56 h at 29.7^0C in corn flour medium of pH 3.65.

Table-10. Regression analysis of second order polynomial model for optimization of biomass yield from mutant *A. carbonarius*

	Coefficients	Standard error	*t*- statistics	*P*- value
Intercept	2.263	0.0294	76.906	0.000**
A_1	0.031	0.018	1.733	0.143
A_2	-0.021	0.018	-1.179	0.291
A_3	0.235	0.018	13.039	0.000**
A_1A_1	-0.255	0.0265	-9.628	0.000**
A_2A_2	-0.225	0.0265	-8.497	0.000**
A_3A_3	-0.063	0.0265	-2.371	0.063*
A_1A_2	0.0025	0.0255	0.098	0.925
A_1A_3	0.05	0.0255	1.961	0.107
A_2A_3	-0.005	0.0255	-0.196	0.852

***Significant at P< 5%level, **Significant at P< 1% level.**

The 3D surface response for independent variables, temperature, pH and fermentation time on biomass yield are shown in **Figures 32 to 34**. The biomass yield was significantly affected by pH, temperature and fermentation time. The 3D response surfaces for the biomass yield indicated that the maximum yield of biomass (2.443g dry biomass/ 100ml growth media) was within the range of process conditions predicted as pH 3.65, temperature, 29.7^0C and fermentation time, 56 h. The validation experiment conducted with the conditions obtained showed a response yield of 2.42 g dry biomass/100ml growth media **(Table-11)**. This indicated that the response model adequately predict the yield. The ARD of the predicted yield from experimental yield for biomass was found to be 1%.

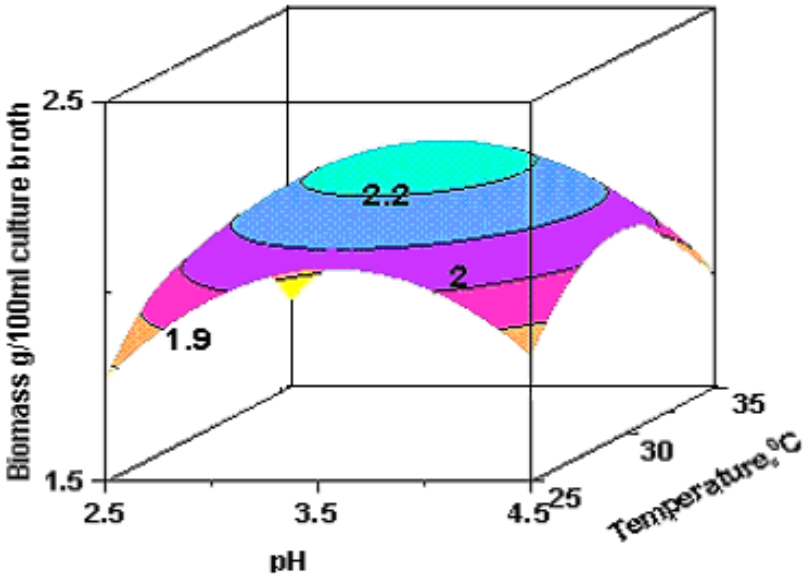


Figure: 32. Three dimensional contour plot showing the effect of pH and temperature on biomass yield (fermentation time 44 h)

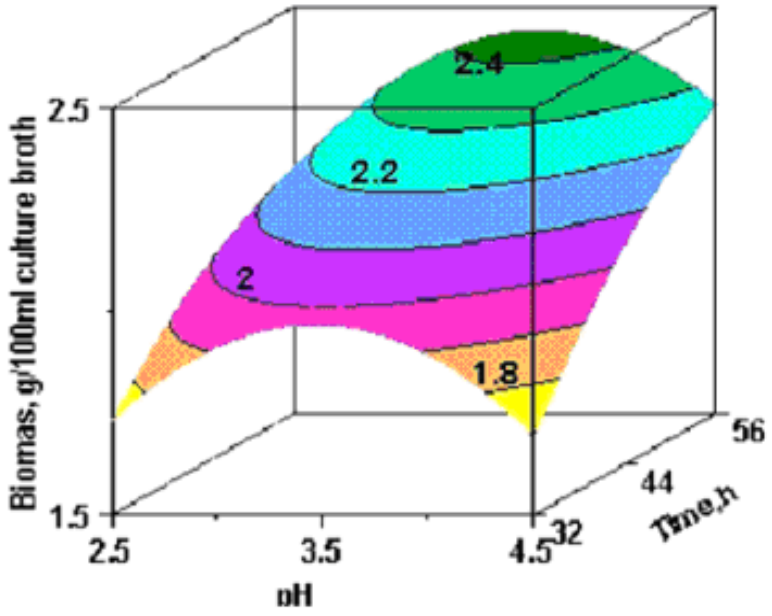


Figure: 33. Three dimensional contour plot showing the effect of pH and Fermentation time on biomass yield (temperature, 30^0C)

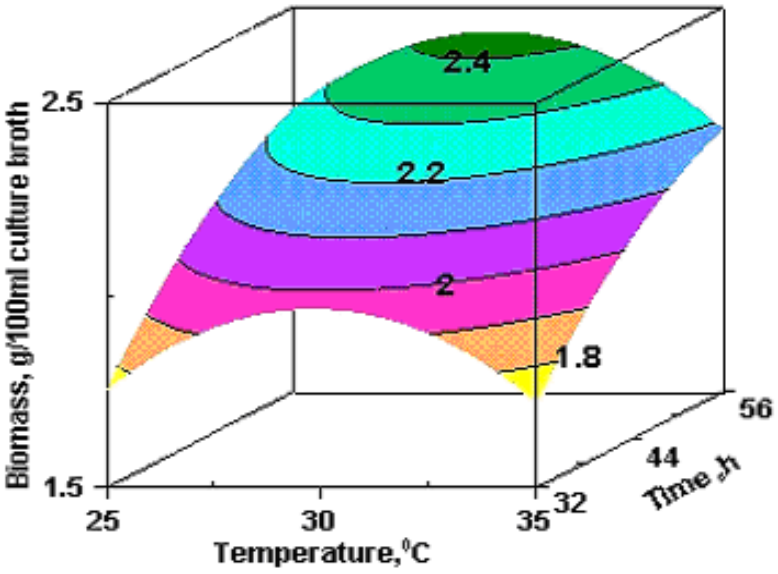


Figure: 34. Three dimensional contour plot showing the effect of temperature and fermentation time on biomass yield (pH, 3.5)

Table: 11. Validation experiment of the process conditions for production of xanthin and biomass as predicted by the model

Dependent variables	Independent variables			Yield		
	pH	Temp. (^{0}C)	Time (h)	Predicted	Observed	Relative Deviation (%)
Xanthin (µg g^{-1} dry biomass)	3.31	29.3	46.7	1283.00	1298.00	0.40
Biomass (dry weight g /100ml culture media)	3.65	29.7	56.0	2.443	2.426	0.69

4.4 OPTIMIZATION OF CARBON SOURCES FOR THE PRODUCTION OF XANTHIN FROM MUTANT *A. CARBONARIUS*

Aspergillii use a wide variety of organic compounds as carbon and nitrogen source (Cochrane 1958). Certain amino-acids serve as poor carbon source for *Aspergilli* even though they are used as nitrogen sources (Kinghorn and Pateman 1977). Carbohydrate utilization is also influenced by its concentration in the medium, trace metals and growth pH (Perlamn 1965). Of all the carbohydrates, glucose, fructose and mannose are readily utilized by the fungus but galactose is less metabolized. Xylose, pentose, arabinose and other pentoses support the growth of the fungus as they are incorporated into pentose phosphate pathway. Glucose reportedly suppresses the uptake of galactose (Berry 1975).

Aspergillus possesses both inducible and constitutive uptake system. Regulation of hexose uptake by metabolic derivatives of acetate i.e. acetyl CoA was demonstrated by Romano and Cornberg (1968). Regulation of main catabolic enzyme or specific uptake system was suggested as a cause for the difference in the growth of *Aspergillus* on different carbon sources (Romano and Cornberg 1968). The most extensively studied form of carbon regulation in *Aspergillus* is carbon repression, wherein, easily metabolized carbon source temporarily prevent the uptake of other less favorable carbon sources (Ronne 1995). Mediation of carbon regulation through carbon repression protein A (CREA) has been demonstrated in the fungus. CREA inhibits transcription of many targeted genes by binding to specific sequences in the promoter region (Kulumberg *et al.* 1993).

A number of studies were carried out on optimization of carbon sources for the production of metabolites in fungi. Sati and Bisht (2006), on utilization of various carbon sources for the growth of waterborne conidial fungi, showed that glucose and sucrose were suitable carbon sources for *Tetracheatum elegans*, *Tetracladium marchalianum*, *Pestalotiopsis submersus* and *Flagellospora penicillioides.* Similarly, another study on effect of starch and sucrose on red pigment production by *Paecilomyces sinclairii* by Cho *et al.* (2001) showed that in sucrose medium the specific growth rate was higher compared to starch medium. However, the specific production rate of red pigments was favored in starch medium. Yet another study on effect of carbon source on production of β-galactosidase from *Aspergillus oryzae* showed that glucose serves as a best carbon source, followed by lactose, maltose and sucrose (Nizamuddin *et al.* 2008).

Study on the mutant *A. carbonarius* showed that aeration, pH, temperature and incubation time were important factors for the production of xanthin (Experiment No. 4.2). Further, most of the work carried out on optimization of cultural parameters showed that, other than the above described media components, carbon source also influences the metabolite production in filamentous fungi. Hence, experiments were carried out to identify a suitable carbon source for increasing xanthin yield from mutant *A. carbonarius*.

The fungus was grown in different carbon sources (3%) like, corn flour, corn starch, sorghum starch, glucose, wheat flour, galactose, xylose, mannose and pectin in a medium containing ammonium salts (Na_2HPO_4 and NaH_2PO_4) as nitrogen source. pH of the culture medium was adjusted to 3.0 with phosphate citrate buffer. After 48 h fermentation, pigment was extracted in ethanol and quantified (Materials and Methods).

Experimental results showed that corn flour as carbon source produced more pigment (1.270 mg/g dry biomass) **(Table 12)**. Yield of xanthin from the fungal biomass grown in media containing corn starch, sorghum starch and glucose were 1.063; 0.756 and 0.584 mg/g dry biomass respectively. Xanthin production was poor when the mutant fungus was grown in wheat flour, mannose, galactose, pectin and xylose.

Table: 12. Effect of carbon sources on the yield of xanthin from mutant *A. carbonarius*

	Carbon source	Biomass yield *	Xanthin yield @	Final pH of the culture filtrate
1	Corn flour	2.78	1.27	2.85
2	Corn Starch	0.99	1.06	2.89
3	Sorghum starch	0.90	0.75	2.91
4	Glucose	1.64	0.58	2.92
5	Wheat flour	2.20	0.05	2.86
6	Galactose	0.98	0.20	2.85
7	Xylose	0.96	0.13	2.95
8	Mannose	0.84	0.12	2.89
9	Pectin	1.22	0.16	2.86

*** dry weight g /100ml media, @ mg g^{-1} dry biomass**

4.5 OPTIMIZATION OF NITROGEN SOURCES FOR THE PRODUCTION OF XANTHIN FROM MUTANT *A. CARBONARIUS*

Fungi while using diverse nitrogen sources for growth express catabolic enzymes of many different pathways. *Aspergillus*, *Neurospora*, and many other fungal species use inorganic nitrate (Cove 1979). Utilization of nitrate requires the de-novo synthesis of nitrate reductase and nitrite reductase, which involves both nitrogen de-repression and specific induction by nitrate (George 1997).

Nitrogenous compounds such as ammonia, glutamine and glutamate are preferentially used by filamentous fungi, and asparagine is a preferred nitrogen source in yeast. However, when these primary nitrogen sources are not available or are present at a concentration insufficient to support the growth, other nitrogen sources like nitrate, nitrite, purines, amides, amino acids and proteins are used by fungi for growth (George 1997).

There are various reports on the effect of nitrogen source on the production of different metabolites in fungi. In a study to optimize lovastatin production by *Aspergillus terreus* using RSM, it was found that, nitrogen was a limiting factor. Concentration of 0.2 M retarded the growth while enhancing lovastatin concentration (Casas *et al.* 2004). Ravindar *et al.* (2006) showed that maximum protein enrichment in microbial conversion of de-oiled rice bran into single cell protein (SCP) occurred when ammonium sulfate was used as nitrogen source. Similarly, the yield of lactic acid from *Rhizopus* was greater when ammonium sulphate was used (Zhang *et al.* 2007).

Carotenoid biosynthesis in immobilized *Geotrichum fujikuroi* mycelia strictly depended on nitrogen limitation in the culture medium. The production occurred at low

(0.1 g^{-1}) nitrogen concentration (Garbayo *et al.* 2003). In the light of these references experiment was carried out to determine the effect of nitrogen sources on xanthin production in mutant *A. carbonarius*.

Nitrogen sources like sodium dihydrogen phosphate, corn step liquor, urea, diammonium hydrogen phosphate, ammonium dihydrogen phosphate, yeast extract, peptone, and ammonium nitrate were used in the experiment. The nitrogen source was added to the growth medium containing 3% corn flour and pH was adjusted 3.0 using phosphate citrate buffer. Biomass was harvested after 48 h fermentation and xanthin extracted from it was quantified (Materials and Methods).

Table-13 shows the xanthin yield from mutant *A. carbonarius* grown in media containing different nitrogen sources. Result showed that ammonium salts were found to be the most suitable nitrogen source with highest (1.256 mg/g dry biomass) xanthin yield followed by ammonium nitrate and urea.

Table: 13. Effect of nitrogen sources on xanthin production from mutant *A. carbonarius*

	Nitrogen source	**Biomass yield ***	**Xanthin yield @**	**Final pH of the culture filtrate**
1	Sodium dihydrogen phosphate	1.21	0.29	2.82
2	Corn step liquor	2.04	0.29	2.83
3	Urea	2.54	0.46	2.94
4	Di-ammonium hydrogen phosphate.	2.35	1.25	2.80
5	Yeast extract and peptone	1.62	0.21	2.84
6	Ammonium nitrate	1.82	0.56	2.90

*** dry weight g /100ml media, @ mg g^{-1} dry biomass**

4.6 EFFECT OF CARBON AND NITROGEN RATIO ON XANTHIN PRODUCITON FROM MUTANT *A. CARBONARIUS*

In submerged fermentation, the ratio of carbon to nitrogen affect fungal morphology and the metabolites it produces (George 1997). Several studies have been carried out on the effect of carbon and nitrogen ratio on fungal metabolism. In *Phaffia rhodozyma,* astaxanthin production was enhanced by an initial high carbon/nitrogen ratio (C/N ratio) in the medium, but cell growth was inhibited by a high glucose concentration (Yamane *et al.* 1976). Likewise, in *Mortierella alpine,* production of arachidonic acid was influenced by consumed carbon to nitrogen in both shake flasks and fermentor studies (Adejoye *et al.* 2006). The study on effect of C/N ratio on the production of lactic acid from *Rhizopus* in submerged fermentation showed that, optimum C/N ratio was in the range of 15:20 with a balance between the amounts of carbon and nitrogen sources (Zhang *et al.* 2007). Similarly, in *Aspergillus oryzae* production of β-galactosidase was optimum when C/N ratio was in the range of 8:10 with glucose and ammonia as the carbon and nitrogen sources respectively (Nizamuddin *et al.* 2008).

In this experiment C:N ratio was optimized for the production of xanthin form mutant *A. carbonarius.* Glucose was used as carbon source and ammonium salts were used as nitrogen source with the molar ratio ranging from 1:1 to 1:12. Mutant *A. carbonarius* was grown at varying nitrogen concentration, keeping glucose concentration constant. Fermentation was carried out for 48 h at 30 ^{o}C. The baffled flasks after inoculation were kept on a rotary platform shaker at 200 rev/min. Biomass was harvested using cheese cloth and xanthin was extracted and quantified spectrophotometrically (Materials and Methods).

C:N ratio affected the pigment yield significantly. Xanthin yield increased gradually when the C:N molar ratio increased from 1:1 to 1:6. However, above 1:6 C:N molar ratio, the xanthin yield decreased (**Table-14**).

Effect of fermentation time, 12 to 72 h was also predicted with optimized C:N ratio of 1:6. The pigment yield with respect to fermentation time is described in **Figure-35**. Pigment synthesis started after 16 h fermentation (0.253 mg/g dry biomass) and gradually increased to reach the maximum after 44 to 48 h fermentation (1.296 mg / g dry biomass). After 48 h fermentation, xanthin yield decreased due to the initiation of sporulation. However the yield of biomass increased up to 62 h fermentation.

Table: 14. Effect of C:N ratio on xanthin yield from mutant *A. carbonarius*

Sl. No	C: N ratio	Biomass yield *	Xanthin yield @	Final pH of culture filtrate
1	1: 12	0.61	0.65	2.92
2	1:11	0.62	0.95	2.95
3	1:10	0.65	1.16	2.92
4	1:9	0.72	1.27	2.95
5	1: 8	0.75	1.28	2.98
6	1:7	0.76	1.29	2.94
7	1: 6	0.78	1.18	2.96
8	1: 5	0.74	1.14	2.95
9	1: 4	0.68	1.12	2.98
10	1: 3	0.68	0.96	2.99
11	1: 2	0.76	0.84	2.95
12	1: 1	0.59	0.72	2.96

*** dry weight g /100 ml media, @ mg g^{-1} dry biomass**

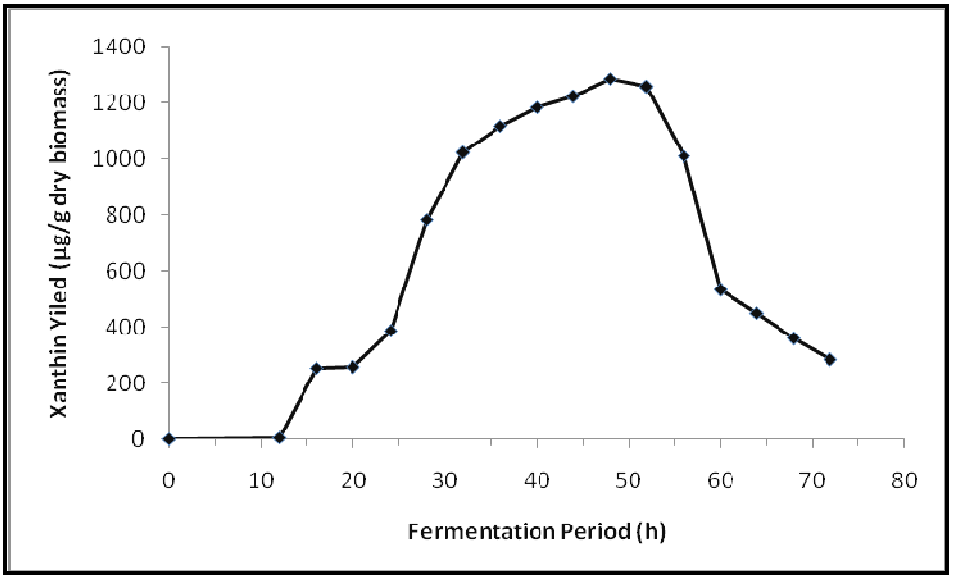


Figure: 35. Xanthin yield from mutant *A. carbonarius* grown in baffled flask at different fermentation periods

4.7 SCALEUP STUDIES FOR XANTHIN PRODCTION USING MUTANT *A. CARBONARIUS*

Studies on optimization of cultural conditions showed pH and aeration affected xanthin production in mutant *A. carbonarius*. In order to carry out a controlled fermentation by monitoring pH and aeration, 1.3 L New Brunswick fermentor was used. The fungal growth medium (1L) made with (g/L) corn flour (40), $(NH_4)_2HPO_4$ (5) and $NH_4H_2PO_4$ (5) adjusted to pH 5.5, was sterilized at 121^oC for 15 minutes. After cooling, the medium was inoculated with 130 mL of 48 h old culture grown in shake flasks. The fungus was grown at 30^oC and the pH was allowed to drop to 3.0 and further maintained during growth, by the addition of 2M $NH_4H_2PO_4$. All through the fermentation period the dissolved oxygen was maintained at 40-60% by agitation.

The xanthin obtained from mycelia of mutant *A. carbonarius* after growth for various periods of time in the fermentor is shown in **Table-15**. Visible xanthin production in mycelia (yellow colour) occurred after 16 h growth. From these mycelia, 0.240 g xanthin could be extracted. Maximum xanthin yield (1.540 mg g^{-1}) was obtained after 36 h fermentation.

Table: 15. Xanthin yield from mutant *A. carbonarius* grown in a fermentor.

Sl. No	Fermentation period (h)	xanthin (mg g^{-1}dcm)
1	16	0.240
2	20	0.256
3	24	0.376
4	30	0.731
5	36	1.540
6	42	1.364
7	48	1.352

BIOACTIVITY OF MUTANT *A. CARBONARIUS* XANTHIN AND IT'S SAFETY EVALUATION

Diets provide a diverse array of antioxidants like vitamins E and C, flavonoids, and carotenoids. Of this, carotenoids are not only essential for human health but are also considered as preventive and therapeutic agents. The consumption of carotenoids decreases the risk of degenerative and cardiovascular diseases. Carotenoids act as antioxidants due to their ability to quench singlet oxygen and to scavenge free radicals (Britton *et al.* 1995). One of the most widely discussed roles of carotenoids is the interaction with free radicals that initiate harmful reactions such as lipid peroxidation (Qian *et al.* 2000). This was evidenced by the reaction of carotenoids with free peroxyl radicals as an additional chain-breaking process (Edge *et al.* 1997). The association of distinct carotenoids with specific tissues in humans and other primates (tissue tropism) suggested a targeted evolutionary role of carotenoids in protection against various reactive oxygen species (Edge *et al.* 1997). Carotenoids can react with ferric ions in metal-induced lipid peroxidation and result in the formation of carotenoid radical cations (Wei *et al.* 1997).

The antioxidant activities of β-carotene incorporated into biological membranes and model membrane systems have been studied extensively (Mann *et al.* 2000). Astaxanthin (3,3$^{!}$-dihidroxy-ß,ß-carotene-4,4$^{!}$-dione) is known for its higher antioxidative activity than ß –carotene, canthaxanthin and vitamin-E (Johnson 2002; Lawlor and O'Brien 1995). A study conducted with Cu^{+} initiated lipid peroxidation on liposomes showed the protection of liposomes by dietary feeding of astaxanthin and canthaxanthin (Rengel *et al.* 2000). Studies have also demonstrated protective action of astaxanthin and

canthaxanthin on plasma, isolated lymphocytes, neutrophils, LDL and microsomal fractions from various tissues (Carpenter *et al.* 1997; Panasenko *et al.* 2000). Dietary feeding of astaxanthin containing yeast suppressed lipid peroxide generation in tissue, normalized liver function and improved muscle pigmentation of trout (Nakano *et al.* 1995).

Astaxanthin is well known to protect membranous phospholipids and other lipids against peroxidation (Naguib 2000). Dietary astaxanthin fight against symptoms of ulcer caused by *Helicobacter pylori* through reduction of gastric inflammation and associated shifts in the inflammation response (Bennedsen 1999). Supplementation of astaxanthin in diet helps protection of retinal photoreceptors in the eyes of rats exposed to acute UV-light injury (Tso and Lam 1996). Xenobiotic metabolizing enzymes were induced in rat liver, lung and kidney during astaxanthin supplementation that helps in preventing carcinogenesis (Gradelet 1998; Jewell C and O'Brien 1999). Astaxanthin has been reported to protect the skin and eggs of salmon against UV-light photo-oxidation (Kritchevsky 1999). *In vitro* studies on human blood cells have demonstrated enhancement of immunoglobulin production in response to T-dependent stimuli by astaxanthin (Jyonouchi *et al.* 1995). Astaxanthin supplementation in rats was found to inhibit the stress-induced suppression of tumor-fighting natural killer cells (Kurihara 2002). Canthaxanthin, at higher concentration (100 μM), inhibits the growth of three tumor cell lines, and stimulates the growth of 3T3 cells (a non-tumor cell line), suggesting that canthaxanthin has a direct effect in inhibiting tumor cell growth (Huang *et al.*1992). Further studies carried out reveled β-carotene and canthaxanthin can also

inhibit malignant transformation induced by 3-methylcholanthrene or X-ray treatment in this same cell line (Punget *et al.*1988).

Colorants have been used for many years in the pharmaceutical industry in order to color medicinal products. Adding a colour makes the product attractive, easier to recognize, and in some cases, it stabilizes light sensitive ingredients by forming an opaque layer (Patnaik *et al.*1997). Food colour is a vital quality attribute and plays an important role in sensory and consumer acceptance of products. Today the food industries have an extensive range of both natural and synthetic colors. In line with the generally observed change in trend from synthetic dyes towards natural colorants, coloring foodstuffs with natural colorants is currently experiencing an annual growth rate of 10–15% in the European market, as the latter category does not require E-number declaration. Hence, natural colorants in foodstuffs are expended to substantially benefit from the fast growing functional food market, especially in the dairy and beverage sectors. Colours extracted from fruits, vegetables and microbial source provide supplementary ingredients of healthy and nutritional value (Patnaik *et al.* 1997). Hence, there is considerable interest for food colors of natural origin worldwide.

The microbial colours have the advantage of being climate independent, require smaller area for their growth and can be produced in any quantity in shorter period (Francis 1987). Microorganisms produce a variety of pigments that includes polyketides, carotenoids, phenazines, acylphenols, pyrones, sclertiorins, anthroquinones but most of these pigments are toxic to humans except for carotenoids, anthroquinones and polyketides. Commercially, microorganisms explored for the production of natural colors are β-carotene from *Blakeslea trispora*, polyketides from *Monascus purpureus*

and *Streptomyces coelicolor*, γ-carotene from *Rhodosporidium*, astaxanthin from *Haematococcus pluvialis* and *Xanthophylomyces dendrorhous* and canthaxanthin from *Brevibacterium* (Patnaik *et al.* 1997; Mohan *et al.* 2006). Further, pigments like carotenoids possess nutraceutical properties which gain more importance in food industries (Fontona 2001).

Natural colorants have to undergo rigorous safety assessments before acceptance for food and pharmaceutical use. Global regulatory authorities require that food derived from biotechnology be as safe as food produced from conventional methods. In the early 1960s FDA issued opinion recognizing that α-amylase, cellulase, amyloglucosidase, catalase, glucose oxidase, lipase and pectinase from *A. niger* can be 'Generally Regarded As Safe' (GRAS) under the condition that non-pathogenic, non-toxigenic strains are used with current good manufacturing practices.

A. carbonarius polygalacturonase is used in food industries for preparation of fruit juices. Since the mutant organism produced xanthins, which has possible applications as food colourant, its antioxidant activity and toxicity of the fungus was studied and the details are described in this chapter.

5.1 ANTIOXIDANT ACTIVITY OF XANTHIN EXTRACTED FROM *A. CARBONARIUS* MUTANT

A compound can exert its antioxidant activity by scavenging radicals, decomposing peroxides or chelating metal ions. Understanding the mode of antioxidant activity or free radical scavenging is essential to establish the bioactivity of the compound. Hence, the antioxidant activity of both pure and crude xanthin was carried out by *in vitro* assays for 1, 1-Diphenlyl–2-picrylhydrazyl (DPPH) free radical scavenging, lipid peroxidation inhibition and metal chelation.

5.1.1 DPPH FREE RADICAL SCAVENGING ACTIVITY

The DPPH test is a very convenient method for screening small antioxidant molecules because the reaction can be observed by TLC, dot blot or spectrophotometric methods. In this assay, the DPPH radicals are scavenged by antioxidant molecules through the donation of hydrogen to form stable reduced DPPH molecule. The antioxidant radical formed are stabilized through the formation of non-radical products.

Analysis of DPPH radical scavenging activity of xanthin extracted from mutant *A. carbonarius* showed that among the different solvent extracts, ethanol extract showed highest DPPH free radical scavenging activity. Crude *A. carbonarius* xanthin showed more activity compared to pure xanthin. The EC_{50} values for pure xanthin and crude pigment were found to be 29.0 and 27.3μg ml^{-1} respectively **(Table-16)**. Syntactic antioxidant Butylated Hydroxy Annisole (BHA) showed an EC_{50} value of 15.33 μg ml^{-1} **(Figures-36&37)**.

5.1.2 LIPID PEROXIDATION INHIBITION ACTIVITY

Lipid peroxidation is free radical mediated propagation for oxidation of polyunsaturated fatty acids involving several types of free radicals. Termination occurs through either enzymatic means or by free radical scavenging by antioxidant.

Lipid peroxidation inhibition assay of xanthin from mutant *A. carbonarius* was carried out by determination of melonaldehyde generation, using egg lecithin liposome model. In this procedure malonaldehyde reacts with thibarbitaric acid to form a reduced substance and the antioxidant activity was monitored by decrease in the absorbance at 515 nm.

Crude pigment extract from *A. carbonarius* showed higher lipid peroxidation inhibition compared to pure xanthin. The EC_{50} value for lipid peroxidation inhibition by the pure xanthin and crude xanthin was found to be 46.5 and 44.2 μg ml^{-1} respectively **(Table-16)**. BHA (control) showed lipid peroxidation inhibition activity with EC_{50} value of 85.5μg/ml.

5.1.3 METAL CHELATING ACTIVITY

In lipid peroxidation assay, iron is used as metal ion catalyst. Thus, in principle, it could happen that the ability of xanthin to inhibit lipid peroxidation was due to complexing iron in a catalytically silent form. In order to evidence the result of lipid peroxidation, the xanthin from mutant *A. carbonarius* was also tested for metal binding capacity. The metal chelating ability of xanthin was assayed as competitive ability of xanthin with ferrozine for binding to ferrous ion and avoiding the formation of coloured complex with absorbance peaking at 562 nm.

Both pure xanthin and crude xanthin showed metal chelating activity with higher EC_{50} values. EC_{50} value for pure and crude xanthin was found to be 125.6µg/ml and 112.6µg/ml respectively **(Table-16)**.

The results of the metal chelating activity suggested that the lipid peroxidation inhibitory activity was due to the combined activity of chain termination by scavenging the peroxyl radicals and iron chelation.

Table: 16. Antioxidant activity of ethanol extract of xanthin from *A. carbonarius*

Compounds	**EC_{50} value (µg ml^{-1})**		
	DPPH radical scavenging activity	Lipid peroxidation inhibition activity	Metal chelating activity
Pure Xanthin	29.0 ± 1.2	46.5 ± 2.3	125.6±2.6
Crude Xanthin	27.3 ± 1.3	44.2 ± 1.8	112.6±1.8
BHA	15.3 ± 0.3	85.5 ± 1.6	111.5±1.2

Values are mean ± standard deviation of triplicate analyses. BHA- Butaylated Hydroxy Annisole.

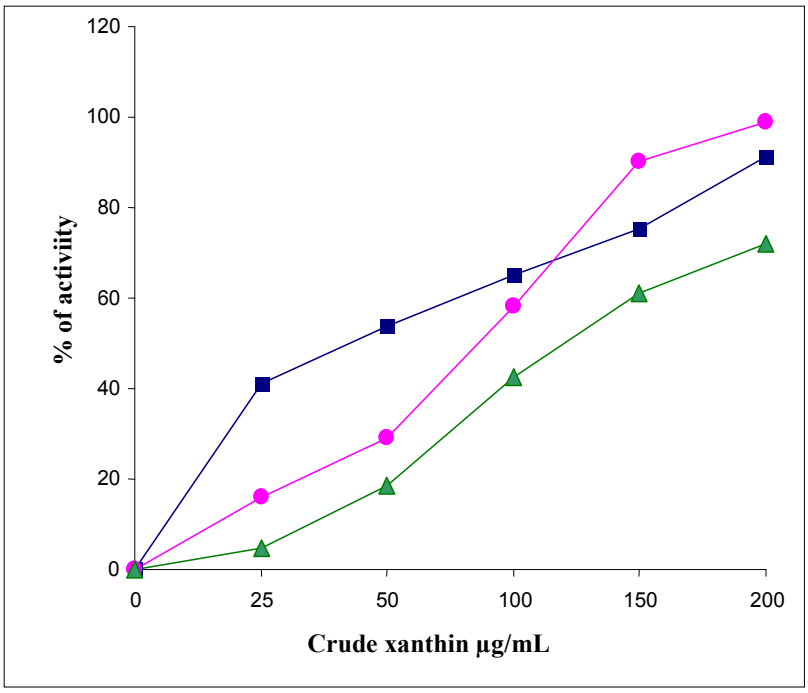


Figure: 36. DPPH free radical scavenging (●), lipid peroxidation inhibition (■) and metal chelating activity (▲)

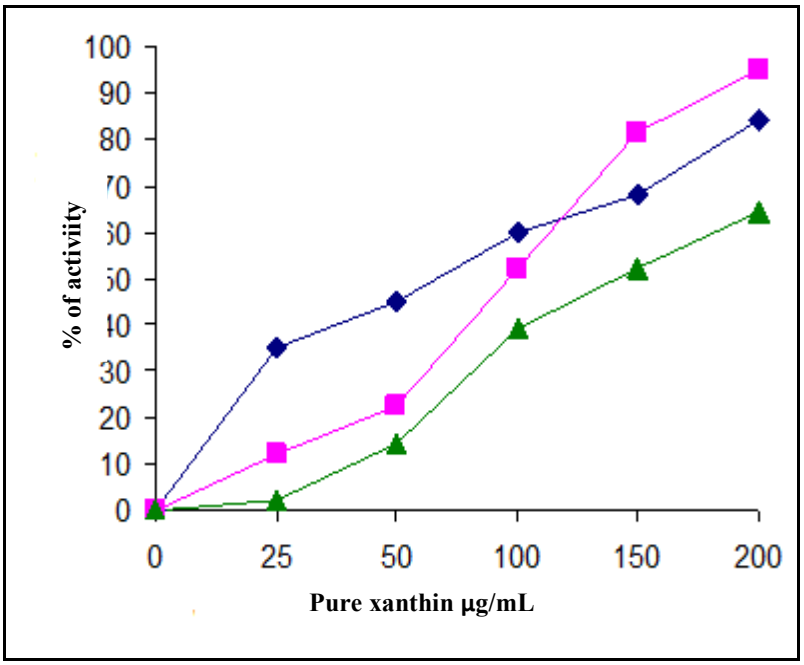


Figure: 37. DPPH free radical scavenging (●), lipid peroxidation inhibition (■) and metal chelating activity (▲)

5.1.4 INHIBITION OF AUTO-OXIDATION OF OILS

There is a strong argument for the use of organic antioxidants from natural sources to prevent deterioration of foods (Kikuzaki& Nakatani 1993). Many fungal metabolites such as carotenoids have also been employed from this point of view (Wang *et al.* 1991).

Based on the results of antioxidant activity of *A. carbonarius* xanthin *in vitro* experiments were carried out to study their effect on preventing auto-oxidation of oils. For this experiment commercial peanut, soybean and palm oil were used. The concentration of the xanthin used was 0.20% w/w of oil. The data on the effect of xanthin on auto-oxidation was obtained by measuring change of peroxide values at 60 ± 2 ^{0}C.

Data showed that both pure and crude xanthin moderately stabilized these oils from autooxidation. Xanthin showed more stabilizing activity on peanut oil compare to soybean and palm oils. Peroxide value of peanut oil with 0.20% xanthin after 20 days of storage was 37.62 and 32.71 meq kg^{-1} for pure and crude pigment respectively. The control samples showed gradual increase in peroxide value from 0.0 to 112.27meq kg^{-1} after 14 days storage **(Figure-38)**. In soybean oil, after 20 days of storage period, peroxide value was found to be 63.45 and 56.90 meq kg^{-1}. Soybean oil with Butylated Hydroxy Toluene (BHT) showed peroxide value of 42.72 meq kg^{-1}**(Figure -39)**. Palm oil peroxide value after addition of 0.2% xanthin was 54.79 and 53.68 (v/v) meq kg^{-1} for pure and crude pigment respectively **(Figure-40)**.

All three pigmented oils used in this study showed higher peroxide values compare to the control (BHT). The results of this experiment showed that pigment was moderately effective in stabilizing oils by reducing auto oxidation.

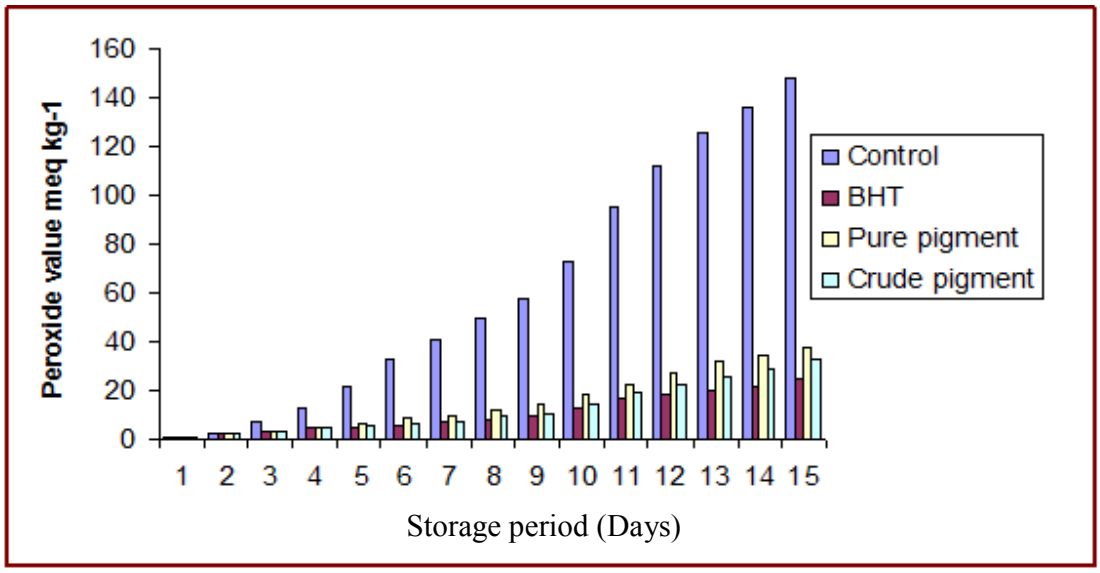


Figure: 38. Effect of xanthin on the formation of peroxides in peanut oil at 60±1°C

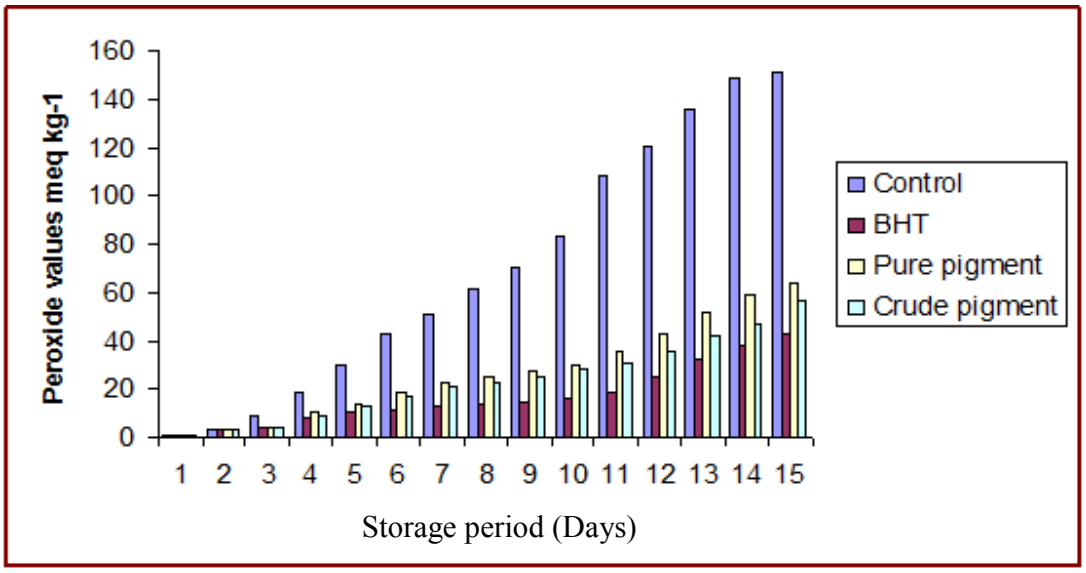


Figure: 39. Effect of xanthin on the formation of peroxides in soybean oil at 60±1°C

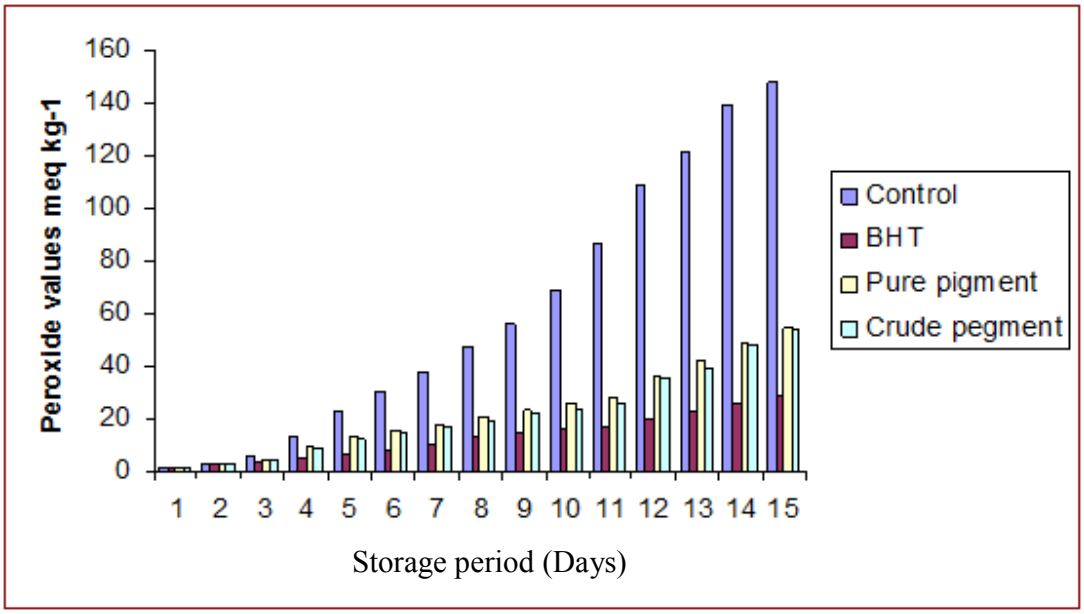


Figure: 40. Effect of xanthin on the formation of peroxides in palm oil at 60±1^{o}C

5.2 ANTIBACTERIAL ACTIVITY OF XANTHIN EXTRACTED FROM MUTANT *A. CARBONARIUS*.

Antibacterial activity of xanthin extracted from *A. carbonarius* biomass was screened by disc diffusion method and minimum inhibitory concentration was determined by broth culture method. Food borne pathogens used in this study were *Bacillus cereus, Pseudomonas aeruginosa, Yersinia enterocolitica, Proteus vulgaris, Listeria monocytogens, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Klebsiella pneumonia* and *Shigella sonnei*. Different concentrations of pure and crude xanthin (25 to 500 μg/ml) were used for minimum inhibitory concentration determination (Materials and Methods).

5.2.1 DISC DIFFUSION ASSAY

Preliminary screening for antibacterial activity by disc diffusion assay showed that the xanthin was effective against some of the tested food borne pathogenic bacteria **(Figures-41 & 42)**. Inhibition zone was significant (12–14 mm) against *Streptococcus pyogenes, Yersinia enterocolitica, Proteus vulgaris* and *Listeria monocytogen* and moderate (8-10 mm) against *Staphylococcus aureus, Klebsiella pneumonia and Bacillus cereus*. The greatest zone of inhibition (14mm) was observed against *Streptococcus pyogenes* and *Yersenia enterocolitica* **(Table-17).**

Table: 17. Antibacterial activity of mutant *A. carbonarius* xanthin

Microorganism	Pure xanthin	Crude xanthin	Oxacillin (control)	Norflo-xacine (control)	DMSO (control)
	Zone of inhibition (mm)				
Bacillus cereus	8.0	8.0	12.0	-	A
Pseudomonas aeruginosa	5.0	6.0	-	26.0	A
Yersinia enterocolitica	13.0	14.0.	-	18.4	A
Proteus vulgaris	12.0	13.0	-	22.0	A
Listeria monocytogen	12.0	12.0	18.2	-	A
Staphylococcus aureus	10.0	10.0	14.0	-	A
Streptococcus pyogenes	12.0	14.0	21.0	-	A
Escherichia coli	2.0	3.0	-	20.0	A
Klebsiella pneumonia	9.0	10.0	-	22.0	A
Shigella sonnei.	5.0	5.0	-	18.5	A

Disc size: 6mm, concentration of xanthin: 30µg /disc, oxcillin 1µg /disc, norfloxacine 10µg /disc,

[A] absence of inhibition.

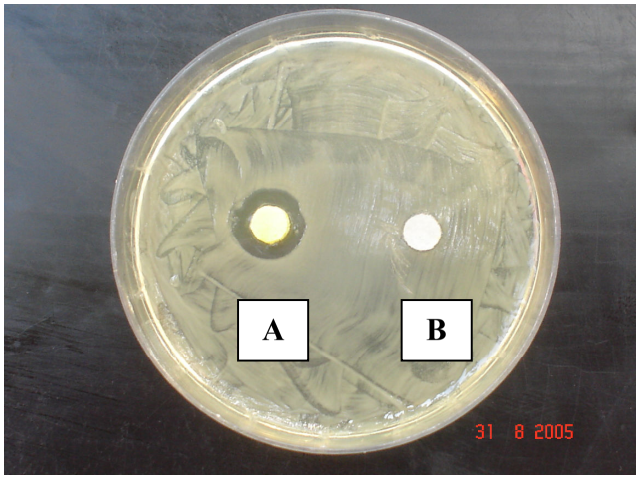
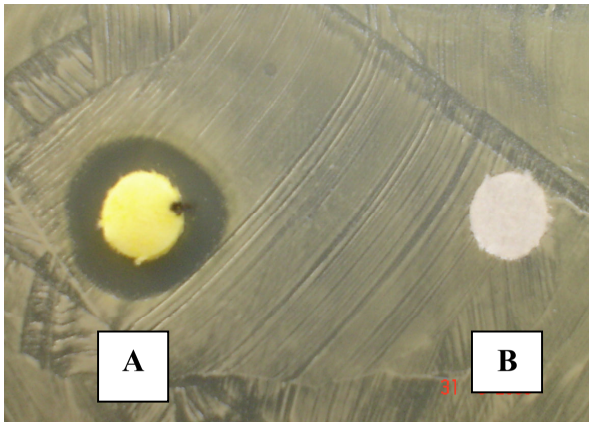


Figure: 41. Effect of xanthin on *Streptococcus pyogenes* growth (A-zone of inhibition, B-control)



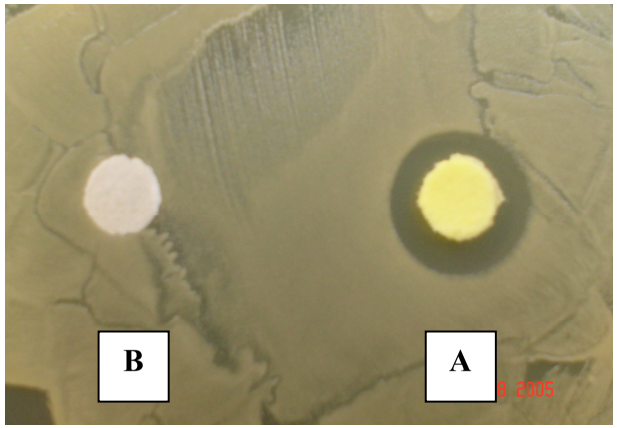


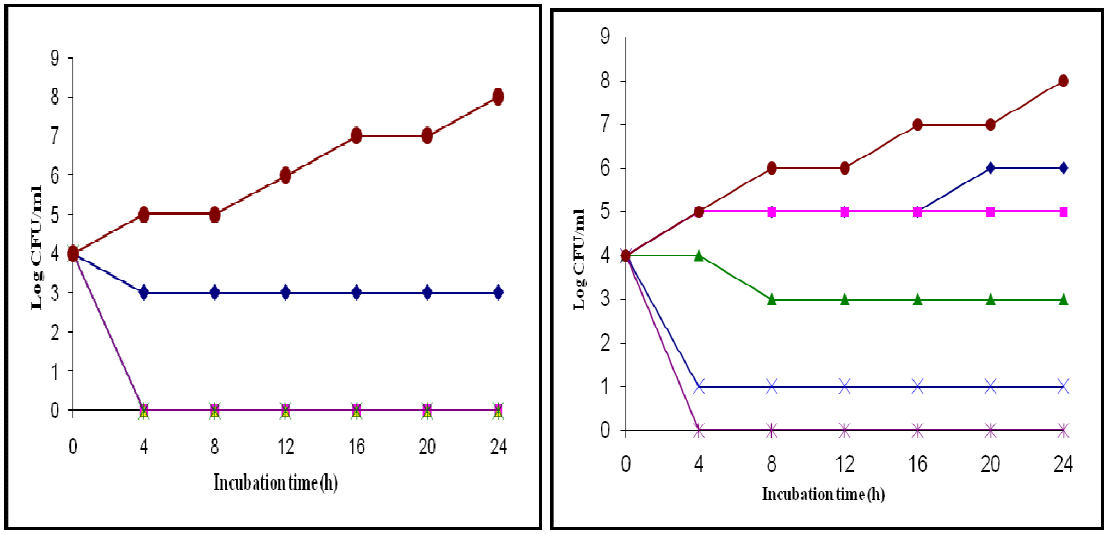
Figure: 42. Effect of xanthin on growth of *Yersinia enterocolitica* and *Proteus vulgaris* (A-zone of inhibition, B-control)

5.2.2 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

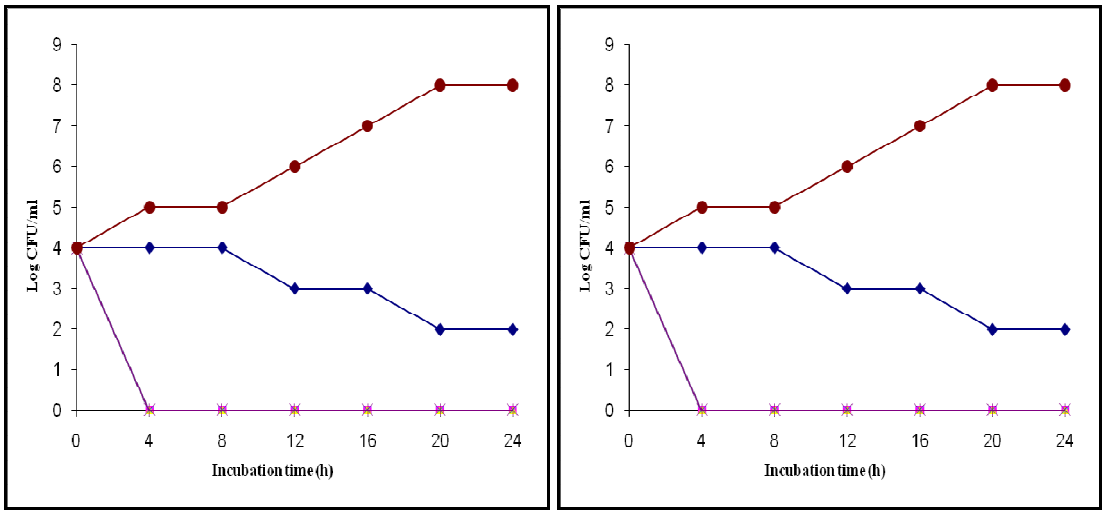
In order to determine the minimum inhibitory concentration (MIC) of xanthin, required to inhibit the growth of bacteria, viability tests were performed. Five food borne pathogens: *Streptococcus pyogenes, Escherichia coli, Staphylococcus aureus, Yersinia enterocolitica* and *Bacillus cereus* were treated with the xanthin (Meterial and Methods).

Xanthin concentration of 50 µg/ml was determined as the MIC for *Streptococcus pyogenes, Bacillus cereus* and *Yersinia enterocolitica*. The MIC for *Staphylococcus aureus* and *Escherichia coli* was higher as 200 µg/ml and 400 µg/ml respectively **(Figure-43)**.

Antibacterial studies showed that there was no uniform response among bacterial strains in terms of susceptibility to pigment. The difference in susceptibility can be attributed to differences in cell wall composition. Results also showed the lysis of bacterial cells in presence of xanthin at higher concentration (400 µg/ml).



Streptococcus pyogenes *E. coli*



Yersenia enterocolitica *Bacillus cereus*

Figure: 43.Minimum inhibitory concentration of xanthin against food borne pathogens (●: Control, ◆: 25µg/ml, ■: 50µg/ml, ▲: 100 µg/ml. X: 200µg/ml, Ж:400µg/ml)

5.2 ACUTE AND SUB ACUTE TOXICITY STUDIES USING MUTANT *A. CARBONARIUS* PIGMENTED BIOMASS

Mutant *A. carbonarius* accumulated a mixture of xanthin in its biomass during growth at acidic pH. Since the pigment had characteristics for use as food colorant and the pigmented fungal biomass was also a good source of protein (22% on dry weight basis). Toxicological evaluation of the organism was carried out with freeze-dried biomass of the mutant *A. carbonarius*.

Acute and Sub-acute studies, by dietary feeding of *A. carbonarius* biomass, were carried out using CFT Wister strain albino rats. The animals were obtained from the stock colony of the CFTRI animal house facility and were kept in separate individual stainless steel cages with screen bottom. Ethical guidelines laid down by the committee for the purpose of supervision of experiments on animals by the Government of India, Ministry of Social Justice and Empowerment. Details and procedure followed for toxicological evaluation were given in section 2.23 of Materials and Methods.

The animal feed used for the experiment is given in **Table-18**. The proximate composition of *A. carbonarius* biomass showed, 78% carbohydrate, 16 % protein, 2 % lipid, 2.0 % minerals, 1.8 % moisture and 0.17 % xanthin.

Table: 18. Composition of basal diet (100 g) of rat

Constituents	Rat diet	Diet with 2% fungal biomass
Crude protein	22.18	22.50
Crude carbohydrate	60.31	61.81
Crude fat	3.38	3.42
Crude fiber	3.35	3.35
Ash	6.28	6.33
Moisture	4.50	4.54

5.3.1 ACUTE TOXICITY STUDIES

Acute toxicity studies evaluate the risk from a single exposure to a substance, typically at a high dose. Acute oral and dermal toxicity studies are frequently designed to express the potency of a substance in terms of a median lethal dose or LD_{50}. The LD_{50} is the dose that is lethal to 50 percent of the laboratory animals in the test. Higher the LD_{50} dose lower the toxicity. The dose is calculated as mg of the test substance/ kg body weight of the tested animals.

Acute toxicity study was carried out using 25 male CFT Wister strain albino rats. Different concentrations (0.5, 1.0, 2.5 and 5.0 g/kg body weight) of *A. carbonarius* pigmented biomass was mixed with diet and fed to experimental rats. Rats were kept under observation for 24 hours for any mortality or hypersensitivity reactions.

Results of acute toxicity study showed that rats fed on *A. carbonarius* biomass in the feed did not develop any clinical signs of toxicity either immediately or during the post treatment even at a highest dose of 5.0 g/kg body weight.

Feeding fungal biomass at different levels did not cause any alteration in food intake and body weight gain of experimental rats compared to control rats. After seven day observation period, relative organ weight of kidney, heart, brain, liver, lungs, spleen, adrenals and gonads of treated groups were comparable to that of controls **(Tables-19&20)**. Further, the hematological profiles **(Table-21)** of both experimental and control group showed no significant differences. Histological examination of vital organs after autopsy did not show any abnormalities in both *A. carbonarius* biomass fed group and control group. Microscopic examination of organs for histopathological alterations did not record any abnormalities in any of the vital organs of the treated animals.

Table: 19. Relative organ weight of rats fed acute dose of *A. carbonarius*

Dose (g/kg body weight)	Relative organ weight (g/100g body weight)							
	Liver	Lungs	Kidney	Heart	Spleen	Testis	Brain	Adrenals
5.0	3.59±0.20	0.44±0.03	0.61±0.01	0.28±0.01	0.22±0.02	1.12±0.06	0.66±0.04	0.02±0.0
2.5	3.70±0.21	0.50±0.03	0.62±0.01	0.30±0.01	0.25±0.02	1.11±0.06	0.65±0.04	0.02±0.0
1.0	3.27±0.20	0.47±0.03	0.62±0.01	0.30±0.01	0.23±0.02	1.05±0.06	0.62±0.04	0.02±0.0
0.5	3.78±0.23	0.52±0.03	0.60±0.01	0.29±0.01	0.22±0.02	1.06±0.06	0.62±0.04	0.02±0.0
Control	3.70±0.20	0.47±0.03	0.61±0.01	0.30±0.01	0.22±0.02	1.11±0.06	0.66±0.04	0.02±0.0

Values are mean ± SEM of five animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 20. Food intake profile (g/rat/day) of male rats in acute toxicity studies

Dose g/kg body weight	Days						
	1	2	3	4	5	6	7
5.0	14.2±0.8	14.9±0.5	14.8±0.6	15.0±1.2	14.7±0.8	14.6±1.0	14.5±0.5
2.5	14.4±0.9	15.0±0.9	15.0±0.8	14.6±1.0	14.7±0.9	14.2±0.5	14.2±0.6
1.0	15.0±1.0	15.0±0.6	15.0±0.9	15.0±0.9	14.6±0.6	14.5±0.6	14.3±0.8
0.5	14.8±1.1	14.8±0.4	15.0±0.5	15.0±1.0	14.9±0.7	15.0±0.5	15.0±0.7
Control	14.6±0.8	14.8±0.5	14.9±0.8	15.0±0.6	15.0±0.4	15.0±0.8	14.8±0.9

Values are mean ± SEM of five animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 21. Hematological profile of male rats in acute toxicity studies

Dose g/kg body weight	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
5.0	15.0±0.6	8.2±0.2	8.6±2.1	38.0±2.6	15.2±1.3	38.5±0.9	72.2±2.2	25.5±2.2	1.4±1.1	1.0±0.0	-
2.5	14.8±0.6	8.5±0.5	6.9±4.8	38.7±1.7	13.5±0.7	40.9±0.9	60.4±3.0	22.7±2.6	1.6±1.4	1.0±0.0	-
1.0	14.8±0.8	9.1±0.4	7.8±2.2	37.0±2.1	13.6±0.8	34.2±0.9	77.2±2.1	22.8±4.2	1.6±0.8	1.5±0.2	-
0.5	14.7±0.9	9.2±0.3	8.7±2.6	38.5±0.6	13.9±1.1	34.3±0.9	77.4±2.0	21.2±6.6	1.5±0.5	1.0±0.0	-
Control	14.9±0.4	9.1±0.6	8.4±1.2	39.3±2.1	14.7±1.0	36.6±1.1	69.0±3.1	29.6±3.6	1.5±0.7	1.0±0.0	-

Values are mean ± SEM (standard error mean) of five animals.
Hb – Hemoglobin , RBC – Red blood cells, WBC - White blood cells, PCV- Packed cell volume, MCH- Mean corpuscular hemoglobin, MCHC - Mean corpuscular hemoglobin concentration, DC - Differential count, L- Lymphocytes, P- Polymorph neutrophils, M- Monocyte, E - Eosinophils, B - Basophils. No significant difference between control and *A. carbonarius* biomass fed groups ($P<0.05$).

5.3.2 SUB-ACUTE TOXICITY STUDIES

The acute toxicity studies determine LD_{50} dose of compound in test animals via a specific route of exposure, but they do not indicate the doze carusing the risk. That determination is made by examining effects seen over a range of doses and durations of time. Subacute studies last for a few weeks to months (~10 percent of the normal life span of the test animal). Exposure routes are identical to those of acute testing programs (oral, dermal, inhalation). In subacute oral toxicity studies, groups of test animals were given various daily doses, from 0.0 to 1000 mg/kg of their body weight. At the end of the designated exposure period, every organ system and physiological parameters are examined to determine differences between exposed and non-exposed test animals. High doses must elicit sub-lethal effects, middle doses must evoke only minimal adverse effects and low doses should trigger no toxic effects. Generally, three to five dose levels are tested in subacute toxicity.

Subacute toxicity studies were carried out by dietary feeding different concentration (0.25 - 2.0% w/w of diet) of *A. carbonarius* biomass for 14 weeks. Results of feeding *A. carbonarius* biomass for longer time did not induced any mortality in experimental animals. The following section explains the results obtained from sub acute toxicity studies.

5.3.2.1 Food intake, body weight and organ weight

In general, incorporation of fungal biomass even at 2% level did not significantly affect the food intake in both the sexes of treated groups and were comparable to that of control group **(Figure- 44&45)**. Food intake profile of all the experimental and control groups were in the range of 5 -20 g between 0 – 14 weeks of time interval. The actual

food intake, mean body weight gain and actual intake of *A. carbonarius* biomass/kg body weight are presented in **Table-22 &23**. No difference in food intake, body weight gain was observed in treated groups of both sexes during the 14 week experimental period. Even though actual biomass intake varied between different groups (0.35 – 3.21 g/kg body weight), it did not influence on differences in body weight gain (36.0 – 298.0 g) of experimental animals **(Figure-46&47)**. All the vital organs after 14 weeks of treatment appeared normal in both *A. carbonarius* biomass fed group and control group. There were no marked differences in mean relative organ weights of various vital organs such as kidney, heart, brain, liver, lungs, spleen, adrenals and gonads between biomass fed rats and control rats **(Table-24&25)**.

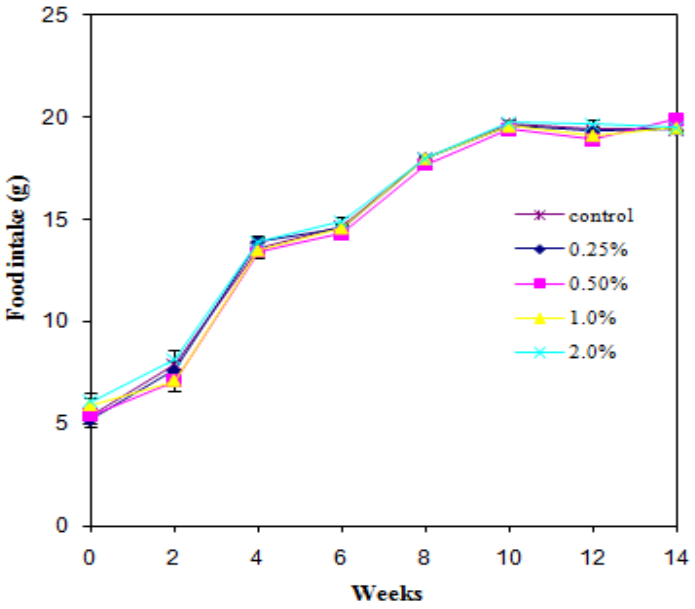


Figure: 44. Food intake profile of male rats fed with dietary *A. carbonarius* biomass for 14 weeks. Values are ± SEM of 5 animals. No significant difference between control and *A .carbonarius* biomass fed groups (P<0.05)

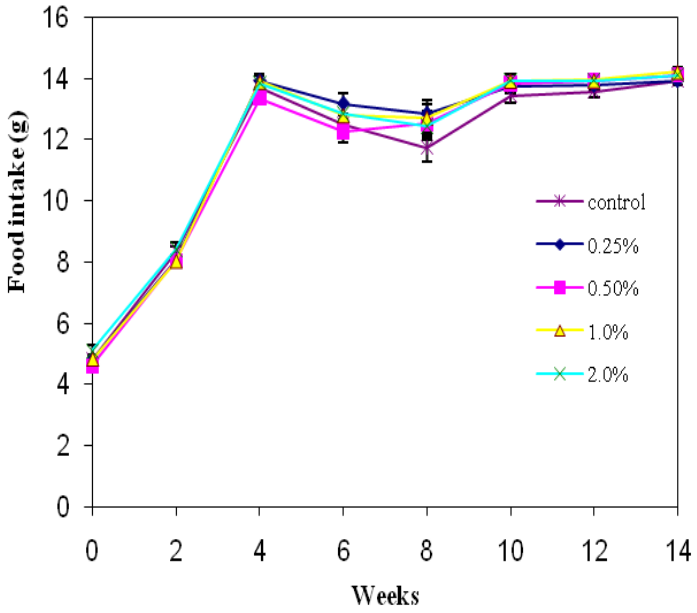


Figure: 45. Food intake profile of female rats fed with dietary *A. carbonarius* biomass for 14 weeks. Values are ± SEM of 5 animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 22. Food intake, Body weight gain and biomass intake of male rats fed with dietary *A. carbonarius* biomass for 14 weeks

Biomass concentration	Control			0.25%			0.5%			1.0%			2.0%		
weeks	food intake (g/rat/day)	body weight (g/rat/week)		food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)
1	5.4	38.2	-	5.2	37.4	0.35	5.4	36.0	0.75	5.9	38.0	1.55	6.1	38.0	3.21
2	6.4	60.9	-	6.4	53.7	0.30	6.2	56.0	0.55	6.5	60.0	1.08	6.8	62.9	2.16
3	7.9	89.8	-	7.6	79.9	0.24	7.1	88.2	0.40	7.5	87.7	0.81	8.2	96.0	1.70
4	10.4	127..0	-	10.2	107.6	0.24	10.3	120.8	0.43	10.5	109.9	0.96	10.6	129.7	1.63
5	13.6	152..8	-	13.9	132.3	0.26	13.4	141.4	0.47	13.5	151.7	0.89	13.9	154.0	1.81
6	13.8	180..9	-	13.6	160.6	0.21	13.5	167.0	0.40	13.6	176.9	0.77	13.8	184.0	1.50
7	14.7	190..6	-	14.6	179.6	0.20	14.3	180.0	0.40	14.6	190.3	0.77	14.9	193.2	1.54
8	16.1	219.0	-	16.5	211.0	0.20	16.8	205.0	0.41	16.5	216.0	0.76	16.8	213.0	1.58
9	18.0	235.8	-	18.0	228.0	0.20	17.7	220.3	0.40	18.0	233.9	0.77	18.0	232.0	1.55
10	18.6	251.3	-	18.4	248.0	0.19	18.8	236.0	0.40	18.6	247.7	0.75	18.8	245.0	1.53
11	19.7	257.3	-	19.6	257.3	0.19	19.4	244.9	0.40	19.6	258.6	0.76	19.7	258.0	1.53
12	19.8	273.4	-	19.6	271.5	0.18	19.5	258.4	0.38	19.8	274.0	0.72	19.9	271.6	1.47
13	19.5	282.0	-	19.3	279.3	0.17	18.9	263.1	0.36	19.1	280.3	0.68	19.6	278.0	1.41
14	19.6	295.3	-	19.8	291.3	0.17	19.9	285.3	0.37	19.2	298.3	0.69	19.9	294.6	1.41

Each value is mean of five animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05)

Table: 23. Food intake, body weight gain and biomass intake of female rats fed with dietary *A. carbonarius* biomass for 14 weeks

Biomass concentration	Control			0.25%			0.5%			1.0%			2.0%		
weeks	food intake (g/rat/day)	body weight (g/rat/week)		food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)	food intake (g/rat/day)	body weight (g/rat/week)	Biomass intake (g/kg bw/rat/day)
0-1	4.8	38.4	-	4.8	36.4	0.33	4.6	36.4	0.63	4.8	37.4	1.28	5.1	38.4	2.66
1-2	6.4	56.1	-	6.3	61.0	0.26	6.5	56.2	0.58	6.8	58.2	1.17	6.9	63.2	2.18
2-3	8.3	87.7	-	8.0	84.7	0.24	8.0	83.0	0.48	8.0	92.4	0.87	8.4	92.4	1.82
3-4	10.2	111.6	-	10.2	110.0	0.23	10.3	107.1	0.48	10.5	114.9	0.91	10.1	122.0	1.66
4-5	13.6	132.7	-	13.2	132.4	0.25	13.3	126.3	0.53	13.8	133.9	1.04	13.8	135.1	2.05
5-6	13.4	142.1	-	13.4	140.0	0.24	13.4	137.0	0.49	13.4	142.4	0.94	11.6	135.1	1.72
6-7	12.8	153.6	-	13.6	155.8	0.22	12.2	154.0	0.40	12.7	156.6	0.82	12.8	156.5	1.64
7-8	12.9.	166.2	-	13.8	155.8	0.22	13.1	167.6	0.39	12.9	169.8	0.76	12.6	144.3	1.75
8-9	11.7	169.6	-	13.8	169.2	0.20	12.5	170.1	0.37	12.7	168.9	0.75	12.4	156.5	1.59
9-10	13.1	176.4	-	13.9	177.7	0.20	12.9	176.4	0.35	13.4	178.7	0.75	13.0	171.2	1.52
10-11	13.4	178.0	-	13.9	178.0	0.20	13.8	176.9	0.39	13.9	179.4	0.78	13.9	171.8	1.62
11-12	13.5	183.3	-	13.8	178.3	0.19	13.8	183.8	0.38	13.8	179.2	0.77	13.8	178.6	1.55
12-13	13.5	187.8	-	13.6	189.0	0.18	13.9	184.8	0.38	13.9	188.0	0.74	13.9	179.8	1.55
13-14	13.8	189.6	-	13.8	191.0	0.18	13.8	189.8	0.36	13.9	186.3	0.75	13.9	184.3	1.51

Each value is mean of five animals. No significant difference between control and *A. carbonarius* biomass fed groups ($P<0.05$).

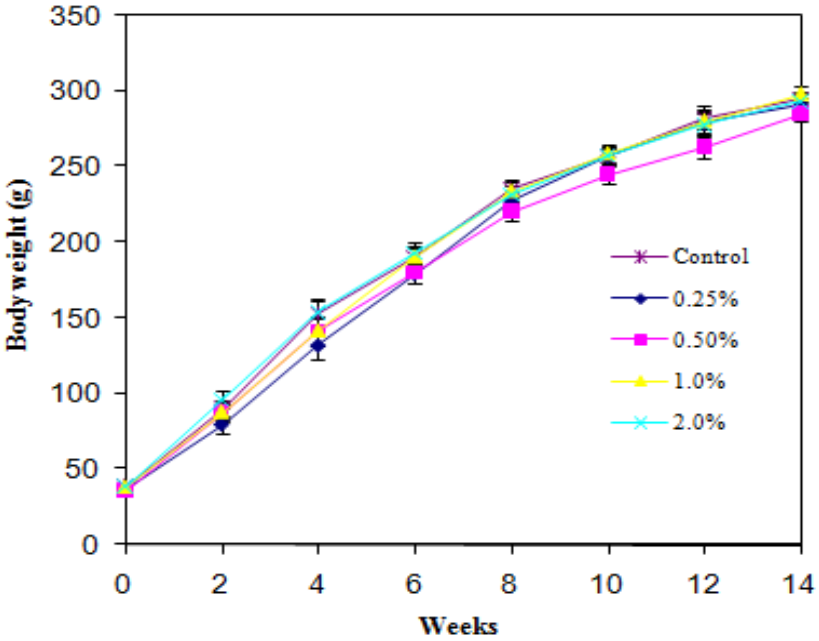


Figure: 46. Mean absolute body weight of male rats fed with dietary *A. carbonarius* biomass for 14 weeks. Values are ± SEM of 5 animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

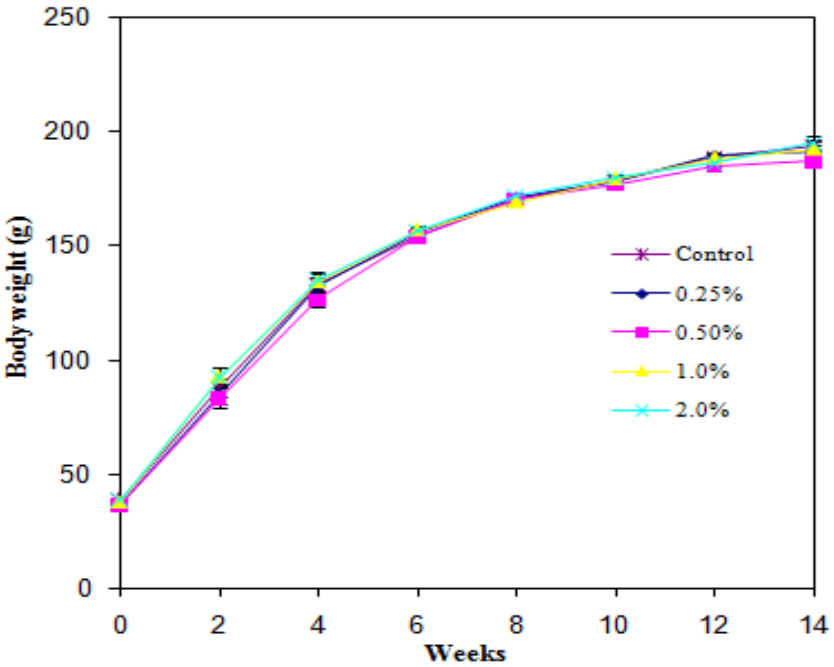


Figure: 47. Mean absolute body weight of female rats fed dietary *A. carbonarius* biomass for 14 weeks. Values are ± SEM of 5 animals. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 24. Effect of feeding freeze-dried *Aspergillus carbonarius* biomass for 14 weeks on relative organ weight (g/100 g body weight) of male rats

Concentration of biomass mixed with diet (w/w).	Liver	Lungs	Kidney	Testis	Spleen	Heart	Brain	Adrenals
Control	3.51±0.12	0.21±0.04	0.64±0.04	1.07±0.09	0.19±0.01	0.30±0.02	0.62±0.03	0.04±0.01
0.25%	3.31±0.10	0.18±0.04	0.69±0.04	0.93±0.08	0.20±0.01	0.27±0.02	0.61±0.03	0.06±0.01
0.5%	3.53±0.12	0.19±0.03	0.64±0.04	0.86±0.09	0.18±0.01	0.27±0.02	0.54±0.03	0.03±0.01
1.0%	3.63±0.11	0.20±0.04	0.65±0.03	0.96±0.09	0.20±0.01	0.26±0.03	0.60±0.04	0.03±0.01
2.0%	3.48±0.12	0.10±0.03	0.59±0.04	1.08±0.08	0.19±0.01	0.26±0.02	0.60±0.03	0.03±0.01

Values are mean ± SEM of five animals. No significant difference between control and *A. carbonarius* biomass fed groups

Table: 25. Effect of feeding freeze-dried *Aspergillus carbonarius* biomass for 14 weeks on relative organ weight (g/100 g body weight) of female rats

Concentration of biomass mixed with diet (w/w)	Liver	Lungs	Kidney	Ovaries	Spleen	Heart	Brain	Adrenals
Control	2.15±0.10	0.31±0.03	0.51±0.04	0.10±0.08	0.15±0.01	0.21±0.02	0.56±0.03	0.03±0.01
0.25%	2.33±0.10	0.35±0.04	0.48±0.04	0.09±0.08	0.13±0.01	0.20±0.02	0.58±0.03	0.04±0.01
0.5%	1.91±0.10	0.36±0.03	0.44±0.04	0.09±0.09	0.14±0.01	0.21±0.02	0.52±0.03	0.03±0.01
1.0%	2.29±0.11	0.33±0.04	0.45±0.03	0.05±0.08	0.12±0.01	0.19±0.02	0.54±0.03	0.03±0.01
2.0%	2.22±0.10	0.31±0.04	0.45±0.04	0.05±0.09	0.12±0.01	0.21±0.02	0.56±0.03	0.03±0.01

Values are mean ± SEM of five animals. No significant difference between control and *A. carbonarius* biomass fed groups

5.3.2.2 Histopathology

Gross examination of vital organs during autopsy did not show any abnormalities that could be attributed to biomass feeding in both sexes of rats. On microscopic examination, no treatment related histo-pathological alterations were observed in the vital organs at any of the feeding levels. Detailed histological examinations of each vital organ showed were as follows:

Liver was characterized by normal hepatic cells with distinct nuclei and normal eosinophilic cytoplasm with normal sinusoids.

Kidney displayed normal renal architecture with normal glomeruli, proximal tubules and collecting ducts. Adrenals showed normal layer of cells in both cortex and medulla.

Normal appearance of heart muscle was observed.

Lung tissue showed fine alveolar sac structures and well defined alveolar ducts.

Spleen showed normal architecture with well defined follicles containing germinal centers.

Brain showed normal cellular architecture both in cerebrum and cerebellar regions.

Testis were with normal somniferous tubules showing different stages of germinal cells, with spermatozoa.

Ovarian tissue showed different stages of follicular development with mature corpus luteum.

5.3.2.3 Hematological profile

After autopsy, blood samples were collected from 5 rats of each group and examined for hemoglobin concentration, Packed Cell Volume (PCV), erythrocytes, leucocytes and differential counts. Mean corpuscular hemoglobin (MCH) was calculated from hemoglobin and erythrocytes counts, where as mean corpuscular hemoglobin concentration (MCHC) was determined from hemoglobin concentration and packed cell volume values. Data on the various hematological parameters (**Tables- 26&27)** showed no significant alteration between control and treatment groups.

5.3.2.4 Serum enzymes

The whole blood taken from each group was allowed to clot and the serum separated was analyzed for serum enzyme activity viz., Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP), Alanine Amino Transferase (ALAT), Asparate Amino Transferase (ASAT). There was no marked alteration in any of the specific activities of enzymes in *A. carbonarius* biomass fed rats **(Table- 28&29)**. Similar results were observed with regard to the level of serum cholesterol as well.

5.3.2.5 Viability test

The viability of the freeze dried *A. carbonarius* biomass before feeding to experimental animals was analyzed by plate count method. Results showed the absence of viable spores in freeze dried biomass. Likewise, no viable cells of *A. carbonarius* were noticed in the faecal matter of rats, fed in any treated groups.

Table: 26. Hematological profile of male rats fed with dietary *A. carbonarius* biomass for 14 weeks

Concentration of biomass mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
2.0%	14.8±1.0	9.9±1.3	8.2±2.1	37.7±2.6	15.18±1.3	39.34±0.9	70.2±2.6	28.5±2.2	1.7±1.1	1±0.0	-
1.0%	14.7±0.6	10.8±1.0	6.6±4.8	35.7±1.7	13.66±0.7	41.09±0.9	68.2±3.7	28.7±2.6	2.0±1.4	1±0.0	-
0.5%	14.0±0.5	11.0±0.7	7.6±2.2	40.0±2.1	13.74±0.8	35.06±0.9	68.5±2.9	31.2±4.2	2.0±0.8	1.5±0.6	-
0.25%	13.8±0.7	10.0±1.0	8.7±2.6	42.5±0.6	13.91±1.1	35.06±0.9	70.2±2.6	30.5±6.6	1.5±0.5	1±0.0	-
Control	14.3±0.3	9.2±1.0	8.0±1.2	39.0±2.1	14.83±1.0	39.41±1.1	69.5±3.3	28.2±3.6	1.5±0.7	1±0.0	-

Values are mean ± SEM of five animals.
Hb – Hemoglobin , RBC – Red blood cells, WBC - White blood cells, PCV- Packed cell volume , MCH- Mean corpuscular hemoglobin, MCHC - Mean corpuscular hemoglobin concentration, DC - Differential count, L- Lymphocytes, P- Polymorph neutrophils, M- Monocyte, E - Eosinophils, B - Basophils. No significant difference between control and *A. carbonarius* biomass fed groups ($P<0.05$).

Table: 27. Hematological profile of female rats fed with dietary *A. carbonarius* biomass for 14 weeks

Concentration of biomass mixed with diet (w/w)	Hb (g/dl)	RBC (10^6/µl)	WBC (10^3/µl)	PCV (%)	MCH (pg)	MCHC (%)	DC (%)				
							L	P	M	E	B
2.0%	14.0±0.9	10.6±1.3	7.2±1.6	38.0±3.9	13.31±1.2	37.22±1.0	67.0±2.0	31.2±6.7	1.6±0.6	2.0±0.7	-
1.0%	14.3±0.7	10.4±1.1	7.0±1.2	39.5±2.6	13.90±1.9	36.38±1.1	71.0±2.4	32.2±6.7	1.3±0.6	1.5±0.7	-
0.5%	13.4±0.6	10.0±1.0	7.3±1.6	39.0±2.0	13.54±1.8	34.38±1.0	69.5±1.3	27.7±1.7	2.0±1.0	1.0±0.0	-
0.25%	13.0±0.3	10.6±0.8	6.9±1.3	38.5±1.9	12.32±1.2	33.8±1.1	70.7±2.5	27.5±2.5	2.0±1.0	1.0±0.0	-
Control	13.3±0.4	9.60±1.1	7.3±1.4	42.5±4.7	13.16±1.9	36.68±0.9	70.0±2.4	28.5±2.4	1.0±0.0	2.0±0.7	-

Values are mean ± SEM of five animals.
Hb – Hemoglobin , RBC – Red blood cells, WBC - White blood cells, PCV- Packed cell volume , MCH- Mean corpuscular hemoglobin, MCHC - Mean corpuscular hemoglobin concentration, DC - Differential count, L- Lymphocytes, P- Polymorph neutrophils, M- Monocyte, E - Eosinophils, B - Basophils. No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 28. Effect of feeding male rats with freeze-dried *A .carbonarius* biomass for 14 weeks on serum enzymes

Concentration of biomass mixed with diet (w/w)	LDH	ALP	ASAT (SGOT)	ALAT (SGPT)	Cholesterol mg/dl
Control	1110.00 ±270.9	564.3 ±60.9	70.45 ±9.82	16.68 ±4.40	67.75
0.25%	1136.87 ±260.9	519.3 ±74.02	74.44 ±3.63	17.79 ±3.74	63.30
0.5%	1181.87 ±271.8	537.1 ±82.04	72.63 ±9.25	19.06 ±4.60	63.25
1.0%	1160.55 ±244.4	525.8 ±56.02	63.20 ±10.46	16.52 ±2.56	68.00
2.0%	1194.42 ±245.4	526.8 ±48.07	68.09 ±9.42	17.79 ±4.20	67.45

Values are mean ± SEM of five animals. LDH - lactate dehydrogenase (U/L), ALP- alkaline phosphatase (U/L), ALAT - alanine amino transferase (U/L), ASAT- aspartate amino transferase (U/L). No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

Table: 29. Effect of feeding female rats with freeze-dried *A .carbonarius* biomass for 14 weeks on serum enzymes

Concentration of biomass mixed with diet (w/w)	LDH	ALP	ASAT (SGOT)	ALAT (SGPT)	Cholesterol mg/dl
Control	1028.00 ±244.9	564.3 ±60.9	68.66±12.12	17.15 ±3.92	64.20
0.25%	1106.91 ±285.09	519.3 ±74.02	72.63±11.34	19.06 ±2.84	60.25
0.5%	1060.44 ±249.19	537.1 ±82.04	77.17±10.7	19.06 ±5.10	63.00
1.0%	1063.00 ±226.19	525.8 ±56.02	66.80±9.31	18.27 ±4.30	60.48
2.0%	1062.42 ±245.4	526.8 ±48.07	76.90±12.4	18.43 ±4.26	62.15

Values are mean ± SEM of five animals. LDH - lactate dehydrogenase (U/L), ALP- alkaline phosphatase (U/L), ALAT - alanine amino transferase (U/L), ASAT- aspartate amino transferase (U/L). No significant difference between control and *A. carbonarius* biomass fed groups (P<0.05).

The results of the safety evaluation study showed that the biomass of mutant *A. carbonarius* UV-10046 containing xanthin did not induce either acute or sub-acute toxic effects in rats. Further, the biomass is well tolerated even at 2% dietary level as evidenced by the absence of any ill effect on growth, organ weight, organ histology, hematological parameters and serum enzyme levels in experimental rats.

DISCUSSION

The mutant *A. carbonarius* was found to incorporate xanthins when grown in shake flasks. Its synthesis and accumulation occurred when the pH of the culture broth dropped to extreme acidity (below pH 4.0). This observation suggested that the xanthin synthesis occurred due to a stress physiology.

Cell membranes which are complex heterogeneous systems to a large extent determine the influences by the external environment such as pH (Alberts *et al.* 1994). Within the cell membrane, the phospholipid molecules interact with the aqueous phases of the inside and the outside of the cell (Neidhart *et al.* 1990; Alberts *et al.* 1994). The microorganisms adjust their membrane lipid composition in response to pH changes to ensure the functionality of enzymes and solute transport (Brown and Minnikin 1973; Russel 1984). Thus for normal cell function, the membrane lipid bilayer has to be largely fluid so that membrane proteins can continue to pump ions, take up nutrients and respire (Berry and Foegeding 1997). Unsaturation of fatty acid chains is the most commonly found change that occurs to increase the fluidity of the membrane because unsaturated fatty acid chains create more disturbances to the membrane then saturated chains. Increased fatty acid unsaturation in response to stress stimuli is known in *Aspergillus niger, Neurospora crassa*, *Penicillium chrysogenum* and *Trichoderma reesei* (Suutari 1995). The results of present investigation showing the production and accumulation of xanthins in the membranes of *Aspergillus carbonarius* mutant during growth in acidic pH (**Experimental 3.0**) suggested alteration in the membrane composition to overcome pH stress. This study identified a bright yellow pigment (**Figure-4**) which upon characterization revealed the production and incorporation of partially saturated

astaxanthin (**Experimental 3.4; Figure-16**) apart from partially saturated canthaxanthin (Kumaresan 2007).

Carotenoids like the above can exert a broad array of functions if they are incorporated into biomembranes (Carmen *et al.* 2000). That the partially saturated astaxanthin associated in this study was a carotenoid shown by NMR spectroscopic analysis and the typical double shouldered peak obtained as a UV-Visible absorption spectrum (**Figure-7**). Thus the membrane of *A. carbonarius* by incorporating the carotenoid acted as barrier to H^+ ion entry for protecting the organism against low pH toxicity when grown in acid environment.

In eukaryotic cell membranes, sterols help to tolerate extreme extra cellular acidity by modulating cell membrane properties. Selective plasma membrane permeability in fungi is associated to higher sterol concentration (mainly ergosterol). In an earlier study Kumarsean (2007), related partially saturated canthaxanthin production in mutant *A. carbonarius* to reduction in sterol content. When the membrane sterol content was quantitated in *A. carbonarius* mutant grown in acid pH, it was found that ergosterol was nearly absent (**Figure-17**). These results suggested the diversion of sterol precursors for the synthesis of partially saturated astaxanthin.

Elucidation of biosynthetic pathway that synthaised partially saturated astaxanthin for incroporation in the cell memranes of *A. carbonarius* mutant

Fungal carotenoids posses four keto groups. These occur as monocyclic or dicyclic compounds with conjugated double bonds. They are synthesized by the mevalonate biosynthetic pathway due to a synthase catalyzed condensation reaction of gernyl-gernyl diphosphate and modification involving different enzymes like, desaturase,

cyclase, ketolase, hydroxylase and glycosilase **(Figure-20).** Since activity of these enzymes can be inhibited by using different inhibitors like, lovastatin, nicotine, norflurazon, diphenylamine, fluridone and pipernylbutoxide, the carotenoid biosynthetic pathway can be elucidated without the need for extraction and solubalization of membrane bound enzymes which are sensitive to detergents (Jin *et al.* 2004; Namthip *et al.* 1997; Agus *et al.* 1993; An *et al.* 1999; Lu *et al.* 1995; Paul *et al.* 1995).

In this study the effect of inhibitors on partially saturated astaxanthin biosynthesis was studied quantifying the xanthin produced. When each of this inhibitor was incorporated in the growth medium, there was concentration dependent decrease in xanthin synthesis (**Figures-21 to 26**). These results suggested that partially saturated astaxanthin was dependent on mevolanate biosynthetic pathway.

Squalene a specific inhibitor of HMG-CoA reductase when used in growth medium also inhibited xanthin synthesis (**Figures- 27 &28**). HMG CoA reductase is also the enzyme that begins the sterol biosynthesis. Since lower concentration of membrane bound sterol were estimated in the xanthin containing mycelia grown at pH 4.0, it appeared as though the mutation induced the diversion of the sterol biosynthetic precursors for xanthin synthesis for protecting the organism against the external high acidic pH toxicity. Thus the fungus was able to grow in growth medium of low pH.

Application of the fungal carotenoids

Carotenoids are a class of wide spread natural pigments reported to have protective role against various diseases. They have antioxidative potency and recently we have shown that partially saturated canthaxanthin of *A. carbonarius* mutant functions as a

retinoid, inducing cell signaling in RAR/RXR containing cancerous cell line to induce apoptosis (Kumaresan *et al.* 2008).

In vitro study using xanthin form *A. carbonarius* mutant, apart from use as food colorant also showed antioxidant properties. It efficiently scavenged free radicals from DPPH in the *in vitro* assay condition (**Table-16).** It also inhibited lipid peroxidation by breaking chain reaction caused due to Fe^{3+} molecules **(Figures-36&37).** Though antibacterial activity was not significant, the assay revealed its ability to inhibit the growth of tested food borne pathogens **(Figures 41 to 43).** Hence, the main protective mechanism attributed to the carotenoid, the antioxidative potency, was also identified in the xanthin of *A. carbonarius* mutant. These studies revealed the fungus was a potential source for use in food and pharmaceutical applications.

Safety of the mutant *A. carbonarius* mycelia

Safety of the pigment bearing mutant *A. carbonarius* mycelia was analyzed for acute and subacute toxicity studies is a prerequisite for food application. The yellow colored freeze dried biomass of *A. carbonarius* was used to feed albino rats for toxicity evaluation.

Acute toxicity study:

Rats fed on *A. carbonarius* biomass did not develop any clinical signs of toxicity either immediately or during the post treatment even at highest dose of 5.0 g/kg body weight. Feeding fungal biomass at different levels did not cause any alteration in food intake and relative weight (**Tables-19&20**). Further, the hematological profile (**Table-21**)

of treated groups was comparable to that of control. No histological alterations were observed at any of the dosage levels.

Sub acute toxicity study:

Dietary feeding of fungal biomass for 14 weeks did not produce any signs of toxicity or mortality during the experimental period at any of the dosage levels. Incorporation of fungal biomass even at 2% level did not significantly affect the food intake in both sexes of treated groups and were comparable to that of control group (**Figures- 44&45**). No difference in food intake, body weight gain was observed in treated groups of both sexes during the 14 week experimental period **(Tables- 22&23)**. There were no marked differences in the group mean relative weights of various vital organs in the biomass treated rats compared to that of control group (**Tables-24&25**). Data on the various hematological parameters (**Tables- 26&27)** showed no significant alteration between control and treated groups.

Gross examination of vital organs during autopsy did not reveal any abnormalities that could be attributed to biomass feeding in both sexes of rats. Further, on microscopic examination, no treatment related histopathological alterations were observed in any of the vital organs irrespective of biomass level. Detailed histological examination of each vital organ showed as follows: Liver was characterized by normal hepatic cells with distinct nuclei and normal eosinophilic cytoplasm with normal sinusoids, Kidney displayed normal renal architecture with normal glomerules, proximal tubules and collecting ducts. Adrenals showed normal layer of cells in both cortex and medulla, normal appearance of heart muscle was observed. Lung tissue showed fine alveolar rac structures and well defined alveolar ducts, spleen showed normal architecture with well

defined follicles, containing germinal centers. Brain showed normal cellular architecture both in cerebrum and cerebellar regions, where as testis showed normal seminiferous tubules showing different stages of germinal cells with spermatozoa, ovarian tissue showed different stages of follicular development with mature corpus leuteum. Analysis of serum clinical enzyme activity viz., Lactate dehydrogenase, Alkaline phosphatase, Alanine amino transferase, Asparate amino transferase (**Tables-28&29**) showed no marked alterations in any of the specific activities of enzymes in *A. carbonarius* biomass fed rats. A similar trend of result was observed with regard to the level of serum cholesterol (**Tables-28&29**). The viability of the freeze dried *A. carbonarius* biomass before feeding to experimental animals was analyzed by plate count method. Results showed that no viable spores were present in freeze dried biomass. Similarly, no viable cells of *A. carbonarius* were noticed in the fecal matter of rats fed in any treatment groups.

There are very few reports in the literature on the safety of fungi for use as food. Toxicological studies carried out on *Rodotorulla gracilis*, a carotenoid and lipid producing yeast was found to be safe in acute and sub-acute toxicity studies in albino rats (Naidu *et al.* 1999a). A technology to cultivate blue green algae *Spirulina platensis* (Becker and Venkatraman 1982) and also methods to isolate the blue colored pigment, phycocyanin, from *Spirulina platensis* (Manoj *et al.* 1994) have been developed at CFTRI, Mysore, India. Further, the safety evaluation of this blue green alga (Krishnakumari *et al.* 1981) and its coloring pigment phycocyanin, has been established in albino rats (Naidu *et al.* 1999b).

The present study is the first step to assess the safety of mycelia of *A. carbonarius* mutant as a source of natural food colorant. The results of the present study in albino rats clearly showed that the biomass of *A. carbonarius* mutant containing xanthin do not induce either acute or sub-acute toxic effects in rats. Further, the biomass is well tolerated even at 2% dietary level as evidenced by absence of any ill effects on growth, organ weight/histology and hematological parameters or serum enzyme levels.

Optimization of xanthin production

Statistical methodologies are applied in biotechnological processes to study the effects and the interaction of cultural parameters which plays a fundamental role in fermentations (Manuel and Antonio 1997). Several successful attempts on the application of Response Surface Methodology in the production of primary and secondary metabolites through microbial fermentation have been reported (Ergun and Mutlu 2000; Escamilla *et al.* 2000; Li *et al.* 2001; Xu *et al.* 2003).

In this study three a level Box-Behnken design was used for optimization of xanthin production by mutant *A. carbonarius* using three independent variables pH, temperature, and fermentation time. **Table-4** showed the concentrations of independent variables at different coded levels employed in the design matrix. Three level Box-Behnken design matrix and the experimental responses of the dependent variables (xanthin and biomass yield) are listed in **Table-5**. The data obtained were used to develop models in which, each dependent variable was obtained as the sum of the contributions of the independent variables through second order polynomial equation and interaction terms.

Based on the experimental response the quantity of xanthin produced by *A. carbonarius* ranged form 54.0 to 851.0 μg/g dry biomass. Runs # 6 and # 13 had the minimum and maximum xanthin production respectively. ANOVA of the regression model for xanthin yield **(Table-6)** demonstrated that the model was significant due to an *F*-value of 106.76 and low probability value (P model >*F* – 0.0005). ANOVA (*F*-test) for the model explained the response of the dependent variable. The experimental yields fitted the second-order polynomial equation as indicated by high R^2 values (0.995). *F*-value obtained showed that the model predicted the experimental results and that the effects of estimated factors were real. The regression coefficients of all the linear term and all quadratic coefficients of X_1, X_2, X_3, were significant at < 1% level and interaction coefficient of X_1 and X_3 was significant at < 5% level (**Table-7**). ANOVA suggested the model to be significant at *P*<0.01. The *P*-values used as a tool to check the significance of each of the coefficients indicated the pattern of interactions between the variables. Smaller value of *P* was more significant to the corresponding coefficient (Liu *et a*l., 2003).

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and effects of interactions of each independent variable, on the response variables (Bocchini *et al*. 2002). The shape of the corresponding contour plots indicated that the mutual interactions between the independent variables were significant or not. Interactions of variables determined the orientation of the principal axes of the contour plots (Cui *et al*. 2005). From the 3D response surface plots and the corresponding contour plots, the optimal values of the

independent variables were observed, and the interaction between each independent variable pair described.

The 3D response surfaces based on independent variables obtained (**Figures-29 to 31**) indicated that optimal levels in coded values for pH and temperature arose between 0 and -1 and for fermentation time 0 and +1. The orientation of the principal axes of the contour plots between the variables pH and temperature, pH and fermentation time indicated that the mutual interactions between these sets of variables had a significant effect on the xanthin yield. When the third independent variable, fermentation time was kept constant at 44 h (**Figure-29**) the interaction between the two variables (temperature and pH) showed that the xanthin yield was sensitive even when pH and temperature were subject to small alterations. Under certain conditions a maximal contour (xanthin, 1283.00 μg/g dry biomass) could be determined, meaning that further change in temperature and pH would not increase the xanthin. The other pair of the independent variables pH and fermentation time showed similar effects while keeping the third independent variable, temperature as a constant at 30 ^{o}C (**Figure-30**). The 3D surface response for temperature and fermentation time on the yield of xanthin (**Figure-31**) where pH was constant showed that the mass yields were obtaineable in the middle level of the process variables. The results showed that as the values of process variables increased, the yield also increased but only up to the midpoint of range of variables and thereafter the yield decreased even though the values of variables increased. The xanthin yield was significantly affected by pH, temperature and fermentation time where pH produced greater effect.

Response analysis reveled the maximum xanthin yield by *A. carbonarius* could be achieved at the conditions when pH of growth media is 3.3, temperature 29.3 ^{o}C and fermentation time 46.7h. Under these conditions the model predicted a xanthin yield of 1283.00 µg/g of fungal dry biomass. The validation experiment conducted at the above optimized conditions resulted in a xanthin yield of 1298.00 µg per gram of dry biomass. The average relative deviation (ARD) of the predicted yield from experimental yield for xanthin was found to be 0.4 % (**Table-11**).

***Optimization of biomass production*: Table-5** shows the considerable variation in the yield of biomass under different cultural conditions along with the predicted values. The biomass yield varied from 1.71 to 2.34 g/100 ml broth. The runs # 6 and run # 3 had the minimum and maximum biomass yield respectively. The results of ANOVA (**Table-9**) for the biomass production of *A. carbonarius* mutant demonstrated that the model is highly significant, as is evident from the calculated *F*-value (36.97), low probability value (P model >F – 0.0005) and high R^2 (98.51%) value. The regression coefficients data along with the corresponding *P*-values (**Table-10**) for mycelia production by *A. carbonarius* mutant showed that the regression coefficients of the linear term X_3 and quadratic coefficients of X_1&X_2 were significant at <1% level. The quadratic term of X_3 and interaction term of X_1 and X_3 were significant at <10% level.

The response analysis reveled that maximum yield of biomass (2.44 g dry biomass/100 ml culture broth) could be achieved when the organism was grown for 56 h in corn flour medium of 3.6 pH at 29.7^{0}C. The 3D surface response for independent variables temperature, pH and fermentation time on the yield of biomass (**Figures-32 to 34**) suggested that biomass yield was affected by all the variables. The 3D response

surfaces for the biomass yield indicated that the maximum yield of biomass could be obtained at pH 3.6, temperature 29.7^0C after 56 h of fermentation. The validation experiment conducted at the optimized conditions yielded 2.42 g dry biomass/100 ml growth medium and the ARD of the predicted yield from experimental yield for biomass was found to be 0.69% (**Table-11**).

The three levels Box Behnken design applied with the aim of optimizing the culture conditions for xanthin production in *A. carbonarius* resulted in increase in xanthin yield (**Table-11**). Evaluating three growth parameters (pH, fermentation time and temperature) in 15 experimental (**Table-5**) conditions provided a better understanding of the physiology of *A. carbonarius* in relation to xanthin production. From 3D response surface plots and corresponding contour plots, the optimal values of the independent variables and the interaction between each independent variables pair were described (**Figures-32 to 34**). This experiment was the first attempt for statistical experimental design to optimize the xanthin production form *Aspergillus carbonarius* mutant. These facts are important in making the whole process economically more feasible for production of food colorant from mutant *A. carbonarius*.

In conclusion it can be stated that the identification and characterization of partially saturated astaxanthin from *A. carbonarius* is new to literature. Experiments suggested that the pigment arose due to mutation in the sterol biosynthetic pathway and diversion of the precursor for the synthesis of xanthins. The antioxidant property of the xanthin revealed its food and pharmaceutical application. The fungal xanthin appeared safe as evidenced by acute and sub-acute toxicity studies in albino rats. In this study fermentation parameters could be optimized for its cost effective production.

SUMMARY

The physiology of *Aspergillus carbonarius* in relation to polygalactruronase production has been the intensive area of research in this laboratory. Temperature tolerant *A. carbonarius* UV 10046 isolated for polygalactruronase over production in shake flasks secreted copious quantities of the enzyme at acidic pH. Studies showed that the enzyme production was induced by acid pH and the intracellular pH homeostasis was maintained with the help of xanthin synthesized and incorporated in to cell membrane when grown at pH below 3.0. Study carried out on the purification and characterization of the xanthin showed synthesis of partially saturated astaxanthin apart form partially saturated canthaxanthin.

The ethanol extract of the pigment was purified by repeated TLC in solvent system, isooctane: acetone: and diethyl ether (6:2:2). The UV-visible spectrum of the pigment in absolute ethanol showed double shouldered peak characteristic of carotenoid structure with λ_{max} at 444 nm. Molecular mass of the pigment was determined by LCMS after confirming its purity by HPLC. Since a major compound fractionated at molecular mass 590, this fraction was identified as compound of interest. Nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C) and 2D COSY combined with DEPT and HSQC that assigned carbon and proton positions and bonding, identified the pigment as partially saturated astaxanthin.

Estimation of total sterol content in pigmented and non-pigmented biomass showed a reduced sterol content and absence of ergosterol in particular with in the pigmented biomass. It appeared that the mutation in *A. carbonarius* resulted in diversion of sterol precursors for the synthesis of xanthin to protect the fungal membrane against

extracellular acidity. Effect of pathway specific inhibitors on xanthin synthesis suggested mutant *A. carbonarius* possibly utilizing mevalonate pathway for xanthin synthesis.

Growth parameters were standardized to improve xanthin yield from mutant *A. carbonarius* under controlled cultural conditions. Response Surface Methodology was adopted using Box-Benkhan design with pH, temperature and fermentation time as selected variables. Under the optimized conditions of pH 3.3; temperature 29.3^0C and fermentation time 46.7 h, model predicted xanthin yield of 1283 μg g^{-1} dry fungal biomas. Prediction by the model was also validated by experimentation.

Studies on xanthin production by *A. carbonarius* in baffled and controlled flask showed aeration was important for xanthin production. Standardization of carbon and nitrogen source for xanthin production showed corn flour and ammonium salts were the best carbon and nitrogen sources respectively. Xanthin yield increased gradually when the C:N molar ratio increased from 1:1 to 1:6.

The fungal origin of xanthin required safety evaluation for application purposes. This study was carried out by feeding CFT-Wister albino rats with pigmented biomass and evaluating the toxicity. Acute and sub-acute studies revealed that the organism was safe since, it did not induce any negative effect on growth, food intake profile, relative organ weight, histology and hematological profile and serum enzyme levels. Xanthin extracted from mutant *A. carbonarius* showed efficient DPPH scavenging and lipid peroxidation inhibition activity with the EC_{50} value of 27.3μg ml^{-1} and 44.2 μg ml^{-1} respectively. Antibacterial activity of xanthin extracted from mutant *A. carbonarius* showed minimum inhibitory concentration ranging from 50 to 400 μg/ml against food borne pathogens.

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PUBLICATIONS

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3. Kumaresan, N., **K, R. Sanjay,** K. S. Venkatesh, K. Ravi-Kumar, G. Vijayalakshmi and S. Umesh-Kumar (2008). Partially saturated canthaxanthin purified from *Aspergillus carbonarius* induces apoptosis in prostrate cancer cell line. *Applied Microbiology Biotechnology* 80:467–473.