Studies on yeast *Rhodotorula*, its carotenoids and their applications.

A

THESIS SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

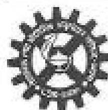
BY

PRAKASH. B. BHOSALE

CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE 411 008 (INDIA)

FEBRUARY 2001

This thesis is dedicated to my parents....



Dr. R.V.Gadre
Scientist

Chemical Engineering Division
National Chemical Laboratory
Dr. Homi Bhabha Road
Pune – 411 008, INDIA

Tel: +91-20-5893300 / 58993400
Extn. 2348
Fax: +91-20-5893041
E-mail: gadre@che.ncl.res.in

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Studies on yeast *Rhodotorula*, its carotenoids and their applications"** submitted by Mr. Prakash B. Bhosale was carried out by the candidate under my guidance at National Chemical Laboratory, Pune. Such material as has been obtained from other sources, has been duly acknowledged in the thesis.

Date: 28th FEB 2001

(Dr. R.V.Gadre)
Research Guide

Acknowledgement

It is my great pleasure to express a deep sense of gratitude to my research guide Dr. R.V.Gadre for his excellent guidance and constant encouragement during the course of this investigation. His idea of giving independence in designing experiments really inspired me many a times.

I am equally obliged to Mr. Sanjay Nene, Head, Biochemical Engineering group for his encouragement and useful suggestions during the progress and completion of this project.

I am grateful to Mr. V. V. Jogdand for his valuable suggestions, constructive discussions, keen interest and constant encouragement during the tenure of this project.

I thank all other Scientists and staff members of Biochemical Engineering Group for their suggestions and constant support in completing this work.

I thank Dr. D. D. Deobagkar, Head, Zoology Department, University of Pune, for critical suggestions during yearly assessments of the work during my tenure as a research fellow.

I also wish to thank Dr K.V.K. Rao, Head, Carcinogenesis Division, Cancer Research Institute, Mumbai, for collaboration.

I am extremely grateful to Dr D. R. Ranade, Agarkar Research Institute, for his valuable suggestions during thesis writing.

I admire the co-operation of Neelima, Deba, Bharat, Subhash, Preeti, Vinod, Rupali, Vidya, Pallavi, Avinash and all other colleagues and friends at Biochemical Engineering Group.

I wish to sincerely thank Rachna, my best friend, whose constant encouragement helped me in finishing Ph.D. work in time.

I would like to express my appreciation to Pradeep, Meena, Nagesh, Shivappa, Ramesh, Aparna, Aslam, Arun, Maneesha, Manish and Sandeep for their constant help and making my stay at hostel lively.

No words can suffice to acknowledge the encouragement and moral support given by my parents. I am grateful to my brothers (Arun and Uday), sisters (Sandhya and Vidya) and bhabhis for their constant support, blessings and love.

I wish to thank Dr. B. D. Kulkarni, Head, Chemical Engineering Division, and the Director, National Chemical Laboratory, for permitting me to work in this laboratory and to submit this work in the form of Ph.D. thesis.

Finally, the financial assistance, from the Council of Scientific and Industrial Research, India, is gratefully acknowledged.

Prakash Bhosale

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Chapter 1

General Introduction

Introduction

Carotenoids are the most pronounced, naturally occurring pigments. They are of great interest in many scientific disciplines because of wide distribution and diverse functions. Owing to their ubiquitous occurrence, diverse functions and interesting properties, carotenoids are subject of interdisciplinary research in biochemistry, biology, chemistry, medicine, microbiology, physics and many other branches of science.

Guibourt first suggested the existence of these pigments in green leaves in 1827. However, the crystalline yellow pigment called "carotene", was later isolated from carrots in, 1837, by Wakenroder (Pfander 1992). The research on carotenoids progressed further when Tswet, in 1911, separated carotenoids by chromatography and Wilstatter and Meig in 1907 demonstrated basic structure of carotenoids to be isoprenoid-derived molecules (Goodwin 1980).

Zechmeister and Polgav (1943) demonstrated chemical structure and spectroscopic behavior of many carotenoids that took research on carotenoid chemistry into storm.

Since then, rapid development in structural studies has revolutionized the utility and studies on carotenoids. The information that follows offers an overview of carotenoids.

Chemical Structures of carotenoids

Carotenoids are hydrocarbons (carotene) and their oxygenated derivatives (xanthophyll). They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule. The two central methyl groups are in 1,6-relationship and the remaining non-terminal methyl groups are in a 1,5-position relationship (Pfander 1992).

All carotenoids are formally derived from the acylic C_{40} H_{56} structure (Figure 1.1) having a long central chain of conjugated double bonds. The carotenoids are derived from this basic structure by 1. Hydrogenation 2. Dehydrogenation 3. Cyclization 4. Oxidation or a combination of these processes. There are reports of carotenoids being derived from certain rearrangements or degradations of basic C_{40} H_{56} carbon skeleton (Pfander 1992).

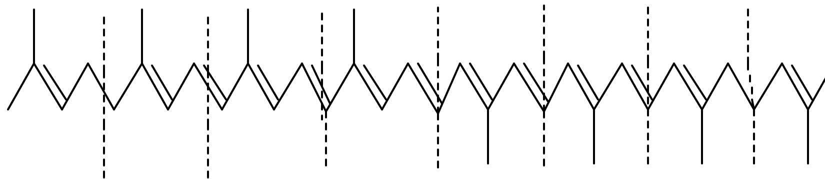


Figure 1.1 Acyclic $C_{40}H_{56}$ structure

Based on chemical structures, carotenoids can be classified in two major categories.

Hydrocarbon carotenoids: These are also called as carotenes. Rules for the nomenclature of these carotenoids are based on the stem name "carotene". The name of a specific compound is constructed by adding prefixes (eta, beta, theta, gamma, kappa etc) to the stem name "carotene" (Figure 1.2). The common examples are β-carotene, α-carotene and lycopene (Figure 1.3).

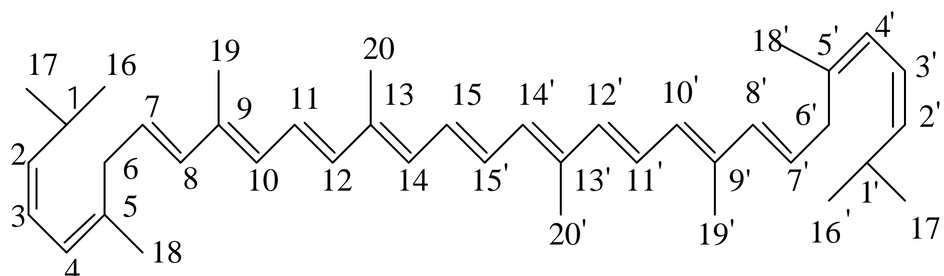
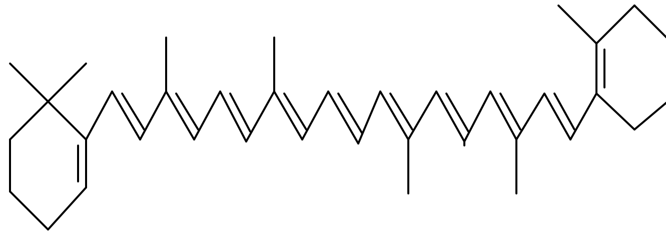


Figure 1.2 Structure and numbering of stem carotene.

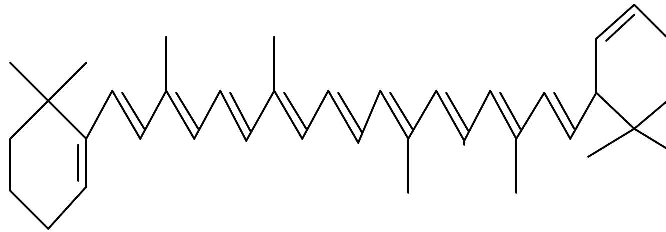
Oxygenated carotenoids (Xanthophylls): They are named according to normal rules of organic chemistry for chemical nomenclature. The functional group most frequently observed are hydroxy, methoxy, carboxy, oxo, aldehyde and epoxy. The common examples are zeaxanthin, spirilloxanthin, echinenone and antheranxanthin (Figure 1.3).

Other commercially important carotenoid includes ketocarotenoids like astaxanthin and canthaxanthin (Figure 1.3).

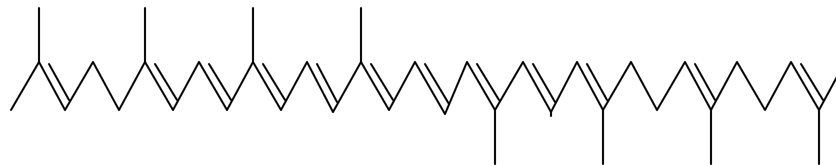
a. β-Carotene (β,β -carotene)



b. α-Carotene (β, ∈ -carotene)



c. Lycopene (ψ, ψ - carotene).



d. Zeaxanthin ([3 R,3' R)-β,β -carotene-3 3'-diol]

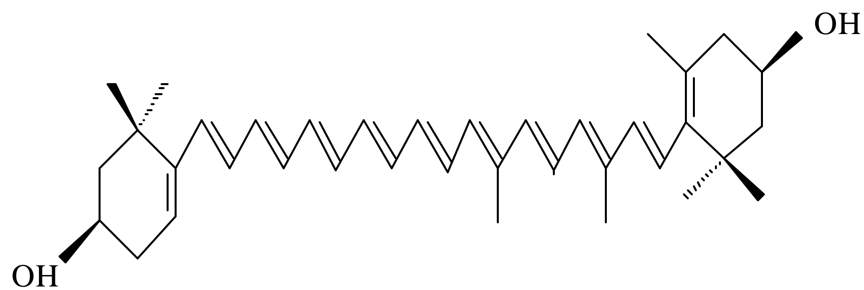
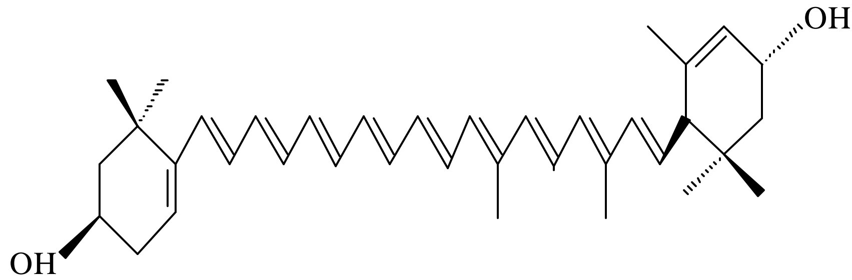
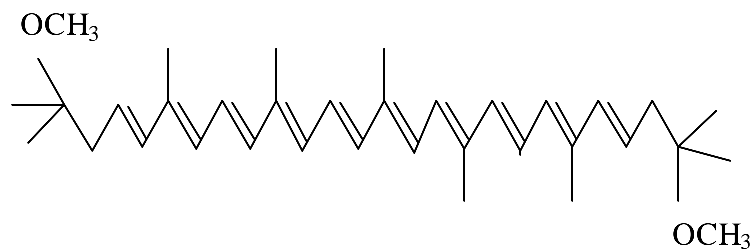


Figure1.3 Structures of important carotenoids (Continued).

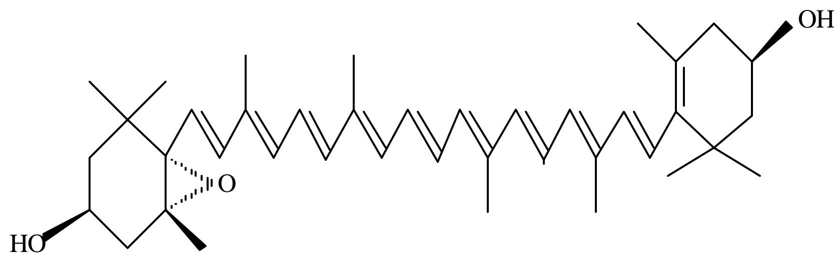
d. Lutein ["Xanthophyll," (3R, 3' R, 6' R)-β, ε-carotene-3-3'-diol]



e. Spirilloxanthin [1,1'-dimethoxy-3,4,3', 4' –tetradehydro-1,2,1',2',-tetrahydro-ψ ψ-carotene)



f. Antheraxanthin (5,6-epoxy-5,6-dihydro-β, β-carotene-3,3'-diol)



g. Echinenone

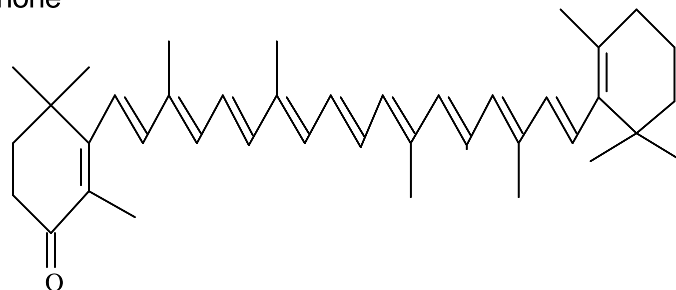
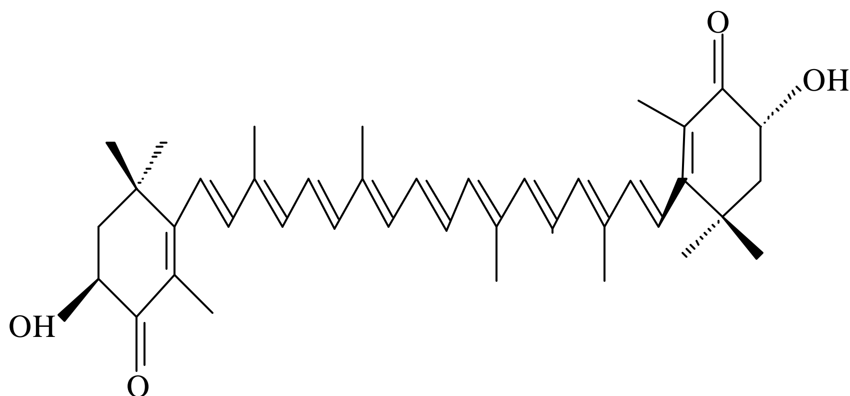
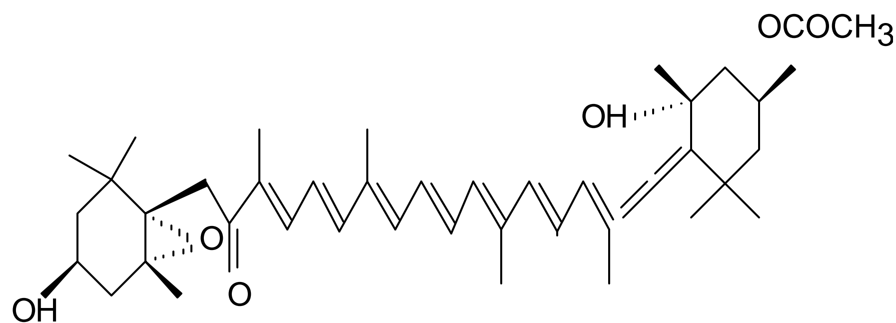


Figure 1.3 Continued....

h. Astaxanthin [(3S,3'S)-3,3'dihydroxy-β,β-carotene-4,4'-dione]



i. Canthaxanthin (β,β-carotene-4,4'-dione)



Carotenoid research is ongoing and researchers are involved in elucidating structures of new carotenoids from various plants and microbial sources as reviewed by Mercadante (1999). The new carotenoids reported recently are listed inTable1.1.

Table 1.1 List of carotenoid structure isolated in the period 1990-2000.

Carotenoids	Source	References
(all-E)-2,1'-dihydroxy-3',4'-didehydro-1',2'-dihydro-b,y-caroten-4-one	Red pigmented strains of bacteria *Deinococcus radiodurans*	Laurant *et al.* 1997
Aromatic carotenoids	Sulfur photosynthetic bacteria	Schaeffer *et al.* 1997
Tobiraxanthins	Seeds of *Pittosporum tobira*	Takashi *et al.* 1997
3,5,6-trihydroxy-5,6-dihydro-b-end groups carotenoids.	Red spice paprika (*Capsicum annuum var. longum*)	Jozsef *et al.* 1998
6-geranylgeranyl 8'-methyl-6,8'-diapocaroten-6,8'-dioate	Seed coat of *Bixa orellana* fruits	Mercadante *et al.* 1999
5,6'-trihydroxy-3'-oxo-6,7-didehydro-5,6-dihydro-10,11,20-trinor-b,e-caroten-19',11'-olide 3-acetate	Oyster *Crassostrea gigas*	Yasuhiro *et al.* 1999
4-keto-19'-hexanoyloxyfucoxanthin	Algae *Emiliania huxley*	Egeland *et al.* 2000

Physical properties of carotenoids

Carotenoids have characteristic absorption spectra because of presence of conjugated ployene system in the molecule and distinct functional groups (Table 1.2). The increase in the position of the absorption maxima due to an additional conjugated double bond varies from 7 to 35 nm. Insertion of other functional group twists the chromophore out of plane and reduces both fine structure and intensity of spectra (hypochromic effect) (Goodwin 1980).

Other techniques like Infrared-spectroscopy (IR), Mass Spectrophotometry (MS), Nuclear Magnetic Resonance spectroscopy (NMR), Optical Rotary Dispersion (ORD), Circular Dichroism (CD) and X-ray Crystallography that exploit physical properties for identification of carotenoids were described earlier by Goodwin (1980).

Table 1.2 Absorption spectrum of some well-known carotenoids in petroleum ether (Reference: Goodwin 1980).

Carotenoids	Absorption maxima (nm)		
Canthaxanthin	-	467	-
α-Carotene	422	444	473
β-Carotene	427	452	477
γ-Carotene	435	461	491
Lycopene	446	472	505
Phytoene	276	286	298
Phytofluene	331	347	367
Zeaxanthin	429	451	479

Stability of carotenoids

Scita (1992) presented detailed systematic studies on the factors that could affect the stability of pure carotenoids under common environmental conditions. In addition to this report, carotenoids are reported to be sensitive to UV light (Shi and Chen 1997, Dzulkefly *et al.* 1999), fluorescent white light (Biacs and Fekete 1998), air (Henry *et al.* 1998), heat and exposure to acid (Ancos *et al.* 1999).

In the process of degradation of carotenoids, several types of chemical reactions were revealed (Berset and Marty 1992)

a. Multiple isomerizations on certain trans double bonds leading to modifications of the spatial conformation of the molecule and thus, modifying the stability.
b. Oxidation of carotenoids molecule occur (epoxy formation, hydroxylation, ketone formation).
c. Oxidative rupture which, progressively shortens the unsaturated chain.

Degradation of carotenoids can be prevented by the use of antioxidants like butylated hydroxy toluene (BHT) and tocopherol (Orset *et al.* 1999). In many food preparations carotenoids are encapsulated or complexed with food grade encapsulating agents like sodium alginate (Go *et al.* 1999), maltodextrin (Desobry *et al.* 1997, Desobry *et al.* 1999), gum arabic (Leach *et al.* 1998) and gelatin (Xuan *et al.* 1998) for enhanced stability.

Biosynthesis of carotenoids

Carotenoids are synthesized in nature by plants and many microorganisms. Acetyl-CoA was demonstrated as the key precursor of carotenoid biosynthesis in fungi, tomatoes and in photosynthetic tissues (Goodwin 1993).

Simpson *et al.* (1964) and later Goodwin (1980a, 1993) reviewed the general pathways for carotenoid synthesis. Carotenoids biosynthesis pathway commonly involves three steps.

A. Formation of isopentyl pyrophosphate (I PP) (Figure 1.4)

The first step involves conversion of Acetyl-CoA to 3-hydroxy-3-methyl glutaryl CoA catalyzed by HMG-CoA synthase. HMG CoA gets converted into a C6 compound mevalonic acid (MVA), the first specific precursor on the terpenoid biosynthetic route (Porter and Spurgeon 1981). Role of MVA in carotenogenesis was demonstrated using radiolabelling experiments by Britton (1988). MVA gets converted into isopentyl pyrophosphate (IPP) by a series of reactions involving phosphorylation by MVA kinase followed by decarboxylatioin (Goodwin 1980).

B. Formation of Phytoene (Figure 1.5)

This involves the isomerization of IPP to Dimethylallyl pyrophosphate (DMAPP) and sequential addition of three IPP molecules to DMAPP. These reactions are catalyzed by prenyl transferase to yield the C20 compound geranyl geranyl pyrophosphate (GGPP).
This pathway is shared with the sterol pathway up to the C15 stage (farnesyl pyrophosphate, FPP). FPP branches off to form squalene, the C30 acyclic precursor of sterols. In carotenogenic system two molecules of GGPP condense head to head to form Phytoene which undergoes desaturation to form lycopene (Goodwin 1993).

C. Cyclization and other reactions of Lycopene (Figure 1.6)

As lycopene is an all-trans compound, the isomerization of the 1st or 2nd double bond of the phytoene must occur at same stage in the desaturation process (Goodwin 1993). Lycopene acts as precursor of cyclic carotenoids and undergoes number of metabolic reactions like cyclization (initiated by H^+ attack) to form β and ∈ rings. However, it undergoes reactions other than cyclization, such as hydration and hydrogenation across the 1st and 2nd double bond.

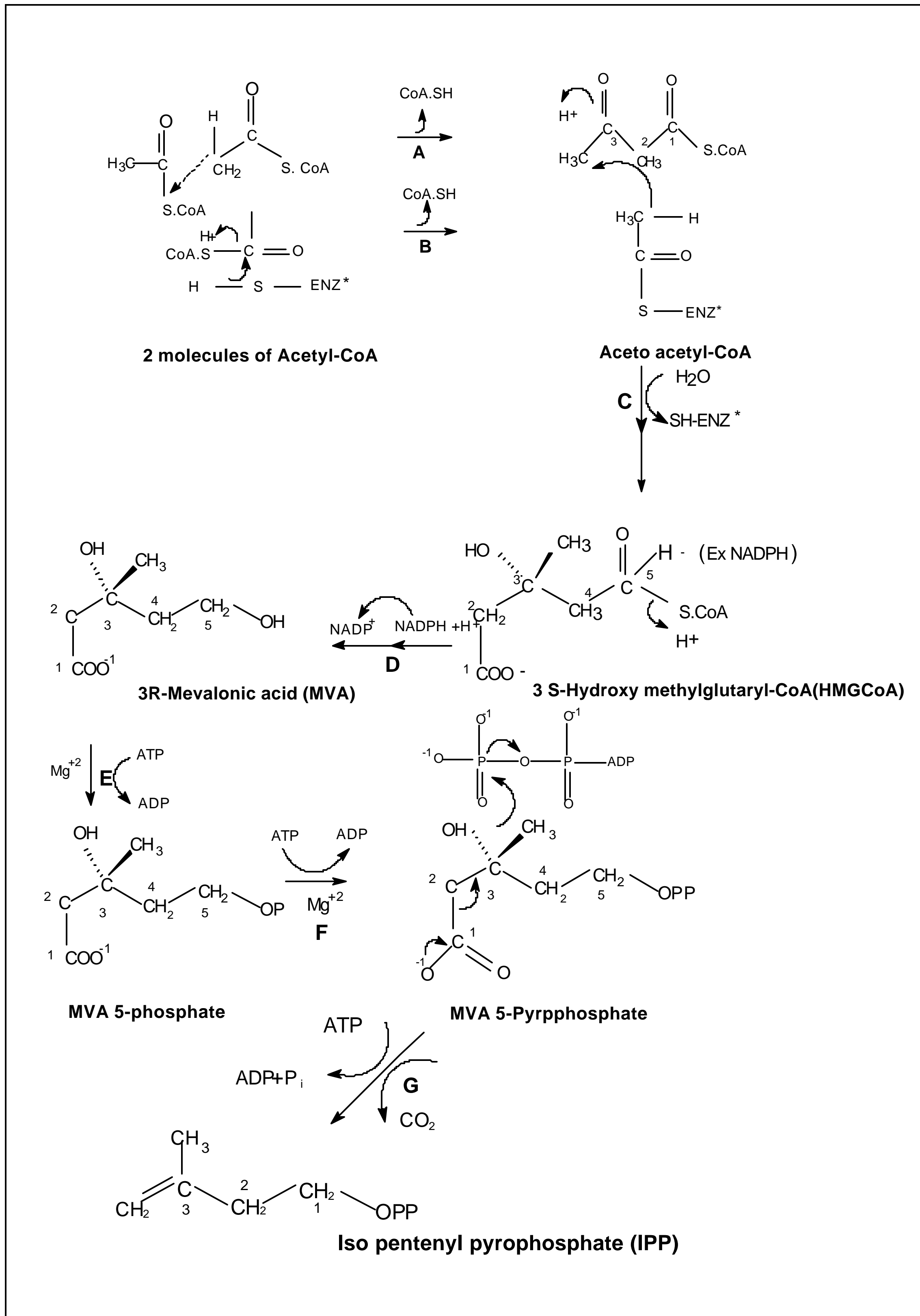


Figure 1.4 General biosynthetic pathway for the formation of isopentyl pyrophosphate (IPP).

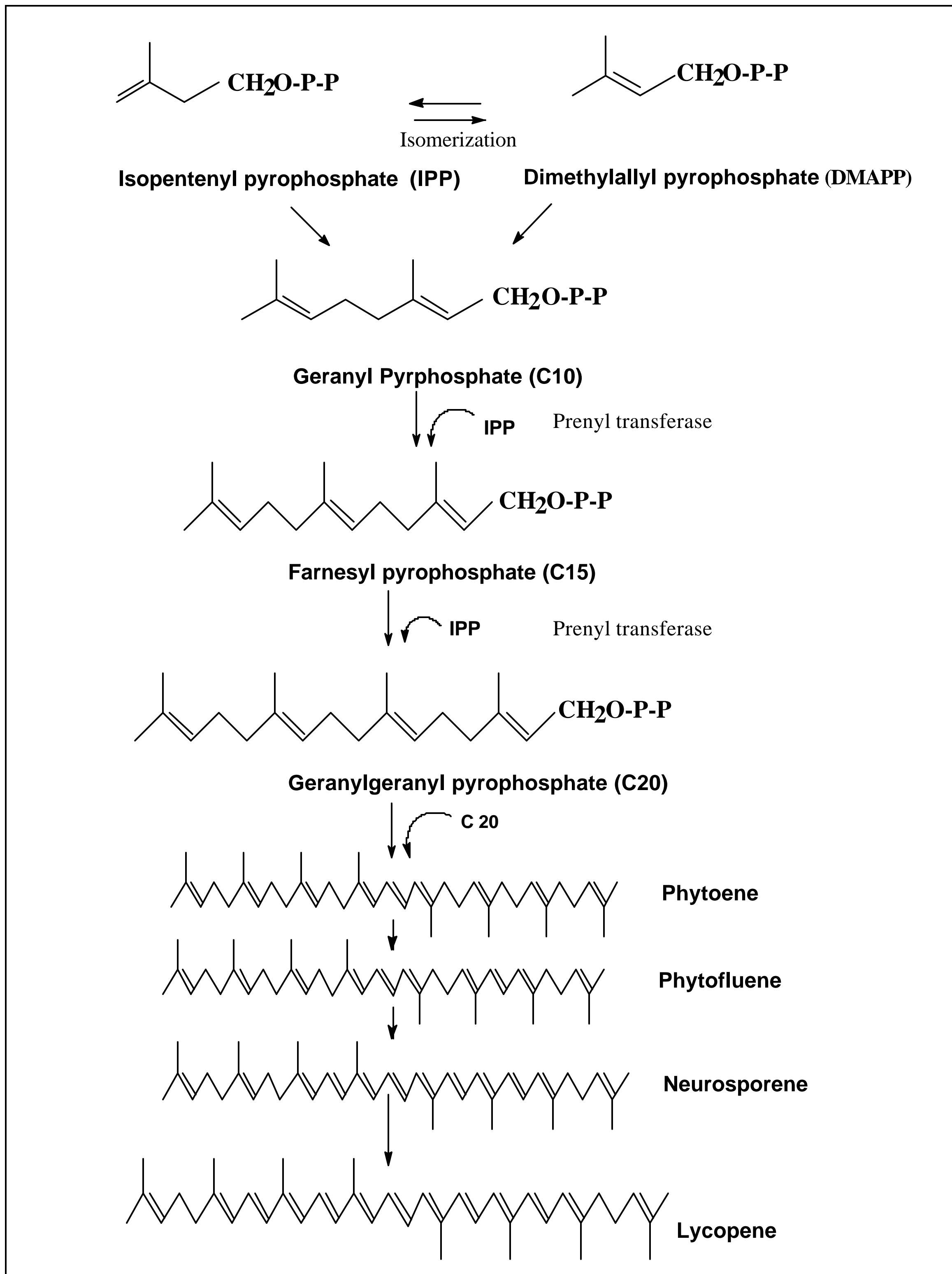


Figure 1.5 Proposed pathway for lycopene synthesis.

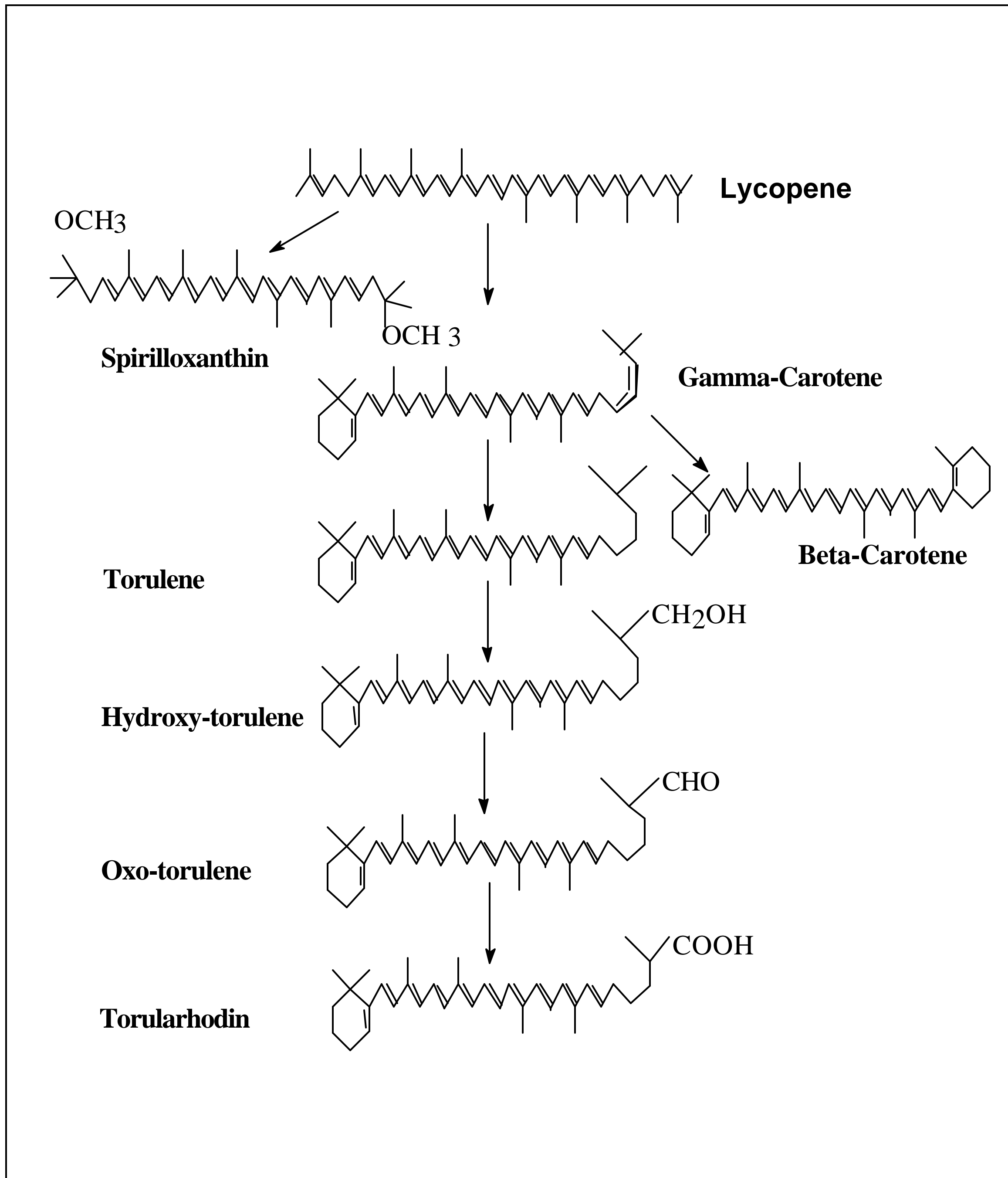


Figure 1.6 Proposed pathways for the transformation of carotenoids (Simpson *et al.* 1964)

Functions of carotenoids

Epidemiological evidence and experimental results suggest that dietary carotenoids inhibit onset of many diseases like arteriosclerosis, cataracts, age related macular degeneration, multiple sclerosis and most importantly cancer, all of which are mainly initiated by free radicals (Peto *et al.* 1981, Henneckens 1997, Stahl and Sies 1996, Greenberg *et al.* 1990).

There are two major theories behind the protective effect of carotenoids.

First is their **Provitamin-A** nature. However, not all carotenoids can be converted to vitamin A. Canthaxanthin, a carotenoid with no provitamin-A activity, has been proposed to reduce the risk of cancer (Edge *et al.* 1997, Mayne and Parker 1989, Mathews-Roth and Krinsky 1987). Therefore, it must be concluded that anticancer effect can be partly attributable to their antioxidant nature.

The second theory is that carotenoids have the ability to act as chain breaking **antioxidants** and thus protect cells against photo-oxidation. The ability of carotenoids to quench singlet oxygen is well known (Foote and Denny 1968, Conn *et al.* 1991, Oliveros *et al.* 1994, Tinkler *et al.* 1994) and reactions with radical species have also been studied (Burton and Ingold 1984, Jorgensen and Skibsted 1993, Bohm *et al.* 1995, Hill *et al.* 1995).

Provitamin-A

The conversion of all trans β-carotene to retinol in the intestinal mucosal cells is the results of the action of several enzymes. β-Carotene 15-15' dioxygenase cleaves the carotene molecule at central double bond that directly forms in two molecules of retinaldehyde (Figure1.7). One molecule of retinaldehyde is subsequently reduced to retinol by retinal reductase enzyme, and other molecule is oxidized to retinoic acid.

Alternatively, enzymatic cleavage of carotene occurs at the 8', 10' and 12' double bonds, which may indirectly produce retinoic acid and retinal in low concentrations (Hinds *et al.* 1997, Wang *et al.* 1991).

Provitamin-A nature of carotenoids depends on structure of individual carotenoids. Theoretically, one molecule of β-carotene can be cleaved into two molecules of vitamin A (Figure1.7). However, inside the human body, β-carotene is only partially converted to vitamin A and the rest is stored as such. Furthermore, the proportion of β-carotene converted to vitamin A in the human body is controlled by the vitamin A status and thus it

cannot cause vitamin A “hypervitaminosis” in humans. Currently available evidences suggests that in addition to being a safe source of vitamin A, β-carotene play many important biological roles that may be independent of its provitamin status. Individual carotenoids have characteristic patterns of absorption, metabolism and tissue storage (Wang 1994). Lutein is reported to be a precursor of vitaminA_2 in fish. Torularhodin and torulene are reported to be provitamin A in vitro studies (Ershov *et al.* 1992).

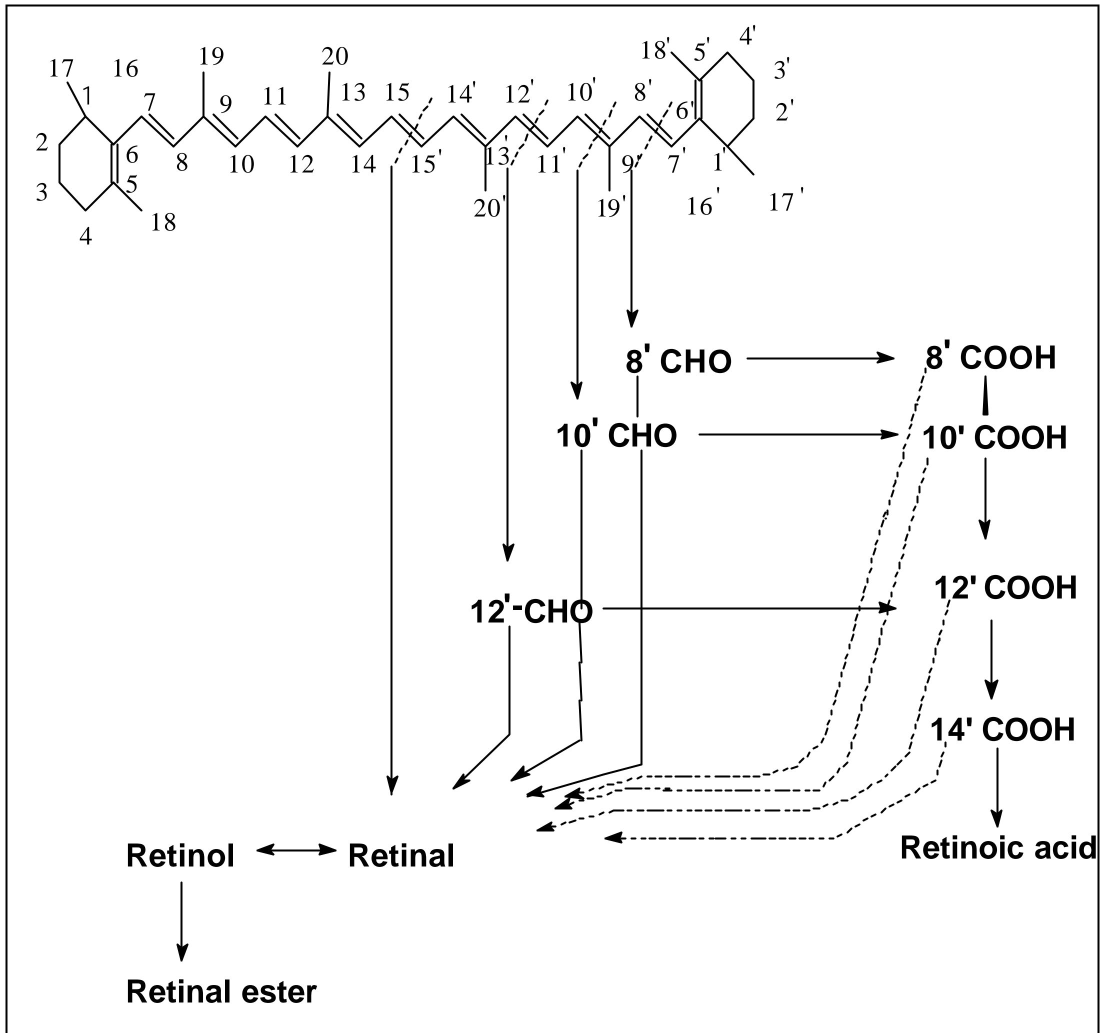


Figure 1.7 Cleavage of β-carotene into vitaminA (Rock 1997)

Antioxidant

There are several mechanisms by which carotenoids can act as antioxidants. These mechanisms can be summarized in the abilities of these molecules to act either as photoprotective agents against the harmful effects of light and oxygen or as compounds

reactive against chemical species generated within cells and able to induce oxidative damage.

The mechanism of its role as an antioxidant is described briefly here.

Reaction with singlet oxygen

Singlet oxygen is generated by electronic energy transfer from the excited state (normally triplet state) of a sensitizer (**S**) to oxygen (Edge *et al.* 1997). Singlet oxygen can lead to deleterious effects including DNA damage, inactivation of proteins and lipid perooxidation. Carotenoids prevent these damages either by quenching triplet sensitizers (**S***) in triplet state and/or singlet oxygen.

The ability of carotenoids to quench singlet oxygen is related to number of conjugated double bonds with a maximum protection offered by pigments having nine or more conjugated double bonds (Edge *et al.* 1997). Quenching ability also depends on the structure and functional groups of carotenoids (Mascio *et al.* 1989). After quenching sensitizer, carotenoid triplet that is formed, readily losses its energy and returns to its original form.

Reaction with oxygen radicals.

Reactive oxygen gets generated in the cell as a result of various metabolic processes or following exposure to Xenobiotics. Reactive oxygen is reported to cleave DNA, peroxidize lipids, alter enzyme activity, deploymerize polysaccharides and kill cells (Brawn and Fridorich 1981, Slater 1982). Carotenoids are known to quench these radicals and prevent degree of nuclear and cellular damage.

Reaction with free radicals

Free radicals are by-products of metabolic process and originate from environmental pollutants such as nitrogen dioxide and ozone in polluted air, heavy metals, and halogenated hydrocarbons, ionizing radiation and cigarette smoke. Free radicals can damage both structure and function of cell membranes, nucleic acids and electron dense regions of proteins. Peroxy radical, owing to its selectivity of reaction and ability to diffuse in biological system are potentially more dangerous than many other types of radicals and is reported to cause heart disease, cancer and process of aging (Edge *et al.* 1997). Carotenoids also have ability to quench these free radicals. Unlike the quenching of singlet oxygen, which leads to energy dissipation as heat, the reaction of a carotenoid

with a free radical will lead to electron transfer or possibly addition reactions (Palozza and Krinsky 1992, Edge *et al.* 1997).

Absorption of carotenoids and their metabolism in human body

β-Carotene along with lycopene, lutein, β-cryptoxanthin and α-Carotene accounts for 90% of circulating carotenoids in body. They are absorbed as such by mucosal cells and subsequently appear unchanged in the circulation and peripheral tissue.

Carotenoids are known to get absorbed only in presence of conjugated bile salts. Body fat stimulates secretion of bile salts. Thus, fat appears to exert the greatest effect on absorption of carotenoids. Non-polar carotenoids like β-carotene are associated with low-density lipo-proteins (L.D.L). Adipose tissue and liver are major storage depots for the carotenoids. High carotenoid concentrations are found in tissues that are rich in L.D.L receptors such as *corpus luteum*.

Provitamin carotenoids get converted into retinol when the need for vitamin A arises. Retinol is then taken into the intestinal mucosal cells by a carrier-mediated process that is facilitated by cellular retinal binding proteins (Omenn *et al.* 1996). Excess intake (30 mg/day) of β-carotene leads to hypercarotemia, or yellowing of skin but it gets resolved soon after reestablishment of normal diet (Edge *et al.* 1997).

Importance of carotenoids

Anticancer potential

Lung cancer

Knekt *et al.* (1999) studied the relation between intake of carotenoids (β-carotene, α-carotene, lutein, cryptoxanthin and zeaxanthin) along with vitamins E and C and the subsequent risk of lung cancer in 4,500 men. These studies (followed for a period of 20 years) showed an inverse correlation between intake of carotenoids, vitamins E and C and the incidence of lung cancer among non-smokers, demonstrating the protective effects of these nutrients against lung cancer. These studies were also supported in many reviews (Pierre *et al.* 1997, Glisson *et al.* 1998, Monika 1999, Laura *et al.* 2000).

The possible protective action of carotenoids can be attributed to their antioxidant nature, especially as singlet oxygen quenchers. However, David (1999a) later suggested that carotenoids might also enhance immune responses in the elderly persons.

Experiments on animals and cultured cells have shown that carotenoids can inhibit the development of cancer by up-regulation of gap junctional communication, induction of detoxifying enzymes, and inhibition of proliferation. Studies on tumor cells indicated that carotenoids could interfere with the growth of transformed cells, suggesting that they may be effective in the treatment of certain types of cancers (Smith 1998).

However, a lung cancer chemoprevention trial (Carotene and Retinol Efficacy Trial (CARET) had to be prematurely terminated when 46% excess lung cancer mortality was found. (Carrie *et al.* 1998, Demetrius 1999). Cancer enhancing actions in lung can be ascribed to the prooxidant action of carotenoid free radicals in damaged cells (Olson 1999).

Liver cancer

In the recent review, β-carotene and other dietary carotenoids are reported as antioxidants that can prevent carcinogenesis in liver by decreasing the levels of free radicals that cause DNA damage (Dawson 2000).

Carotenoids like α-carotene, lycopene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin, astaxanthin were reported to have anticancer effect in animal systems (Shamaan *et al.* 1997, Yasuhiro *et al.* 2000). Youping *et al.* (1997) reported that carotenoid-rich foods (carrots, tomatoes and orange juice) substantially inhibited biochemical and cellular events, which were thought to play a role in the early stages of hepatocarcinogenesis in animal model.

Several researchers have studied protective effects of carotenoids in chemically induced hepatocarcinogenesis in animal systems (Ong *et al.* 1994, Sarkar *et al.* 1995, Moreno *et al.* 1995, Pierre *et al.* 1997a, Youping *et al.* 1997, Rizzi *et al.* 1998). These studies concluded that protective effects of carotenoids were mainly attributed to their antioxidant nature. In one report, lycopene was reported to have modulating effect on the liver cytochrome P 450 2E1 enzyme activating DEN (Pierre *et al.* 1997). Sarkar *et al.* 1997 reported that β-carotene acts as chemopreventive agent protecting DNA chain breaks in the DEN induced hepatocarcinogenesis. In the earlier studies, β-carotene was also reported to enhance the immune response of splenic T lymphocytes (Ong *et al.* 1994).

Stomach and bronchus cancer

Gey *et al.* (1987) reported direct correlation between mortality rate due to cancer and low plasma levels of carotene. This study was conducted in 3000 men, followed for 15 years. Cancers of the bronchus and stomach were reported to be associated with low carotene levels. Other studies which support the protective effect of carotenoids in stomach cancer include reports on Japanese (Satoshi *et al.* 2000) and Chinese (Jun-Yao 1998) intervention trials. β-Carotene was mainly used as carotenoid for the studies. However, other carotenoids like astaxanthin and canthaxantin were also reported to offer protection against stomach cancer in experimental animals (Santamaria *et al.* 1985, Yoshiyuki *et al.* 1999).

Nutrition intervention trials in Linxian, China indicated that carotenoids are active only at the very late stages (progression) of a direct carcinogenic process in the stomach (Jun-Yao 1998). Cancer of the stomach is mainly caused by consumption of salted and pickled foods containing carcinogens. Carotenoids offer protection against these carcinogens because of their antioxidant nature.

Head and neck cancer

Role of carotenoids in the prevention of head and neck cancers in patients with and without second primary tumours was studied by earlier researchers (De Vries and Snow 1990, De Vries *et al.* 1999, Vakoulovskaia *et al.* 1997). Both groups of patients had decreased levels of β-carotene suggesting role of carotenoids in offering prevention against secondary cancers.

Prostate cancer

Prostate cancer has one of the highest incidence rates of all cancers (Monika 1999). Epidemiological and animal studies suggest a direct relationship between high dietary intakes of carotenoids from fruits and vegetables and decreased risk of several malignancies, including prostate cancer (Giovannucci *et al.* 1995, Williams *et al.* 2000).

Lycopene, a non-provitamin A carotenoid with potent antioxidant activity was clearly linked to protection against cancers (Clinton *et al.* 1996). Reports on the link between intake of lycopene containing tomato and reduced risk of prostate cancers are many (Giovannucci and Clinton 1998, Peter *et al.* 1999).

Breast cancer

The relation between carotenoids consumption at various times in life and breast cancer risk was assessed by conducting a case-control study within a population-based cohort of women screened for breast cancer in Sweden. These studies indicated that intake of diet containing high amount of β-carotene reduces the chances of getting breast cancer. However, these studies failed to give critical time period of intake during such diets are more relevant in preventing breast cancer. Lycopene, β-carotene, α-carotene, lutein, astaxanthin, α-fucoxanthin, halocynthaxanthin, β-cryptoxanthin and peridinin are reported to impart strong anticancer activity because of their antioxidant activity against breast cancer (Jaervinen *et al.*1997, Sang-Pill *et al.* 1998, McEligot *et al.* 1999).

Earlier researchers (Sharoni *et al.* 1997) reported positive role of carotenoids against breast cancer in mammalian systems but it was contradicted immediately by Moon and Constantinou (1997). Poor absorption and low levels of carotenoids that reach the target tissues complicate the interpretation of data in rodent models of mammary carcinogenesis.

Skin cancer

The skin possesses an elaborate antioxidant defense system to deal with UV-induced oxidative stress. However, excessive exposure to UV and harmful chemicals can overwhelm the cutaneous antioxidant capacity, leading to skin cancer. There are several reports, which provide plausible mechanisms for benefits of carotenoid supplementation against skin cancer (Santamaria *et al.*1982, Kornhause *et al.* 1986, Steenvoorden *et al.* 1997, Hsueh *et al.* 1997). Animal studies have indicated that carotenoids can delay or prevent induction of sarcomas and skin cancer in mice exposed to chemical carcinogens (Lambert *et al.* 1990, Lambert *et al.* 1994, Nishino 1997). Carotenoids are reported to block photocarcinogenic enhancement by chemical carcinogen in mice.

Medicinal applications

Age related macular degeneration

Age-related macular degeneration (AMD) is the most common cause of blindness at old age. This disease affects the macula densa, the small central part of the retina, responsible for the most acute vision. The macula degenerates when capillaries proliferate under the retinal pigment epithelium, sometimes growing into the subretinal space.

Seddon *et al.* (1994) correlated the disease with dietary carotenoid intake with AMD. Carotenoids like lutein and zeaxanthin were found to be associated with reduced risk of AMD. These two carotenoids are obtained primarily from dark green leafy vegetables such as spinach and collard greens. The lens and cornea of human eye filter out ultraviolet light, but still visible blue light reaches the retina. This near-UV radiation can cause photodamage that contributes, over the time, to degeneration of the macula. Lutein and zeaxanthin enter the eye from the plasma and selectively accumulate in the retina, where, they filter out visible blue light. These carotenoids may also protect against peroxidation of fatty acids in the photoreceptor membrane, and thus protects the blood vessels that supply the macular region.

Photo protectant

It was reported earlier that β-carotene offers photoprotection to individuals with porphyria. The effect in other photosensitivity disorders such as Xeroderma pigmentosum (XP) has not been well studied, but the benefit does not appear as substantial (Mathews-Roth 1997). Gensler and Holladay (1990) reported that carotenoids (Canthaxanthin) along with retynyl palmitate could prevent photo-induced cancer and UV-induced immune suppression in mice. Interest in systemic photoprotection continues to develop with the recent report (Gonzalez 1997) of substance called PL found to triple the tolerance to UV light in healthy volunteers.

Application in Cosmetics

A cosmetic preparations comprising the carotenoids likes β-carotene, lycopene, lutein, phytoene and phytofluene were reported to be effective in preventing various kinds of damage resulting from oxidation and exposure to UV light (Etienne *et al.* 2000, Frank *et al.* 2000, Nobuhiko *et al.* 2000). Their utility is also being proven as skin-care cosmetics, foundations and sunscreens to inhibit skin aging due to sunburn (Biesalski and Gollnick 1998, Hong and Jihong 1999). These preparations are either derived from microbial sources like *Dunaliella* (Etienne *et al.* 2000) or non-microbial sources like palm (Masahiko *et al.* 1994).

Application in food industries

Carotenoids are popular in food industries as colourants and vitamin A sources (Bloukas *et al.* 1999, Yuki 1999). Current trend in fermentation biotechnology towards the development of processes for high-value products including high-value food additives

have been reviewed recently (Sang-Pill *et al.* 1998, Chen 1999) and it reveals inclination of food industries towards microbial sources of carotenoids.

Carotenoids, mainly β-carotene, astaxanthin, α-carotene, fucoxanthin, halocynthaxanthin and peridinin, are used as food colorants owing to their antioxidant nature and provitamin A activity. Carotenoids are usually fortified in the food preparations (mainly in baby and infant food) to improve their vitamin content (Rosa *et al.* 1999, Olaf *et al.* 2000, Dorota *et al.* 2000). However, carotenoids are sensitive to food-processing conditions hence, they are normally encapsulated with agents like cyclodextrins (Lajos *et al.* 1998).

Feed additive

Carotenoids play important role in animal health by inactivating harmful free radicals produced in normal cellular activity and in stress. Carotenoids like astaxanthin and β-carotene were also reported for prevention of gastric ulceration of stressed rats (Yoshiyuki *et al.* 1999). Pure carotenoids or carotenoid-containing preparations are also playing important role as feed additive for pigmentation in aquaculture (Harpaz *et al.* 1999).

Astaxanthin is the major carotenoid used for pigmentation of fishes and salmons. Yeast *Phaffia rhodozyma* is widely used as astaxanthin source in aquaculture industries (Johnson *et al.* 1977). Biotechnology companies active in the pigment business have devoted considerable research and development efforts to this organism (Nelis and De Leenheer 1991).

Carotenoid sources

Fruits and vegetables

Human body cannot synthesize β-carotene *de novo*. Carrot root (*Daucas carota*) is undoubtedly among the most widespread and important sources of β-carotene. Besides carrot, various green leafy vegetables also have considerable amount of carotenoids although their presence is visibly hindered by chlorophyll. β-Carotene is also present in most of the fruits but its concentration varies with the varieties. Tomato, banana, berries, mango, orange, papaya and starfruit are common fruits that have β-carotene ranging between 40 and 615 μg per 100 g fresh weight of the fruit. Apricots, sweet potato, egg yolk, butter, shrimp, lobster, salmon and trout owe their colour to carotenoids (Ong and Tee 1992).

Microbial sources of carotenoids

Despite the availability of variety of natural and synthetic carotenoids, there is currently renewed interest in microbial sources of carotenoids (Nelis and DeLeenheer 1991, Ausich 1997).

A process for production of β-carotene from a hyper producing fungus (*Blakeslea trispora)* was reported by Ceigler *et al.* (1963) but was later abandoned because it could not compete with synthetic manufacturing. Yet, carotenoid production from microbial sources is now again getting renewed interest owing to public awareness against "synthetic food additives".

Microbes producing carotenoids are listed in Table 1.2. There are several microbes like algae, fungus, yeasts and bacteria reported to produce carotenoids and are discussed below

Algae

Production of β-carotene by an alga *Dunaliella sp.* is a well-developed technology (Ben-Amotz 1998, Ben-Amotz 1999). *Dunaliella* is a unicellular alga that lacks rigid cell wall and produces high level of β-carotene when grown under high salts and high light conditions (Haouazine *et al.* 1999). Several processes based on commercial application of carotenoid containing preparations from *Dunaliella sp* have been patented recently (Tanaka 2000, Tanaka 2000a).

Presently, commercial production is being carried out in large open ponds situated near salt lakes in Australia (Betaten pvt. Ltd., Whyala and Hutt Lagoon at South Australia) and in USA (Nucara True Aroma Therapy, Iowa). The compositions made out of algae are suitable for oral administrations or as dietary supplements (Honda 1997). Capsules and tablets of its dried biomass are used in Japan as a source of β-carotene.

Another alga *Haematococcus pluvialis* is also under study as a potential industrial source of astaxanthin (Kopecky *et al.* 2000, Sang-Pill *et al.* 1998). Although algae are found to be rich in carotenoids, their production on commercial scale is found to be difficult due to several factors as mentioned below.

1) Production of pigments by microalgae, particularly *Dunaliella sp*, requires maintenance of stress conditions of salt concentration and intense light.

Table 1.2 A comprehensive overview of carotenoids producing microorganisms.

Carotenoids	Microbial sources (Reference No.)
Antheraxanthin	*Erythrotrichia carnea (36).*
Astaxanthin	*Haematococcus pluvalis (35), Ankistrodesmus sp., Mychonastes zofingiensis, Chlorella fusca, Pleurastrum sarcinoideum (21).* *Xanthophyllomyces dendrorhous# (7,42), Candida utilis (47), Phaffia rhodozyma (19), Rhodotorula sp. (11).* *Mycobacterium lacticola, Brevibacterium sp (31),Agrobacterium aurantiacum, Escherichia coli# (27).*
b- Carotene	*Dunaliella salina (21,35), Dunaliella bardawil (5), Dunaliella parva (3), Dunaliella bardawil (4), Spirulina platensis (44), Botryococcus braunii (16), Scenedesmus obliquus (24), Chlamydomonas reinhardti (25), Gonyaulax polyedra (7), Erythrotrichia carnea (37), Laurencia sp.(38), chrysophycean algae (29), Cladophora glomerata Kutz (17), Ulva pertusa (26), Prochloron sp.(29).* *Blakeslea trispora (18). Phycomyces blakesleeanus(36).* *Rhodotorula strains (2,33,46), Phaffia rhodozyma (10,20), Sporobolomyces pararoseus (39), Candida utilis (47), Sporobolomyces shibatanus (28), Rhodosporidium diobovatum (39), Sporobolomyces pararoseus T (22).* *Rhodobacter sphaeroides (45), Agrobacterium aurantiacum, Zymomonas mobilis, Escherichia coli# (27).*
g-Carotene	*Cladophora glomerata Kutz (13).*
a -Carotene	*Dunaliella salina (32), Dunaliella bardawil (4).* *Phaffia rhodozyma (2)*
Canthaxanthin	*Xanthophyllomyces dendrorhous (40)* *Corynebacterium michiganese, Brevibacterium sp, Rhodococcus maris (31)*
Cryptoxanthin	*Erythrotrichia carnea (36), Spirulina Species (9), Cladophora glomerata Kutz (17)*
Echinenone	*Botryococcus braunii (16), Spirulina Species (9)*
Fucoxanthin	*Fucus serratus (13), Ascophyllum nodosum, Pelvetia canaliculata, Laminaria digitata (13), Laurencia nidifica (37), chrysophycean algae(29)*
Loroxanthin	*Botryococcus braunii (16)*
Continue on next page...	

Lutein	*Botryococcus braunii (43), Scenedesmus obliquus (24), Dunaliella parva (3), Dunaliella bardawil (4), Spongiococcum excentricum, Chlorella pyrenoidosa (31).*
Lycopene	*Blakeslea trispora (15,9).* *Xanthophyllomyces dendrorhous # (42), Candida utilis # (14).* *Rhodopseudomonas capsulata (46) Agrobacterium aurantiacum, Escherichia coli# (26), Streptomyces chrestomyceticus (30).*
Neoxanthin	*Botryococcus braunii (43), Dunaliella bardawil (4).*
Phoenicoxanthin	*Phaffia rhodozyma(2).*
Spirilloxanthin	*Pseudomonas radiora (32).*
Torularhodin	*Cladophora glomerata Kutz (17).* *Wangiella dermatitidis (8).* *Rhodotorula sp. (33), Phaffia rhodozyma (34), Rhodosporidium diobovatum (28), Sporobolomyces pararoseus T(22)*
Torulene	*Wangiella dermatitidis (8)* *Verticillium agaricinum (11)* *Rhodotorula sp.(31) Phaffia rhodozyma (34) , Rhodosporidium diobovatum (40), Sporobolomyces pararoseus T (22)*
Violaxanthin	*Botryococcus braunii (43)*
β-Zeacarotene	*Phaffia rhodozyma(1)*
Zeaxanthin	*Dunaliella parva (3), Erythrotrichia carnea (36), Dunaliella bardawil (4), Spirulina Species (9), Prochloron sp. (30).* *Flavobacterium sp (31).*

References: (Please see chapter 10 for details of references)

1. An and Johnson. 1990	13. Haugan and Liaaen 1992	25. Masahiko *et al.* 1991	37. Terje *et al.* 1984
2. An *et al.* 1999	14. Hiroshi *et al.* 1998	26. Michio *et al.* 1980	38. Unn *et al.* 1979
3. Andrew *et al.* 1990	15. Ivakin *et al.* 1998	27. Misawa and Shimada 1998	39. Usatai 1999
4. Ben-Amotz 1982	16. Izumi *et al.* 1998	28. Mukugaichi and Kakutani 1981	40. Vaskivnyuk and Getman 1998
5. Ben Amotz 1996	17. Jelena and Dragutin 1977	29. Nancy *et al.* 1981	41. Vazquez and Santos 1998
6. Chen *et al.* 1998	18. Jiang *et al.* 1998	30. Nancy *et al.* 1978	42. Verdoes *et al.* 1999
7. Ernani *et al.* 2000	19. Johnson *et al.* 1977	31. Nelis and Deleehneer 1991	43. Volova *et al.* 1998
8. Geis *et al.* 1984	20. Kim *et al.* 1998	32. Orset *et al.* 1999	44. Xin *et al.* 1998
9. Georges 1979	21. Kopecky *et al.* 2000	33. Perrier *et al.* 1995	45. Yu *et al.* 1998
10. Gigard *et al.* 1994	22. Korolerva *et al.* 1979	34. Poluyakh *et al.* 1991	46. Yueying *et al.* 2000
11. Hari *et al.* 1992	23. Laurant *et al.* 1997	35. Sang-Pill *et al.* 1998	47. Yutaka *et al.* 1998
12 Hasiao ad Moeller *et al.* 1984	24. Lichtenthaler *et al.* 1995.	36. Shlomai *et al.* 1992	

# Indicates recombinant strain

2) Algae are photosynthetic autotrophs and need light for their growth and pigment accumulation.

3) They have a peculiar requirement of CO_2 and oxygen during day and night cycles of growth, respectively.

4) The growth rate of algae is low and it takes long time (often a few weeks) for sufficient quantity of the cell mass growth and pigment accumulation.

5) The algal cultures are prone to contamination by bacteria and protozoa that can cause serious problems in their cultivation on a larger scale.

6) *Dunaliella* is a microalga that does not have rigid cell wall and thus needs special care during handling and growth. In addition, the cultures can not be agitated and this can cause non-uniform conditions in the larger cultures.

Fungus

A fungus *Blakeslea trispora* is capable of producing high amount of β-carotene (Chen *et al.* 1998). The detailed studies on production parameters have been reported earlier by many researchers (Feofilova *et al.* 1994, Finkelstien *et al.* 1993, Kim *et al.* 1996, Seon-Won *et al.* 1997). Carotenoid containing preparations derived from *Blakeslea trispora* have been used poultry (Fisinin *et al.* 1982). However, the production of carotenoids by *Blakeslea trispora* is dependent upon sexual mating of two compatible strains during fermentation (Vasilchenko *et al.* 1989, Morozova *et al.* 1996). In addition to that, the growth of *Blakeslea trispora* in fermenters becomes viscous and needs considerable energy input to keep it aerobic and well mixed.

Yeasts

Progress on research of astaxanthin producing red yeast *Phaffia rhodozyma* has been reviewed recently (Mingjun 2000). Production of β-carotene by a mutant of *Phaffia rhodozyma* was also reported recently (Girard *et al.* 1994, Kim *et al.* 1998).

The red-pigmented yeast, *Phaffia rhodozyma*, containing high concentration of astaxanthin was evaluated as a dietary pigment source for egg yolk of laying hens fed on a low carotenoid diet (Akiba 2000). However, *Phaffia rhodozyma,* has tough cell wall which difficult to disintegrate or digest biologically and this limits its utility as carotenoid containing whole cell product (Hari *et al.* 1992)

Rhodotorula glutinis often called "pink yeast" is a free living, non-fermenting, unicellular yeast found commonly in nature. *Rhodotorula* is well known for its characteristic

carotenoids "torulene and torularhodin". It also accumulates β-carotene and in α-carotene minute quantities (Perrier *et al.* 1995). Torulene and torularhodin are reported to have provitamin-A activity (Euginea *et al.* 1997).

Production of carotenoids by yeast has several advantages over to algae and fungus as follows.

1) They are unicellular microorganisms and can be grown in bulk quantity.
2) Their growth rate is high and production of large quantities of cell mass is relatively easy.
3) The cells are tough and can be grown in conventional fermenter to desired cell densities.
4) The cells mass in dried form can be used for various pharmaceutical products and as feed additive.

Strain improvement for hyper production of carotenoids from microbes.

Improving the efficiency of carotenoid biosynthesis can increase carotenoid production by microbes. Carotenoid biosynthesis is governed by the level and activity of carotenoid biosynthetic enzymes and the total carbon flux through the synthesizing system. Thus, hyper production can be achieved by altering the level and the activity of these enzymes either by **mutation** or **by use of recombinant DNA technology** (Schmidt-Dannert 2000, Sandmann 2001).

Mutation

Most of the mutagenesis programs for enhanced carotenoids producing strains were focussed on hyper production of commercially important carotenoids like β-carotene and astaxanthin. Mutations were carried out using ultraviolet rays or chemical mutagens like Nitrosoguanidine (NSG), Ethyl methyl sulphonate (EMS), Diphenylamine (DPA) and Antimycin. Literature available on mutations in carotenoids producing microorganism indicates that *Phaffia rhodozyma* and *Blakeslea trispora* are two most frequently used microorganisms for strain improvement (Retamales *et al.* 1998, Ren and Yin 1998). *Phaffia rhodozyma* was reported to produce higher amount of β-carotene because of blocked astaxanthin biosynthesis (Kim *et al.* 1998, Gigard *et al.* 1994). Astaxanthin producing stable mutants of *Phaffia rhodozyma* were also developed by nitrosoguanidine and UV treatments (Johnson *et al.* 1991, Prevatt *et al.* 1991). β-Carotene superproducing mutants of *Phycomyces blakesleeanus* were also

investigated (Murillo Araujo *et al.* 1982, Salgado *et al.* 1989). There are scanty reports on mutagenesis of yeast *Rhodotorula* for carotenoid production (Sakaki *et al.* 2000).

Recombinant DNA technology

There are two basic requirements for exploitation of recombinant DNA technology for carotenoid production. Firstly, the availability of genes for the enzymes to produce carotenoids. And secondly, the system to introduce these genes into the desired host as have those genes incorporated and expressed in stable manner through many generations of the cells.

The genes for the enzymes required for transforming acetyl CoA to phytoene and further to lycopene and β-carotene have been isolated and (Schmidt-Dannert 2000). Previous researchers have obtained stable transformed strain of fungus *Phycomyces* for β-carotene production (Cerda-Olmedo *et al.* 1993).

Astaxanthin producing stable transformants were obtained after genetic transformation of two *Phaffia rhodozyma* mutants (Martincz *et al.* 1998). Ausich *et al.* (1996) claimed production properties of yeast *Saccharomyces cerevisiae* developed by recombinant DNA technology. Carotenoid production from *Escherichia coli, Zymomonas mobilis and Saccharomyces cerevisiae* was also claimed. All of which were incorporated with the carotenoid producing genes isolated and characterized from bacterium *Erwinia uredovovo* (Sandmann *et al.* 1992).

Presently, all the tools essential for the use of recombinant DNA technology for carotenoid production have been identified but it is necessary to develop target specific strategies for production of specific carotenoids. Stable transformats can be coupled with fermentation studies to develop complete processes (Sandmann *et al.* 1999) for carotenoid production.

Carotenoid production from *Rhodotorula spp.*

Brief history of genus Rhodotorula (Phaff and Fell 1970)

Harrison in 1928 established the genera *Rhodotorula* for the asporogenous, pigment-forming yeasts.

Lodder (1934) pointed out that the numerous colour intermediates (between red and yellow) made it impossible to place certain strains with any degree of confidence in genus *Rhodotorula*. She therefore brought all asporogenous yeasts with clearly visible pigments into the genus *Rhodotorula*.

Historical review of genus *Rhodotorula*, prior to above-mentioned developments, reveals that earlier all non-fermentative yeasts were grouped under genus *Cryptococcus*.

Nakayama *et al.* (1954) presented evidence for separation of *Cryptococcus* and *Rhodotorula* on the basis of the manifestation of carotenoid in these organisms. However, because this property was dependent on concentration and composition of carotenoids along with the environmental conditions, it was strongly contradicted.

Hasegawa *et al.* (1960) proposed separation of *Cryptococcus* and *Rhodotorula* on the basis of spectrophotometric estimation of carotenoid and later proposed division of *Rhodotorula* into two subgenera, *Flavotorula* (for colonies that are reddish to pale yellow or pale orange) and *Rubrotorula* (For red to orange colonies). But this concept also was not accepted because of the great variability in the types and concentration of carotenoid pigment in different strains and with changes in cultural conditions.

Phaff and Spencer (1966) suggested separation of *Cryptococcus* and *Rhodotorula* genera based on the utilization of carbon compounds, especially inositol. The simplicity of the inositol test together with its likely metabolic significance induced researchers to consider it as major diagnostic criterion to separate both genera.

General occurrence of Rhodotorula

Rhodotorula strains are of wide occurrence in nature and have been isolated from a variety of environments like flowers, seawater (Indian Ocean and Great Salt Lake, Utah), marshy areas as well as body surfaces and intestines of marine animals like shrimps (Phaff and Ahearn 1970). Kunkee and Amerine (1970) reported occurrence of *Rhodotorula* on grapes must, grape surface, in dry and sweet wines along with that

some of dairy products like cream and butter also records presence of natural *Rhodotorula* strains (Walker and Ayres 1970).

Significance of carotenoids form Rhodotorula

Various researchers studied *Rhodotorula* strains for carotenoid production and quantitaion and it was universally concluded that **torulene** and **torularhodin** are two distinct characteristics carotenoids produced by this yeast and along with them, **b-carotene** and **g-carotene** are also produced in minute quantities.

Torulene is reported to improve the color and other organoleptic qualities of eggs and meat (Harasawa *et al.* 1984) whereas torularhodin-containing eye-drop preparation is reported to prevent opacity of eye lens (Setsuo and Sakaki 1996). Both carotenoids are reported to have provitamin-A property also (Ershov *et al.* 1994, Eugienia *et al.* 1997). There are few reports, which highlight the importance or significance of these carotenoids for yeasts and are described below.

a. Protection against electric field (Voronin *et al.* 1981)

Exposure of *Rhodotorula gracilis* to the electric field (1 kW/cm frequency) enhanced growth and produced a higher biomass yield than control cultures. The carotenoid content of the cells that were resistant to electric field at any frequency (1-7 kW/cm) was 30% higher than that of control cells, suggesting a protective role of carotenoids not only against visible light but also against the electric field.

b. Role of carotenoids in preventing oxidative damage (Moore *et al.* 1989)

Rhodotorula mucilaginosa, was cultured with duroquinone (DQ) (100 mM), a redox-cycling quinone known to generate intracellular singlet oxygen or were grown in a hyperoxic atmosphere (80% O_2) under conditions where carotenoid concentration were altered either intracellularly or extracellularly. Neither of these oxidative challenges affected cell growth unless carotenogenesis was blocked by the addition of diphenylamine (50 mM). In the diphenylamine-treated non-pigmented cells, growth was completely inhibited by DQ and by hyperoxia. Carotenoids, therefore, are able to prevent oxidant-induced cytotoxicity in *R. mucilaginosa*.

d. Animal studies with Rhodotorula

Animal studies were performed using carotenoid-containing *Rhodotorula*. Feeding experiments showed that supplementation of the diet with this yeast could maintain the

normal growth rate of rats. No toxic effect due to yeast supplementation could be detected (Bhuyan *et al.* 1985).

Spray dried biomass of *Rhodotorula rubra* 120r was used as fodder additive in laying hens nutrition. It increased the Hoffman-La Roche egg's color index, improved the carcasses and body fat, organoleptic qualities and detected a rise in body and egg weight. The dried product prepared from *Rhodotorula* was found to be nontoxic and had a good tolerance in laying hens during prolonged administration (Eugenia *et al.* 1997).

d. Radioprotective properties

The lipo-carotenoid complex of *Rhodotorula glutinis* is reported to produce a normalizing effect on the parameters of the lipid transport system and peroxide oxidation of blood serum lipids in irradiated rats (Zalashko 1997).

Carotenoid production from Rhodotorula

Research on carotenoid production from *Rhodotorula* was carried out initially by Nakayama *et al.* (1954), Peterson *et al.* (1954, 1958) and Simpson *et al.* (1964). They reported that *Rhodotorula* produces predominantly three carotenoids viz. torularhodin, torulene and β-carotene in various proportions dependent on environmental and cultural conditions.

Later, utility of *Rhodotorula* with references to its nutritional properties and carotenoid production was established (Atamanyuk and Vakar 1976, Slyusarenko *et al.* 1977, Stabnikova and Drobot 1978, Atamanyuk *et al.* 1979, Stabnikova *et al.* 1979).

Advancement of knowledge of carotenoids as food and feed additives, especially as an anticancer agent (Peto *et al.* 1981), encouraged research on economic production of carotenoids from microbes. In this regard, the use of low cost media components as nutrient source, which included various industrial by-products, was reported (Atamanyuk *et al.* 1981, Martelli *et al.* 1990, Martelli *et al.* 1992, Martin *et al.* 1993, Shih and Hang 1996, Buzzini and Martini 1999). Frengova *et al.* (1994) reported the co-cultivation of *Rhodotorula* with lactobacillus in ultrafiltered whey. Later, optimization of cultural conditions for cocultivation led to substantial increase in carotenoid production described by Frengova *et al.* (1995) and Frengova *et al.* (1997).

Various methods of media-manipulations and cultural conditions were also suggested for improvement of carotenoid content (Burgerova 1981, Tada and Shiroishi 1982, Costa *et*

al. 1984, Atamanyuk and Razumarskii 1974, Atamanyuk *et al.* 1982, Costa *et al.* 1987, Nam and Rhee 1991, Oramas *et al.* 1992, Zhang *et al.* 1998, Sakaki *et al.* 1999).

Most of the studies were of academic significance. However, an attempt to commercialize the carotenoid production from *Rhodotorula* strains was also reported mainly in two steps.

a. *Optimization of media components:*

   Selected *Rhodotorula* strains were investigated for optimization of media components either by conventional method (Mahattanatavee *et al.* 1991, Zhang *et al.* 1995, Zhang *et al.* 1998, Yueying *et al.* 1999) or by statistical methods (Chen and Zhang 1997, Govindaswamy *et al.* 1999, Buzzini 2000).

b. *Strain improvement* :

   Rhodotorula strains were subjected to chemical mutagenesis (Kawai *et al.* 1994, Sakaki *et al.* 2000) for improvement of carotenoid production. Selected *Rhodotorula* strains were also grown in fermenters (Atamanyuk *et al.* 1981, Kawai1994) and downstream processing for carotenoid production was also performed by conventional methods (Perez and Maria 1989).

Several screening methods and new strains of *Rhodotorula* were also reported in the last decade (Hari *et al.* 1992, Perrier *et al.* 1995, Buzzini and martini 1999, Yueying *et al.* 2000).

Scope of this thesis

Increasing population and limitation in cultivable land and water supply for conventional agricultural land had forced mankind to search for alternative food resources. Microorganisms are being investigated as potential food either directly or as feed in poultry and aquaculture.

From last two decades emphasis is paid on yeasts as alternative sources of protein foods. Asporogenous yeast, *Rhodotorula* has drawn interests as feed additive in live stock, poultry and fish industries because of its well-known characteristic carotenoids *viz.* torularhodin, torulene and β-carotene. However, as seen from the summary of research on *Rhodotorula* and its carotenoids, low total carotenoid content and β-carotene proportion limits its industrial exploitation. Hence, there is a need to improve the total carotenoid and β-carotene proportion in *Rhodotorula.* Along with this, it is essential to develop an economic process by using industrial by-products as nutritional sources for yeast growth.

The following is the scope of the work.

- Screening of wild *Rhodotorula* strains for carotenoid production.
- Shake flask studies on growth and carotenoid production by a selected strain of *Rhodotorula glutinis* and strain improvement for increased β-carotene content by mutations.
- A study on the selected mutant in shake flask by factorial approach for optimization of β-carotene production rate and scale up investigations in suitable bioreactors of 14 and 200 L.
- Replacement of conventional carbon and nitrogen sources with industrial by products like sugar cane molasses and corn steep liquor.
- Investigation on the growth of the selected mutant in seawater-based medium for easy extraction of carotenoids.
- Product formulation by spray drying of mutant yeast cell mass under different temperature regime and investigation of stability of carotenoids within the cell mass under different environmental conditions.
- Determination of suitability of *Rhodotorula* as a feed additive in rat liver carcinogenesis model.

Work is presented in following chapters...

Chapter 2. This chapter describes screening of wild strains of *Rhodotorula sp.*, selection of UV mutants of *Rhodotorula glutinis* NCIM 3353 that are efficient β-carotene producers. Investigation of a selected mutant "mutant 32" for its biotechnological and biochemical properties is also described.

Chapter 3. This chapter describes improvement of carotenoid production by mutant 32 in two steps. First, effect of media constituents on carotenoid proportion and content was studied. A two-level, three-variable factorial approach was used to optimize the medium for production of carotenoids and secondly, the effect of cultural conditions on carotenoids production was investigated. Mutant 32 was further grown in fermenter to achieve high cell density and hyper carotenoid production under optimized conditions.

Chapter 4. The objective of the work presented in this chapter was to determine the utility of sugar cane molasses and corn steep liquor as nutrient source for carotenoid production from *Rhodotorula glutinis* mutant 32. Using the optimized conditions, it was grown in fermenters to scale up the process of carotenoid production.

Chapter 5. In this chapter growth and carotenoid synthesis by *Rhodotorula glutinis* mutant 32 in a medium prepared in seawater is described. Effect of seawater on content and proportions of individual carotenoids were studied. Along with that the effect of pH of medium prepared in seawater on extractability of pigments is also described.

Chapter 6. In these studies an attempt was done to reuse the fermented broth after microfiltration as water source for carotenoid and cell mass production.

Chapter 7. In this chapter, suitability of membrane microfiltration for concentration of *Rhodotorula* cell mass is presented. Concentrated cell mass was spray dried in presence of exogenous antioxidants and stability of dried preparation under different light and temperature are described.

Chapter 8. Chemopreventive and anticarcinogenic effects of feed additive preparation of spray dried yeast *Rhodotorula glutinis* NCIM 3353 (RG) containing carotenoids is described in this chapter. Spray dried *Rhodotorula* cells were supplied in diet of Wistar (WR) strain rats. N-nitrosodiethylamine (DEN) was used as a carcinogen to induce

preneoplastic lesions in liver. (This work was done in collaboration with Cancer Research Institute, Parel, Mumbai, India).

Chapter 9. The last chapter summarizes the conclusions of our work and offers suggestions for future studies in this area.

References

References are listed as **Chapter 10**

Chapter 2

Screening, mutation and properties of selected mutant of Rhodotorula sp. for hyper carotenoid production

Summary

Wild strains of *Rhodotorula spp.* were studied for carotenoid production (mg/l), cellular carotenoid accumulation (mg/g), proportion of the three carotenoids (%, w/w) and cell mass yield (g/l). *Rhodotorula glutinis* NCIM 3353 produced 2.2 mg total carotenoid per liter of culture broth in 72 h and was selected for further studies. Total carotenoid content in the cell mass of this strain was 0.122 mg/g and β-carotene accounted for 14% (w/w) of the carotenoids.

Rhodotorula glutinis NCIM 3353 was subjected to UV mutagenesis for strain improvement. The best mutant in each mutagenesis experiment was selected for further strain improvement. Out of 2051 isolates screened, a yellow coloured "mutant 32" produced 15 fold (33 ±2 mg/l) more total carotenoids than the parent strain, in 72 h. β-Carotene was the major carotenoid produced and exhibited 120 ±2 fold (2.048 ±0.05 mg/g) more β-carotene production than the parent culture in 32 h, which was 88 ±5% (w/w) of total carotenoid content.

Carotenoid production profile of mutant 32 indicated that the volumetric β-carotene production (mg/l) and cellular accumulation (mg/g) was growth associated. Selected mutant was also studied for its biochemical and nutritional properties.

Introduction

Yeasts are more convenient than algae and fungi for large-scale production in fermenters due to their unicellular nature and comparatively higher growth rate. Asporogenous yeast, *Rhodotorula*, is known to produce characteristic carotenoids viz. torulene, torularhodin and β-carotene in various proportions. The quantity of β-carotene in naturally occurring strains of *Rhodotorula glutinis* is, however, very less and its proportion to other pigments is also considerably low (Peterson *et al.* 1954, Peterson *et al.* 1958, Perrier *et al.* 1995, Buzzini and Martini 1999). Low content of β-carotene makes the process of carotenoid and feed additive preparation from this yeast unattractive. Previously, a few researchers have attempted mutation in *Rhodotorula sp.* to increase yield of carotenoids (Sakaki *et al.* 2000, Kawai *et al.* 1994). However, such mutations were focussed on increasing total carotenoid content which comprised mainly of torulene and torularhodin. The published literature for mutation of *Rhodotorula* for **b-carotene** production is scanty.

During pigment biosynthesis, γ-carotene acts as a branch point carotenoid from where hydrogenation leads to β-carotene formation whereas hydroxylation followed by carboxylation leads to formation of torulene and torularhodin, respectively (Simpson *et al.* 1964). In order to get β-carotene hyper producing mutant, there was a need to block the biochemical pathway that leads to torularhodin and torulene synthesis. In this chapter, **screening** of wild strains of *Rhodotorula sp.* is described, followed by **selection** of UV mutants of *Rhodotorula glutinis* NCIM 3353, that are efficient β-carotene producers. Finally, the study of a selected mutant for its **biotechnological and biochemical** properties is also presented.

Materials and methods

Reagents and chemicals

The media ingredients were purchased from HiMedia (Mumbai, India). HPLC grade acetonitrile, ethyl acetate, methanol, tetrahydrofuran and iso-propanol were obtained from E. Merck (Mumbai, India). Chloroform, petroleum ether (40-60 °C) and n-hexane of spectroscopic grade were from S. D. Fine Chemical Co. (Boisar, India). Trans-β-carotene (C 9750) and α-carotene (C 0251) used as reference compounds for HPLC were from Sigma Chemical Company, USA.

Yeast strains

Rhodotorula strains listed in Table 2.3 were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India.

Medium

All the parent strains and mutants of *Rhodotorula glutinis* NCIM 3353 were maintained on YM agar containing (g/l) glucose 35, malt extract 30, yeast extract 20, peptone 10, K_2HPO_4 3, KH_2PO_4 3, $MgSO_4 \cdot 7H_2O$ 0.2 and agar 20, at pH 6.0.

The liquid basal medium for shake flask studies contained (g/l) glucose 25, yeast extract 10, K_2HPO_4 2, KH_2PO_4 2 at pH 6.0.

UV Mutagenesis

Cells of *R. glutinis* 3353 in mid-logarithmic phase (Absorbance$_{500nm}$ 39) were suspended in sterile 0.85% (w/v) NaCl solution and shaken gently to separate the mature daughter cells from mother cells. The suspension was then placed in sterile petri dishes and

irradiated in a hood equipped with UV light source with maximum energy at 220-280 nm at a distance of 8 cm, for time intervals ranging from 5 to 300 seconds. After irradiation, the suspension was diluted appropriately, spread on yeast extract glucose agar plates and the plates were incubated in dark. The UV exposure time was selected to get 10% survival. The selected UV irradiation time was used for further mutations. This was done for all step mutants, subsequently.

Mutant screening

The isolated colonies were inoculated in 5 ml liquid medium in 150 mm X 25 mm test tubes (Borosil, Mumbai) and incubated at 28 °C for 18 h on a rotary shaker at 240 rpm. These liquid cultures were added to 100 ml medium in 500 ml Erlenmeyer flasks and incubated at 28 °C for 72 h.

Initially, mutants were screened spectrophotometrically. Absorbance was measured at 500, 484 and 452 nm depending upon the absorbance maxima of the pigments extracted from 1 ml of culture broth. Selected isolates were further screened using HPLC, for individual carotenoid content (mg/g and mg/l) and their proportion.

One best isolate from each mutation stage was selected for subsequent strain improvement. The satisfactory mutant obtained from final mutation stage was studied for growth and pigment production.

Pigment extraction from the yeast cells

Method A

One hundred ml culture broth was centrifuged at 10,000 *g* for 10 min at 5 °C. Supernatant was discarded and media components in the cell pellet were washed away by repeated suspension in sterile physiological saline and centrifugation. The cell mass was subjected to mechanical disruption using Braun's homogenizer (Braun, Melsungen, Germany, Model number 01822) in the presence of 10 ml cold, oxygen-free acetone. Ten subsequent acetone extracts were pooled and centrifuged to remove insolubles. The combined acetone extract was transferred to 250 ml separating funnel, 20 ml of n-hexane (cold and oxygen-free) was added to it and mixed well. Twenty six ml cold distilled water was added to the mixture to cause phase separation. If necessary, a few drops of saturated NaCl solution were added to break the emulsion. Hexane hyperphase was made to pass through glass wool pack having anhydrous Na_2SO_4. The pigments were concentrated by rotary vacuum evaporation and then stored at -20 °C, before

analysis. The pigments were reconstituted in suitable solvent for further analysis. The volumes of solvents were adjusted proportionately, according to the volume of culture broth to be processed.

Method B

One ml aliquot of the culture broth was centrifuged. The pellet obtained was washed twice with distilled water and dehydrated with 1 ml dry acetone. Cells were disintegrated for pigment extraction in a mini bead beater (Biosep products) with 1 ml mixture of acetonitrile, isopropanol and ethylacetate (40:40:20). The extract was centrifuged and supernatant filtered through 0.45 μm membrane filter, before quantitation by HPLC.

Pigment separation and purification

Fifty ml hexane extract containing mixture of the carotenoids viz. torularhodin, torulene and β-carotene (obtained from extraction with method A) was washed two times with 25 ml 90% methanolic potassium hydroxide (0.1N), to remove torularhodin. The methanolic potassium hydroxide solution containing torularhodin was first neutralized and later acidified with the 0.02N HCl. Acidified torularhodin was then extracted out in 30 ml petroleum ether, which was then dried under vaccum.

Torularhodin-free hexane extract, containing torulene and β-carotene, was then subjected to column chromatography under dim light and nitrogen atmosphere using MgO (light) and Celite in the proportion of 2:1(w/w). Thick slurry (80 g/l powder mixture in hexane) was used to pack the column (12 cm bed height) under vacuum. Cold and oxygen-free hexane was used for column packing and washing. Ethanol was used for elution of the pigments. Purity of these purified carotenoids was confirmed by thin layer chromatography, spectroscopy, HPLC and by checking the absorbance maxima as described below.

Thin Layer Chromatography (TLC)

Carotenoids were chromatographed using pre-coated TLC plates (silica gel 60, Merck, Germany) and a mixture of petroleum ether: acetone (80:20, v/v) as the mobile phase. Presence of carotenoids was detected by developing plates in iodine chamber and individual spots were identified by calculation of R_f.

Spectroscopic estimation

The purified and dried torularhodin was taken in chloroform while torulene and β-carotene were taken in hexane for spectroscopic quantitative analysis at 515, 485, 452 nm using $E^{1\%}_{cm}$ values of 1932, 2680 and 2500, respectively (Frengova *et al.* 1994, Perrier *et al.* 1995). The spots were also eluted in HPLC mobile phase and used as reference torularhodin and torulene during HPLC analysis.

High Performance Liquid Chromatography (HPLC)

Instrument

The High Performance Liquid Chromatography equipment (Thermo Separation Products Inc. U.S.A.) had four-channel solvent degasser SCM 1000, a quaternary low pressure gradient pump P4000, a UV-Visible Forward Optics Scanning Detector Spectra Focus UV3000, chromatography software PC1000 Ver 3.0.1A and a Rheodyne 7725 injector with 20 μl loop. The pigment analysis was performed on reversed phase C_{18}, ODS-2 (LiChroCART, LiChrospher 125-4); particle size 5 μm; pore size 80 angstrom analytical column.

Method development

Different mixtures of HPLC-grade solvents procured from Merck, India were studied as mobile phases at a flow rate 0.7 ml/min (Table 2.1). The column was maintained at 25 ^{o}C in a column oven. The UV-VIS detector was operated in scanning mode in 390-700 nm wavelength range for method development and at 457 nm, subsequently. TLC-purified torulene and torularhodin along with reference α-carotene and β-carotene as well as all the samples were run in triplicates.

The optimal mobile phase was an isocratic mixture of acetonitrile: isopropanol: ethyl acetate in a proportion of 40:40:20, at a flow rate of 0.7 ml per min. The injection volume was 20 µl. The automatic peak purity index determinations were made by on-line spectral scans of all the peaks of standards and samples. Quantitation was based on the peak area. A graph of β-carotene concentration (mg/l) and peak area was plotted. Samples were diluted, whenever necessary, to bring the response in the range.

Absorption spectrum

The absorption spectrum of the purified pigments and carotenoid mixtures extracted from cell mass were taken in hexane, using a Shimadzu UV-240 ultraviolet-visible recording spectrophotometer. The spectra were also compared and confirmed with spectrum obtained with on-line Unicam UV-2 ultraviolet-visible recording spectrophotometer.

Cell growth measurement

Cell growth was monitored by measurement of turbidity at 500 nm with a UV-Visible spectrophotometer (Shimadzu UV-240, Japan). Samples were diluted suitably (so as to have absorbance between 0.2 to 0.8) with distilled water and absorbance measured immediately at 500 nm.

For dry cell weight (CDW) estimation, the sample (usually 5 or 20 mL) was centrifuged at 10,000 *g* for 10 minutes, washed twice with distilled water by suspension and centrifugation, supernatant discarded and then the cell pellet was taken to constant weight in an oven at 80 °C.

Characterization of mutant 32

Growth and carotenoid production

Growth and carotenoid production by the parent culture and mutant 32 were studied in shake flasks in the basal medium, in quadruplets. Samples were withdrawn at regular intervals and analyzed for pH, dry cell weight and carotenoid content as described earlier.

Effect of oxidative stress

In the presence of white light, methylene blue acts as a singlet oxygen generator. To study the effect of oxidative stress, methylene blue at 10 mg/l concentration was added to the basal medium. The degree of tolerance of each strain to the oxidative stress was studied by the effect on carotenoid content (mg/l, mg/g) and final dry cell mass (g/l, after72 h).

Effect of UV light

One ml samples of the parent strain and mutant 32, broth grown in shake flask containing the basal medium, were withdrawn at 12 h time intervals and suspended in ten ml of sterile 0.85% (w/v) physiological saline. The suspension was shaken gently to separate the mature daughter cells from mother cells. The suspensions were suitably

diluted (between 10^4 and 10^8 CFU /ml) and plated on yeast extract glucose agar plates. The plates were irradiated in a hood equipped with UV light source with maximum energy at 220-280 nm from a distance of 8 cm for time intervals ranging from 5 and 100 seconds. After irradiation for predetermined time, the plates were incubated in dark for 72 h. Colony forming units (CFU) were measured and compared with the plates not exposed to UV.

Effect of Diphenylamine (DPA)

The effect of diphenylamine (DPA) on pigmentation of culture, carotenoid production (mg/g, mg/l) and cell growth (g/l) of the parent strain and mutant 32 was examined by culturing cells in shake flask in the presence of different concentrations of diphenylamine (between 5 and 120 mg/l).

Results and discussion

TLC and absorption maxima.

The thin layer chromatography revealed R_F values of the pigment spots to be similar to those published earlier for torularhodin, torulene and β-carotene (Perrier *et al.* 1995). Close agreement was obtained between the absorption maxima of the isolated pigments and published data as presented in Table 2.1 (Simpson *et al.* 1964, Frengova *et al.* 1994).

Table 2.1 Thin layer chromatography and absorption maxima of purified pigments.

Carotenoids	R_F value	Absorption maximum #
Torularhodin	0.23	515
Torulene	0.82	485
β-Carotene	0.93	452

Determinations for torulene and β-carotene were made in hexane. Torularhodin was in $CHCl_3$.

HPLC of carotenoids

High performance liquid chromatographic methods for the separation of carotenoids were first reported in 1970 (Craft 1992). The sophistication in HPLC methods for carotenoid has evolved as HPLC columns and equipment has improved. A variety of reversed phase HPLC methods have been published earlier in the literature. The most common column packing material used for carotenoid separation is octyldecylsilane (ODS or C 18). This is a popular column packing because of its compatibility with most solvents, usefulness for entire polarity range of carotenoids and wide commercial availability.

Mobile phases have made major influence on carotenoid separations. Majority of mobile phase alternations have resulted in development of better methods. Carotenoids are insoluble in water and therefore should be avoided or only to be incorporated sparingly as a solvent modifier. From column evaluation studies, it was found that the recovery of carotenoids could be improved by adding modifiers.

Various solvent systems from the available literature (Craft 1992) were tested for separation and quantitation of carotenoids from *Rhodotorula sp.* (Table 2.2). Mobile phase comprising acetonitrile: propanol: ethyl acetate (40:40:20) was selected for further studies (Weissenberg *et al.* 1997).

Absorption maxima of β-carotene, torulene and torularhodin were found to be at 457, 485 and 492 nm, respectively, similar to those described by Perrier *et al.* (1995). During HPLC analysis, the main carotenoids from *Rhodotorula* strains *viz.* torularhodin, torulene and β-carotene, eluted at 2.29, 3.2 and 3.9 min, respectively, (Figure 2.1) and the peaks had satisfactory purity indices of 987, 956 and 974, respectively. The spectral scanning performed in visible range showed excellent peak purity for carotenoids confirming good resolution, from a relatively crude sample preparation. The triplicate chromatography runs gave excellent reproducibility. The facility of scanning detector greatly enhanced the reliability of HPLC analysis as it could perform on-line spectral analysis that eliminated problem of co-elution of two or more compounds. It also helped in absorption maxima determination during method development. The selected method finally had several advantages over the conventional spectroscopic methods. In spectroscopic methods the sample has to pass through acetone-water, n-hexane, absolute ethanol and then back to hexane, before analysis. This can cause loss of material during phase separation and degradation due to the exposure to light and atmospheric oxygen. It also involves a time consuming column chromatography, before measurement of absorbance for quantitation.

During the analysis, the standard as well as samples were dissolved in mobile phase made up of relatively high boiling solvents as compared to n-hexane. This avoided a practical problem of volume error arising due to volatile nature of hexane or petroleum ether.

In addition, the procedure of taking the samples in hexane for injection developed by Nam *et al.* (1988) and later used by Kim *et al.* (1996) was found to develop a substantial and rapid increase in back pressure in the column, presumably due to the insufficient compatibility of n-hexane and mobile phase. Use of the mobile phase itself to dissolve samples ensured compatibility of compounds being analysed with mobile phase and avoided possible precipitation of non-compatible solutes in the HPLC system. The technique prevented rapid changes in column backpressure, resolved the carotenoids very well, and most importantly had a very short chromatographic run time that permitted analysis of large number of samples.

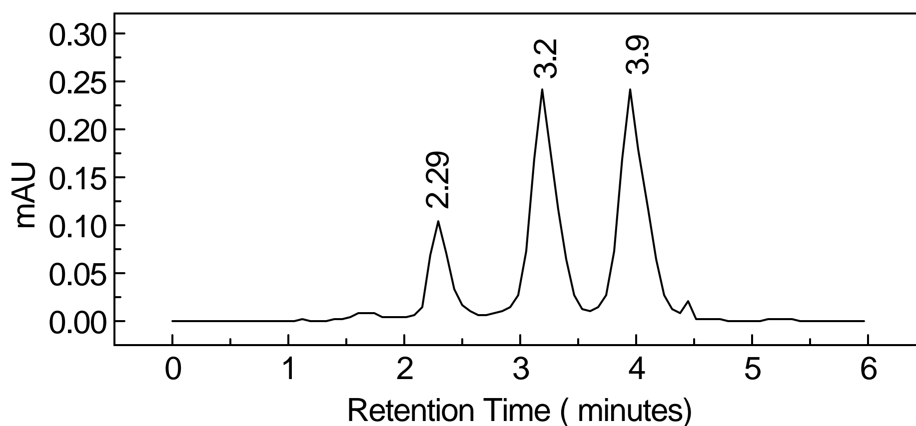


Figure 2.1 HPLC chromatogram of standard mixture of torularhodin (R.T 2.29 min, 5 mg/l), torulene (R.T. 3.2 min, 30 mg/l) and β-carotene (R.T 3.9 min, 30 mg/l).

Linear calibration curve of β-carotene concentration (between 0.35 and 200 mg/l) versus peak area was obtained (Figure 2.2) which was used for calculating β-carotene concentration in the samples.

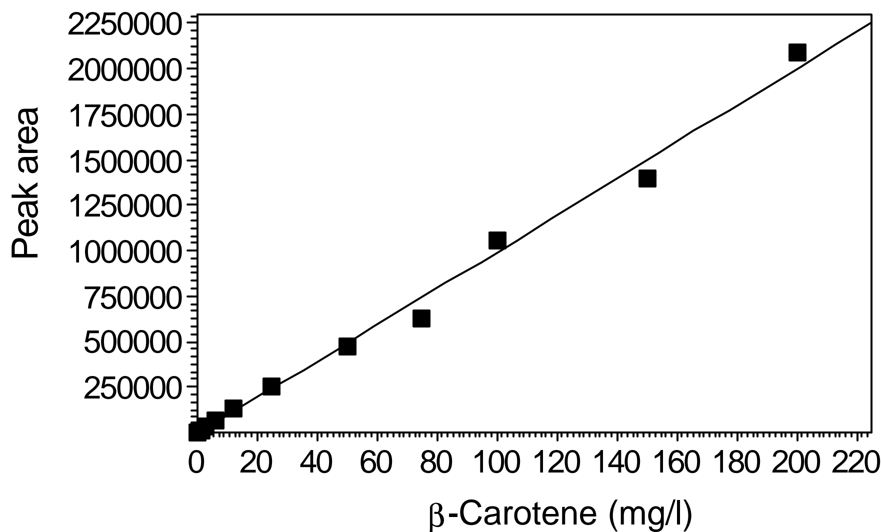


Figure 2.2 Calibration curve of β-carotene concentration (mg/l) and peak area.

Table 2.2 Effect of different mobile phases for separation of carotenoids from *Rhodotorula glutinis.*

Mobile phase	Solvent for sample	Carotenoids (Retention time, minutes)	**Advantage /** disadvantages
Acetonitrile: Tetrahydrofuran: Water (50:38.5: 11.5)	Petroleum ether	Trdin (6.2), Tlene (8.3), αC(12.0), βC (12.49)	Petroleum ether can have evaporation loss leading to error in quantitation**.**
Acetonitrile: Tetrahydrofuran: Water (52:35: 13)	Mobile phase	Trdin (2.5), Tlene (3.3), αC (3.8), βC (3.96)	**Quick and rapid.** THF can cause peroxidation of carotenoids.
Acetonitrile: Methanol (50:50)	Mobile phase	Trdin (6.8), Tlene (8.7), αC (13.6), βC (14)	Slow and high back pressure.
Acetonitrile: Propanol: Ethyl acetate: (40:40:20)	Mobile phase	Trdin (1.9), Tlene (3.4), βC (3.9).	**Rapid and quick, No peroxidation problems, No backpressure problems.**
Acetonitrile: Methanol: THF (50:45:5)	Mobile phase	Trdin (6.13), Tlene (14), αC (17.4), βC (19.3).	**No peroxidation problems, No backpressure problems.** Slow.
Methanol:Tertahy drofuran (95:5)	Mobile phase	Trdin (5.3), Tlene(12.3), αC(13.5), βC(14.3)	**No peroxidation problems, No backpressure problems.** Slow.

Trdin: Torularhodin, Tlene: Torulene, αC: α-Carotene, βC: β-Carotene.

Screening of wild Rhodotorula strains

Rhodotorula strains obtained from National Collection of Industrial Microorganisms (NCIM) were screened for total carotenoid production (mg/l and mg/g) and growth (CDW, g/l). It was observed that most of the strains produced torulene and torularhodin as major carotenoids and the amount of β-carotene produced was very less (Table 2.3). The low content of β-carotene would make the process of carotenoid production from these yeast strains unattractive, in spite of the advantages mentioned in chapter 1. Hence, it was necessary to improve the selected yeast strain for hyper production of β-carotene.

Table 2.3 Screening of wild strains of *Rhodotorula sp.* for carotenoid production.

Organisms	Dry cell weight (g/l)	Total carotenoids		β-Carotene	Proportion β-carotene: torulene: torularhodin (% w/w)
		mg/l	mg/g	mg/l	
R. glutinis NCIM 3168	16	0.60	0.037	0.072	12:55:33
R. glutinis NCIM 3169	15	0.40	0.026	0.048	12:59:29
R. glutinis NCIM 3170	14	0.57	0.040	0.068	12:54:34
R. glutinis NCIM 3353	18	2.20	0.122	0.308	14:53:33
R. rubra NCIM 3171	16	0.67	0.042	0.047	7:57:36
R. rubra NCIM 3173	17	0.82	0.048	0.098	12:48:40
R. rubra NCIM 3172	15	0.52	0.035	0.067	13:47:40
R. rubra NCIM 3174	15	0.72	0.048	0.086	12:48:40
R. rubra NCIM 3175	12	0.66	0.055	0.026	4:60:36
R. rubra NCIM 3325	16	0.91	0.057	0.064	7:57:37
R. rubra NCIM 3260	13	0.41	0.032	0.012	3:64:33
R. rubra NCIM 3395	18	--	--	--	--
R.rubra NCIM 3560	3	0.01	0.003	0.003	3:69:28
R.aurantica NCIM 3425	7	--	--	--	--
R.graminis NCIM 3426	13	0.74	0.057	0.022	3:63:34
R.marina NCIM 3415	14	--	--	--	--
R.minuta NCIM 3350	15	--	--	--	--

Rhodotorula glutinis NCIM 3353 was chosen for mutation from the strains studied based on final dry cell mass (g/l) and carotenoid production (mg/l, mg/g) and most importantly, relatively more β-carotene content in the wild strains studied.

Mutation

A large number of colour variants appearing white, intense pink, yellow and brown were isolated after UV mutagenesis of *Rhodotorula glutinis* NCIM 3353 (Figure 2.3, 2.4).

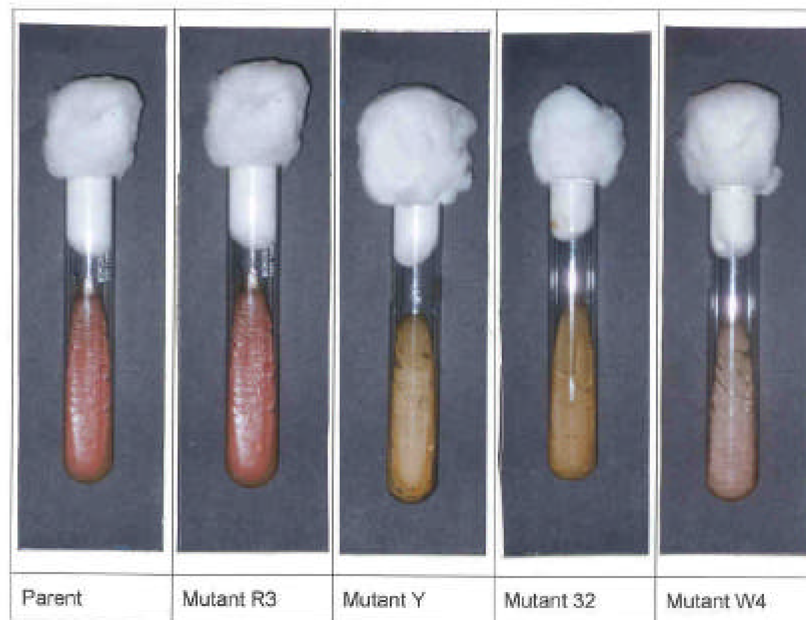


Figure 2.3 Photographs of the parent strain *Rhodotorula glutinis* NCIM 3353 and mutant cultures.

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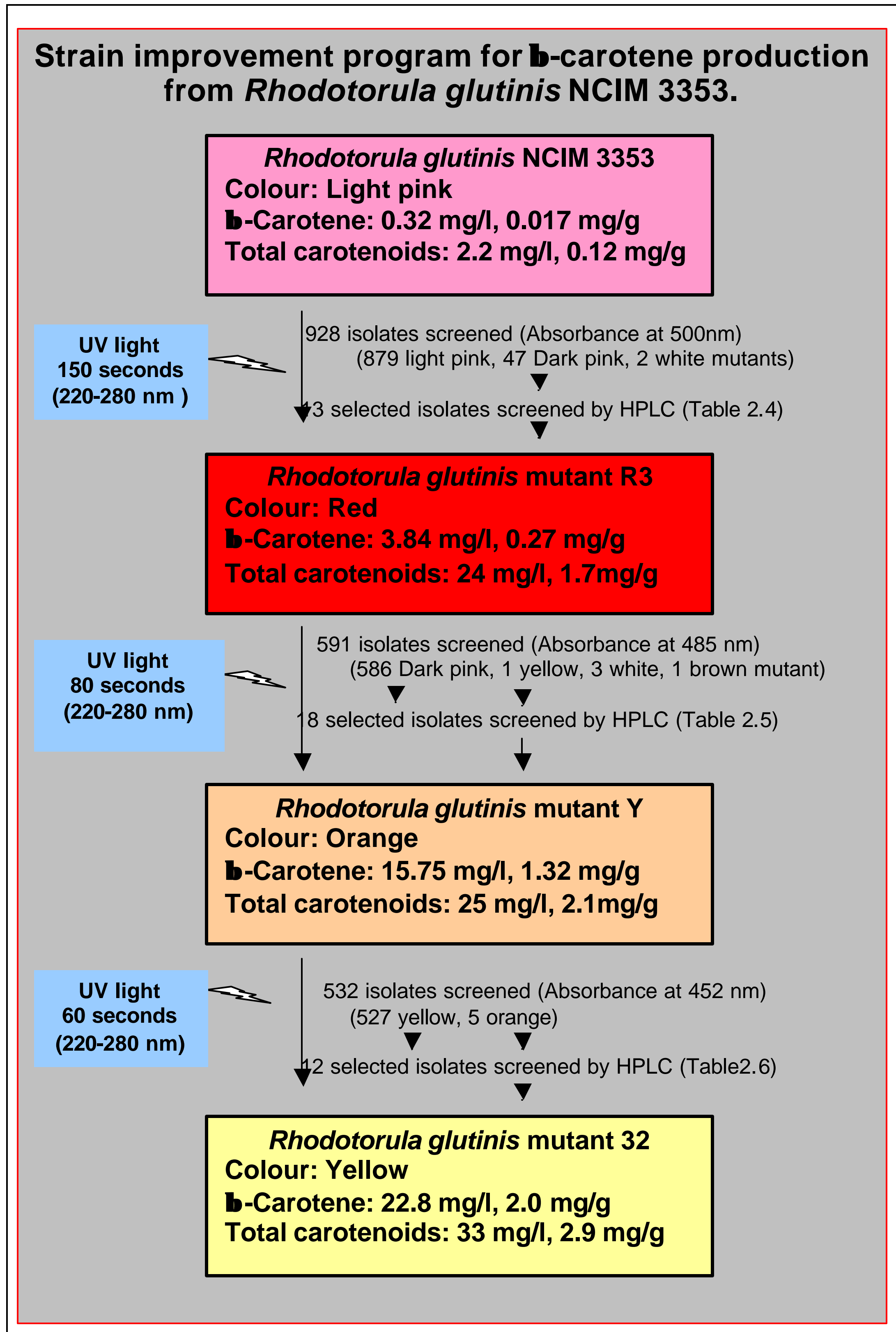
Figure 2.4 Improvement of *Rhodotorula glutinis* NCIM 3353 by UV mutagenesis.



Mutation step 1

A total of 928 isolates were screened spectrophotometrically by measuring the absorbance of pigment extract at 500nm during first mutation stage. A mutant labeled, as “R3” was the best among the thirteen selected mutants examined for growth in shake flask and pigment estimation by HPLC (Table 2.4). HPLC analysis of selected mutants indicated that there was marginal improvement in the proportion (%, w/w) of β-carotene whereas torularhodin and torulene represented the major carotenoids. The final cell mass of R3 achieved after 72 h was 14 g/l with 24 mg/l volumetric total carotenoid production. Mutant R3 showed 12.8 and 16.1 fold improvement over the parent strain in the volumetric production (mg/l) and cellular accumulation (mg/g) of β-carotene, respectively, and was selected for further mutation.

Table 2.4 HPLC analysis of selected mutants during Step 1 mutagenesis of *Rhodotorula glutinis* NCIM 3353.

Parent/Mutants	Dry cell weight	Total Carotenoids		β-Carotene	Proportion β-carotene: torulene: torularhodin (% w/w)
	± 2 g/l	± 2 mg/l	± 0.02 mg/g	mg/l	
Parent	**18**	**2.2**	**0.12**	**0.30**	**14:53:33**
Mutant R 1	15	2.9	0.19	0.46	16:64:20
Mutant R 2	14	2.3	0.16	0.29	13:59:28
Mutant R 3	**14**	**24.0**	**1.70**	**3.84**	**16:81:3**
Mutant R 5	13	4.3	0.33	0.65	15:62:23
Mutant R 7	14	3.8	0.27	0.57	15:53:32
Mutant R 10	17	5.5	0.32	1.54	28:49:23
Mutant R 21	13	4.0	0.31	0.64	16:63:21
Mutant R 23	14	6.7	0.49	0.94	14:67:19
Mutant R 25	12	4.1	0.35	0.62	15:56:29
Mutant R 26	13	5.8	0.45	0.93	16:57:27
Mutant R 31	17	5.3	0.32	0.85	16:66:19
Mutant R 32	13	4.3	0.33	0.69	16:65:19
Mutant R 39	9	4.4	0.50	0.66	15:29:56

Mutation step 2

Using R3 mutant for further strain improvement, 10% survival was obtained after 80 seconds UV exposure. Out of 591 isolates screened spectrophotomerically, by measuring the absorbance of pigment extract at 485 nm, selected 18 mutants were examined by HPLC analysis (Table 2.5). HPLC analysis indicated significant improvement in the β-carotene proportion and quantity in the mixture of carotenoids.

The yellow mutant labeled as Y was found to accumulate (mg/g) 77.7 times more β-carotene as compared to *Rhodotorula glutinis* NCIM 3353 and exhibited complete absence of torularhodin. The volumetric production (mg/l) of β-carotene was observed to be 49 times more than the parent culture.

Mutation step 3

Yellow coloured mutant Y was used for further improvement. Out of 532 mutants screened spectrophotometrically (absorbance at 452 nm) in the last mutation stage, mutant 32 accumulated (mg/g) 118 times more β-carotene than *Rhodotorula glutinis* NCIM 3353. Volumetric carotenoid production was found to be 22.8 mg/l.

HPLC analysis of 12 selected mutants revealed that β-carotene was the major carotenoid and torulene and torularhodin were the two minor carotenoids produced (Table 2.6). This best β-carotene accumulator, mutant 32, was selected for all further β-carotene production studies.

Table 2.5 HPLC analysis of selected mutants during Step 2 mutagenesis of *Rhodotorula glutinis* NCIM 3353.

Parent/Mutants	Dry cell weight	Total Carotenoids		β-Carotene	Proportion
	± 2 g/l	± 2 mg/l	± 0.02mg/g	mg/l	β-carotene: torulene: torularhodin (% w/w)
Parent	**18**	**2.2**	**0.12**	**0.32**	**14:53:33**
Mutant R3	**14**	**24.0**	**1.7**	**3.84**	**16:81:3**
Mutant 1	16.2	39.3	2.4	12.2	31:65:4
Mutant 3	7.4	6.4	0.9	3.4	53:34:13
Mutant 8	14.2	19.2	1.36	6.4	33:55:12
Mutant 11	14.1	27.3	3.45	7.8	29:66:5
Mutant 10	11.4	14.9	1.32	5.8	39:52:9
Mutant 15	14.0	22.8	1.63	8.4	37:55:8
Mutant 21	14.8	20.9	1.42	6.5	31:64:5
Mutant 25	11.5	12.4	1.07	4.8	39:52:9
Mutant 35	12.1	17.3	1.43	7.5	43:47:10
Mutant 38	9.6	15.7	1.64	5.6	36:54:10
Mutant 39	8.7	19.3	2.22	7.7	40:47:13
Mutant 40	12.5	18.5	1.48	7.0	38:51:11
Mutant 41	8.12	17.8	2.19	6.4	36:58:6
Mutant 100	15.0	23.1	1.5	5.7	25:70:5
Mutant Y	**12**	**25**	**2.10**	**15.7**	**63:37:0**
Mutant W2	17	No pigment		--	--
Mutant W3	18	No pigment		--	--
MutantW4	20	No pigment		--	--

Table 2.6 HPLC analysis of selected mutants during Step 3 mutagenesis of *Rhodotorula glutinis* NCIM 3353.

Parent/ Mutants	Dry cell weight	Total Carotenoids		β-Carotene	Proportion β-carotene: torulene: torularhodin(%, w/w)
	± 2 g/l	± 2 mg/l	± 0.2 mg/g	mg/l	
Parent	**18**	**2.2**	**0.12**	**0.32**	**14:53:33**
Mutant R3	**14**	**24.0**	**1.7**	**3.84**	**16:81:3**
Mutant Y	**12**	**25.0**	**2.1**	**15.8**	**63:37:0**
Mutant 3	16.5	20.3	1.3	14.2	70:22:8
Mutant 5	5.2	16.9	3.3	10.9	65:25:10
Mutant 30	11.3	20.1	1.8	14.0	70:25:4
Mutant 32	**11.3**	**33.0**	**2.9**	**22.8**	**69:27:4**
Mutant 33	12.7	22.5	1.7	15.7	70:26:4
Mutant 34	14.3	20.2	1.4	14.5	71:25:4
Mutant 40	12.2	23.2	1.9	17.2	74:20:6
Mutant 41	13.1	19.7	1.5	14.2	72:25:3
Mutant 45	13.1	19.3	1.5	13.2	72:22:6
Mutant 46	12.3	17.3	1.6	11.8	68:25:7
Mutant 39	11.4	17.3	1.5	11.6	67:28:5
Mutant 49	15.2	23.9	0.6	15.1	63:26:11

Selected mutants were studied for biochemical characterization and morphological characters. All of them exhibited remarkable similarity (Table 2.7, 2.8) with the standard description of *Rhodotorula sp*. (Fell *et al.* 1984).

Table 2.7 Properties of selected mutants

A. General properties

Properties	Parent	Mutant R3	Mutant 39	Mutant Y	Mutant W4	Mutant 32
Colour (Liquid growth)	Pink	Red	Pink	Yellow	Cream	Orange
Main Carotenoid	Torulene (53%)	Torulene (81%)	Torularhodin (56%)	Torulene (63%)	----	β-carotene (69%)
$\mu_{max}(h^{-1})$	0.61	0.48	0.57	0.53	0.53	0.39
Lag phase	0.5h	2h	5h	4h	1h	6h
CDW (g/l) After 72h	18	13	12	12	12	13

B. Morphological characterization

Shape	Oval	Oval	Oval	Oval	Oval	Oval
Size (Diameter)	≥ 1mm	≥ 1mm	≥ 1mm	≥ 1mm	≥ 1mm	≥ 1mm
Reproduction	Bipolar budding	Bipolar budding	Bipolar budding	Bipolar budding	Multilateral budding	Bipolar budding
Spore formation	-ve	-ve	-ve	-ve	-ve	-ve
Pseudo mycelium	-ve	-ve	-ve	-ve	-ve	-ve
Sediment formation	-ve	-ve	+ve	+ve	+ve	+ve
Pellicle formation	-ve	-ve	-ve	-ve	-ve	-ve
Fermentation	- ve	-ve	-ve	-ve	-ve	-ve
NO_3 assimilation	+ve	+ve	+ve	+ve	+ve	+ve

- ve: Negative test, +ve: Positive test

Table 2.8 Nutritional and biochemical properties of the parent *Rhodotorula glutinis* 3353 and selected mutants.

Tests	Parent	Mutant R3	Mutant R39	Mutant Y	Mutant W4	Mutant 32	Standard# test
Urease test	+	+	+	+	+	+	+
Assimilation of carbon compounds							
Citric acid	++	++	+	+	+	+	V
Succinate	+	+	+	+	+	++	+
Erthritol	-	-	-	-	-	+	-
Arabinose	++	++	++	+	+	++	V
Mannitol	-	+	++	++	++	++	+
Maltose	+	+	+	+	+	+	+
Cellubiose	+	+	+	+	+	+	+
Soluble starch	+	++	+	++	+	+	V
D-Xylose	+	++	+	+	+	+	+
Lactose	-	-	-	-	-	-	-
Glucose	++	++	++	++	++	++	++
Sucrose	++	++	++	++	++	++	++
Ribose	++	++	++	++	++	++	V
Galactose	++	++	++	++	++	++	++
Raffinose	++	++	++	++	++	++	++
Inositol	-	-	-	-	-	-	-

Reference Fell *et al.* (1984), ++ indicates the assimilation was rapid and complete.

Characterization of mutant 32

Mutant 32 was studied for its characteristics with reference to the parent culture *Rhodotorula glutinis* NCIM 3353.

Biosynthetic pathway

Proposed biosynthetic pathway for carotenoid syntheses in yeast *Rhodotorula glutinis* (Simpson *et al.* 1964) shows that γ-carotene is at the branch point and acts as a precursor for β-carotene and torulene. Hydroxylation and oxidation of torulene by mixed function oxidase (Figure 2.5) leads to formation of torularhodin (Goodwin 1980a).

Mutant 32 produced minute quantity of torularhodin and probably had an adversely affected oxidase activity. However, although there was some correlation seen between decrease in torularhodin and increase in β-carotene content, the increase in β-carotene was several folds.

Previous researchers have attempted to block the pathways of other carotenoids at a point wherein the flow towards β-carotene biosynthesis was enhanced. This was achieved either by genetic transformation (Martincz *et al.* 1998) or by use of ethyl methane sulfonate (EMS) and nitrosoguanidine (NTG) (Gigard *et al.* 1994, An 1996) in *Phaffia rhodozyma*. However, there are no reports for such studies in the pink yeast, *Rhodotorula.*

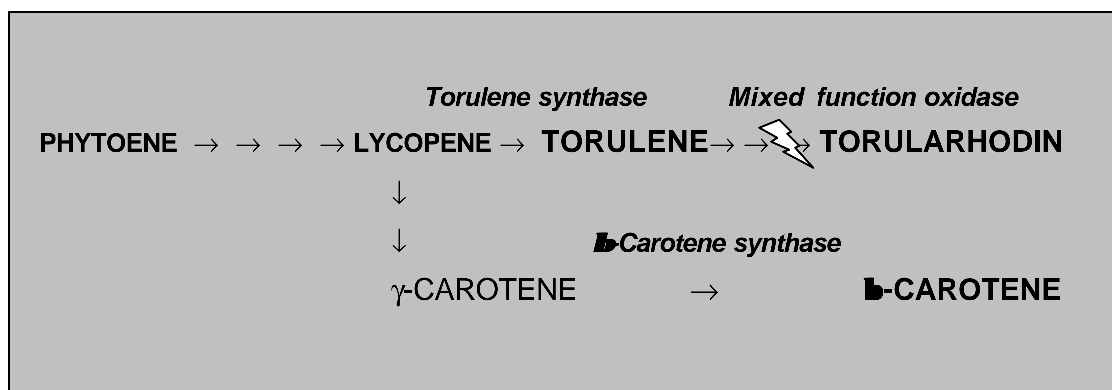


Figure 2.5 Proposed biosynthetic pathway for carotenoid production by *Rhodotorula glutinis* (Simpson *et al.* 1964).

Stability of mutant 32

The microbial strains, particularly hyper producing mutants, have to be genetically stable for any fermentation process to become industrially feasible. It was observed in the present studies that the selected mutants, including mutant 32, were stable over a period

of at least one year. They were maintained on MGYP slopes by monthly subculturing. Carotenoid content and growth behavior were checked during each transfer. It was observed that mutant 32 was stable with respect to its carotenoid production properties.

Absorption spectrum of carotenoid mixture

The characteristic absorption spectrum of a carotenoid is the consequence of the conjugated polyene system present in the molecule and of the various additional features. In the mixture of carotenoids, the pattern of absorption maxima depends on the main carotenoid present in the mixture. Mutant 32 produced β-carotene as a major carotenoid, hence, the main peak of absorption maximum was observed to be close to that of wavelength maximum of β-carotene (452 nm). Similarly, The parent culture *Rhodotorula glutinis* NCIM 3353 that produced torulene and torularhodin as major carotenoids, showed major peak of absorption maxima at 492 (Figure 2.6), which was closely related to that of torulene (485nm) and torularhodin (500nm).

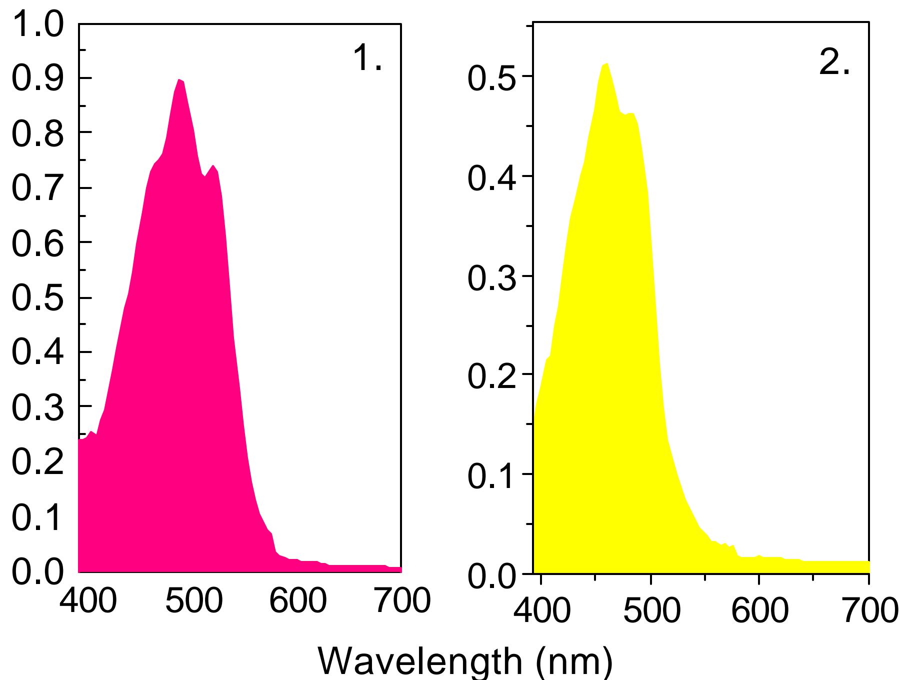


Figure 2.6 Scanning of carotenoid mixtures obtained from **1.** *Rhodotorula glutinis* NCIM 3353 (Pigment from 1 ml broth taken in 1 ml hexane) and **2.** Mutant 32 (Pigment from 0.25 ml culture broth taken in 1 ml hexane).

Growth properties

In shake flask, it was observed that mutant 32 had an extended lag phase (6 h) as compared to the parent culture (2 h). The mutation probably had a negative effect on the specific growth rate (μ_{max}) of mutant 32, which was found to be decreased to 0.22 h^{-1} as compared to 0.56 h^{-1} of the parent culture. After 72 h of incubation, final cell mass concentration achieved by mutant 32 in basal medium was 11.3 ±2 g/l as compared to 19.1 ±2 g/l by parent culture (Figure 2. 7).

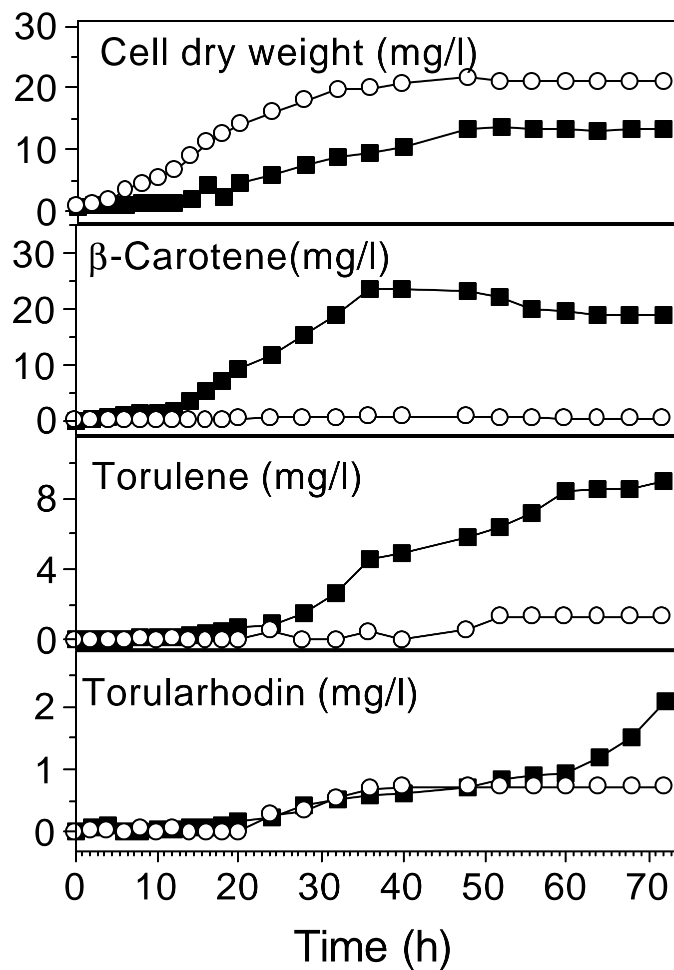


Figure 2.7 Growth and carotenoid production from parent culture, *Rhodotorula glutinis* NCIM 3353 (○) and mutant 32 (■).

Carotenoid production

The carotenoid production (mg/l) was found to be associated with increase in cell mass during the growth phase, both in the parent culture as well as in mutant 32 (Figure 2.7). Mutant 32 produced β-carotene as the major carotenoid. During exponential growth phase (between 12 and 32 h), considerable increase in volumetric β-carotene production rate (0.88 ±0.07 mg/l/h) was observed in mutant 32 as compared to the parent culture (0.017 mg/l/h, between 12 and 20 h). Maximum cellular accumulation (mg/g) of β-carotene by mutant 32 was found to be 2.086 ±0.144 mg/g at 20 h, after which it was found to be constant till the end of incubation period (72 h). Carotenoid production profile (Figure 2.7) indicated that along with β-carotene, mutant 32 also produced torulene and torularhodin in minor quantities.

Previously reported mutants of yeast, *Phaffia rhodozyma* produced 0.54 mg/g β-carotene, in 120 h The present mutant 32, reported here, produces 3.9 fold more β-carotene in much shorter time (32 h) and thus, has a clear edge over the report published earlier (Girard *et al.* 1994).

At the end of the incubation period (72h), the proportion of β-carotene, torulene and torularhodin was 69, 27 and 4% (w/w) respectively, of the total carotenoid content (30.07 mg/l). However, β-carotene represented 88% (w/w, 19.7 mg/l) of the total carotenoids at 32h.

In case of parent culture, β-carotene content was 37% (w/w, 0.7mg/l) at 36 h (Figure 2.8) that later decreased to 13% (w/w) of total carotenoid content (2.34 mg/l) at the end of fermentation.

Effect of oxidative stress.

Singlet oxygen can be generated by exposure of methylene blue (MB) to white light. It was observed that MB affected maximum specific growth rate (μ_{max}) of parent culture as well as mutant 32. In the presence of light and MB, parent culture showed 78% (w/v) decrease in dry cell mass (g/l) as compared to 20% (w/v) in case of mutant 32.

The protective role of carotenoids against oxidative stress in yeast (*Rhodotorula sp* and *Phaffia rhodozyma*) was reported earlier (Santopietro *et al.* 1998, Sakaki *et al.* 2000) and that could be a reason for some degree of tolerance exhibited by mutant 32 under oxidative stress in this study.

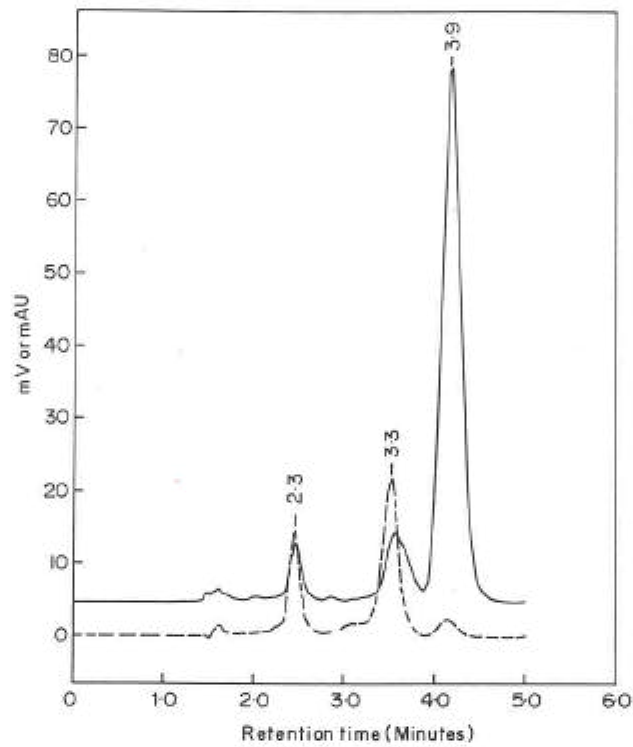


Figure 2.8 HPLC chromatogram of carotenoids from parent culture *Rhodotorula glutinis* NCIM 3353 (---) and mutant 32 (—) at 36h. Torularhodin, torulene and β-carotene eluted at 2.3, 3.3, 3.9 minutes, respectively.

Effect of MB on carotenoid content indicated little change in volumetric production (mg/l). However, in the presence of MB, increase in cellular accumulation of carotenoids (mg/g) was observed in the parent as well as the mutant 32 (Table 2.9). In both cultures, MB did not affect the ratio between carotenoids (β-carotene, torulene, and torularhodin). These observations were similar to those made by Zalashko *et al.* (2000).

Table 2.9 Effect of MB on the parent culture *Rhodotorula glutinis* 3353 and mutant 32.

Strain	Test /Control	Dry cell weight g/l ± 0.8	μ_{max} h^{-1}	Total carotenoids mg/l	Total carotenoids mg/g	Proportion β-carotene: torulene: torularhodin (% ,w/w)
Parent	Control	18	0.61	2.2	0.12	14:53:33
	Light +MB	4	0.13	2.3	0.58	16:52:32
	Dark +MB	7	0.14	2.4	0.34	17:49:34
	Light control	17	0.81	2.1	0.12	19:53:28
	Dark control	14	0.42	1.7	0.12	13:53:34
Mutant 32	Control	11.3	0.39	33	2.9	69:27:4
	Light +MB	9	0.29	37	4.1	68:25:7
	Dark +MB	7.8	0.29	38	4.9	67:26:7
	Light control	12	0.51	39	3.3	71:23:6
	Dark control	10	0.12	33	3.3	58:29:3

The property of acquiring resistance to MB-derived singlet oxygen was also observed recently for hyper torularhodin producing mutant of *Rhodotorula glutinis* (Sakaki *et al.* 2000).

Effect of Diphenylamine (DPA)

Diphenylamine is a common inhibitor of phytoene desaturase in many carotenoid-producing microorganisms (Villadsen 1992). Surprisingly, yeast *Phaffia rhodozyma* is reported to have increased astaxanthin and β-carotene content in the presence of DPA (Chompolkulwong *et al.* 1997, Sanpietro *et al.* 1998).

In the present study, DPA was found to inhibit the growth of mutant 32 as compared to parent culture, which was evident from the higher degree of decrease in the final dry cell mass of mutant 32 in presence of DPA (Table 2.10).

In the presence of DPA, the decrease in total carotenoid content (mg/l) was also observed in both cultures, similar to the earlier observations in *Rhodotorula glutinis* and *Sporobolomyces shibatanus* (Kakutani 1967, Kirillova and Zaichenko 1996).
With increase in the selected DPA concentration, increase in β-carotene proportion by 10± 3% (w/w) was seen in parent (upto 90 mg/l) as well as in mutant 32 (upto 30 mg/l). The results were similar to the earlier report for *Rhodotorula gracilis* (Protiva *et al.* 1959).

Effect of UV light on cell inactivation

Effect of UV light on carotenoid production was observed in mutation studies done in early part of this chapter. With reference to cell viability, it was observed that mutant 32 was more sensitive to inactivation by exposure to UV light as compared to parent culture. Results were in contradiction to the observation made by earlier researchers (Hitoshi 1973, Santopietro *et al.* 1998) wherein un-pigmented *Rhodotorula glutinis* and *Phaffia rhodozyma* respectively, were observed to be more sensitive to the UV light exposure. However, in both parent strain as well as mutant culture more resistance to inactivation by UV light was observed in the late hours of growth indicating some degree of protection presumably due to the carotenoid accumulation (Table 2.11).
Carotenoids are known to offer protection against UV radiation (Fritz *et al.* 1998). Preparation from *Dunaliella sp.* was also claimed recently for the use in cosmetic for prevention of damage resulting from exposure to UV light (Etienne *et al.* 2000). Sensitivity of *R. glutinis* mutant 32 to UV light observed in the present investigation, irrespective of considerably higher β-carotene accumulation, needs further investigation.

Table 2.10 Effect of DPA on the growth and carotenoid production from parent culture and mutant 32.

DPA (mg/l)	Colour	Dry cell weight g/l	Total carotenoids mg/l	Total carotenoids mg/g	β-Carotene mg/l	Proportion β-carotene: torulene: torularhodin (%, w/w)
Parent culture (*Rhodotorula glutinis* NCIM 3353)						
5	Light pink	15.9	1.44	0.09	0.14	10:50:40
10	Light pink	15.9	0.96	0.06	0.12	13:69:18
20	Light pink	15.9	0.92	0.05	0.13	14:68:18
30	Light pink	15.8	0.89	0.06	0.12	14:67:17
50	Light pink	16.1	1.01	0.06	0.17	17:77:6
70	Light pink	16.1	0.93	0.06	0.23	25:68:7
90	cream	15.6	0.36	0.02	0.09	26:68:6
100	cream	15.3	0.38	0.03	0.07	19:75:6
110	White	15.1	0.18	0.01	0.02	12:55:6
120	white	16.0	--	--	--	--
Control	**Light pink**	**18.0**	**2.2**	**0.012**	**0.31**	**14:53:33**
Mutant 32						
5	Yellow	11.0	24	2.2	17.3	72:26:2
10	Yellow	10.1	16	1.6	12.3	77:20:3
20	Yellow	10.1	15	1.3	11.6	77:19:4
30	Yellow	10.0	7	0.7	5.8	81:16:3
50	Yellow	10.0	5	0.5	3.1	62:36:2
70	Yellow	10.2	0.2	0.02	1.2	61:36:3
90	White	9.2	-	-	--	--
100	White	8.3	-	-	--	-
110	White	7.2	-	-	--	-
120	White	6.2	-	-	--	-
Control	**Yellow**	**11.3**	**30**	**3.0**	**21.0**	**70:27:3**

Table 2.11 Effect of UV light on inactivation of Mutant 32 and *Rhodotorula glutinis* NCIM 3353 cells (CFU/ml).

Viability of cells under different dose of UV light (CFU/ml)					
Dose (Seconds)→ Age of Culture (h) ↓	0	20	40	60	80
0	[a]60×10^3	27×10^3	15×10^3	0	0
	[b]57×10^3	31×10^3	11×10^3	0	0
12	16×10^5	14×10^5	4×10^5	0	0
	90×10^5	20×10^5	4×10^5	0	0
24	62×10^7	14×10^7	4×10^7	1×10^7	0
	92×10^7	21×10^7	11×10^7	4×10^7	2×10^7
36	87×10^9	21×10^9	3×10^9	2×10^9	0
	87×10^9	22×10^9	3×10^9	2×10^9	4×10^7
48	102×10^9	33×10^9	2×10^9	2×10^9	0
	103×10^9	32×10^9	3×10^9	2×10^9	4×10^7
60	126×10^9	25×10^9	3×10^9	2×10^9	0
	129×10^9	42×10^9	3×10^9	3×10^9	2×10^9
72	124×10^9	37×10^9	5×10^9	3×10^9	1×10^9
	137×10^9	48×10^9	4×10^9	3×10^9	2×10^9

a: mutant 32 **b:** Parent strain (Both values are mean values of two sets)

Conclusion

In the present studies, seventeen wild strains of *Rhodotorula spp.* were screened for carotenoid production. *Rhodotorula glutinis* NCIM 3353 was selected for mutagenesis to improve β-carotene production. Considerable success was achieved in the overproduction of β-carotene from a mutant 32 obtained by UV mutagenesis of *Rhodotorula glutinis* NCIM 3353. Mutant 32 was observed to be a stable mutant and showed favorable properties for its application in β-carotene production.

References

References are listed in **chapter 10.**

Chapter 3

Optimization of growth parameters for production of carotenoids

Summary

Production of carotenoids from *Rhodotorula glutinis* mutant 32 was optimized with respect to medium ingredients and growth parameters. Initially, the mutant was grown in media containing different carbon, nitrogen sources and metal salts. Of the various carbon and nitrogen sources studied, the best carotenoids yield (31 ±3 mg/l) was obtained when glucose and yeast extract were supplied as carbon and nitrogen sources, respectively. Addition of divalent cation salts further increased the total carotenoid content to 66 ±2 mg/l with β-carotene as the major component, being 55 ±2%, w/w.

Selected medium components were optimized (quantitatively) in shake flask, using two level, three-variable factorial design. Optimized medium containing (g/l) glucose 46, yeast extract 11.74, and threonine 18, along with Ca $(OH)_2$ and $MgSO_4.7H_2O$, resulted in 129 ±2 mg/l of total carotenoid content in the mutant. The medium optimization resulted in four-fold increase in volumetric production (mg/l) and two-fold increase in cellular accumulation (mg/g) of carotenoids. Mutant 32 was further studied for growth and carotenoid production under different cultural conditions like pH, agitation, temperature and exposure to light using optimized medium. Temperature and light were observed to be the most influencing factors. High cell densities were obtained using fed-batch (CDW ≥ 100 g/l) and membrane cell recycle reactor (CDW ≥ 84 g/l) in "optimized medium" wherein threonine was replaced with soyapeptone (10g/l). The carotenoid yields obtained by fed-batch and membrane cell recycle reactor run were as high as 462 ±5 mg/l.

Introduction

Optimization of growth medium and cultural conditions is necessary in microbial fermentations to fully exploit the potential of selected microbial strains (Parekh *et al.* 2000). The conventional method of "one variable at a time" used for optimization of medium is usually time consuming and often fails to assess the additive response of the variables (Sakaki *et al.* 1999). Factorial approach for optimization is convenient and can yield several fold improvements in the process as demonstrated in several cases earlier (Florencio *et al.* 1998, Gawande *et al.* 1998).

Low carotenoid yield and lack of information about optimization of medium and fermentation conditions for carotenoid production by yeast limits the possibilities of their commercialization (Nelis and Deleenheer1991). Buzzini (2000) has presented medium

optimization for carotenoid production by a strain of *Rhodotorula glutinis* but the improvement was marginal. The effects of cultural conditions on carotenoid production from yeasts are reported by many researchers (An and Johnson 1990, Frengova *et al.* 1994, Meyer and Du preez 1994, Buzzini and Martini 1999). Earlier, Costa *et al.* (1987) demonstrated several methods of medium manipulation for improving concentration of β-carotene in *Rhodotorula* strain. However, the quantities of the β-carotene reported in these studies were very low.

In this chapter, improvement in carotenoid production by mutant 32 is presented in the following steps. The yield of carotenoids (mg/l, mg/g) could be enhanced considerably by the techniques presented in this chapter as compared to all those reported earlier in literature.

1. Effect of medium components on carotenoid proportion was studied initially.
2. A two-level, three-variable factorial approach was used to optimize the medium for production of carotenoids.
3. The effect of cultural conditions on carotenoids production by mutant 32 was investigated in shake flask.
4. Mutant 32 was further grown in fermenter to achieve high cell density and higher carotenoid production, under optimized conditions.

Materials and methods

Inoculum

A 10% (v/v, wherever not mentioned) inoculum of mutant 32 in log phase, grown in basal medium was used throughout the studies (A_{500} nm 42, Dry cell weight 10 g/l) as described in chapter 2.

Effect of media components

To see the effect of C: N ratio, mutant 32 was studied for growth and carotenoid production in the liquid basal medium in which glucose content was kept constant (25 g/l) and yeast extract content was varied to give C: N ratio between 10 and 70.

Subsequently, different carbon and nitrogen sources were studied in such a way that the C: N ratio in the final medium was 10. This C: N ratio was selected based on experimental results. For studies on the effect of other carbon sources, glucose was replaced by the indicated carbon source (Table 3.3). Similarly, for studying effect of nitrogen sources (including amino acids), yeast extract was replaced by the indicated

nitrogen source (Table 3.4, 3.5). Effect of addition of salts was examined at a concentration of 100 mg/l of the individual salts mentioned in Table 3.6.

Optimization studies in shake flask

The experimental medium containing varying concentrations of glucose, yeast extract and threonine in addition to (g/l) Ca $(OH)_2$ 0.1, KH_2PO_4 0.2, $MgSO_4.7H_2O$ 0.05, was used for optimization. These components were selected on the basis of results obtained after studying various carbon and nitrogen sources s well as metal salts. The pH of the medium was 6.0 (unless otherwise mentioned). All experiments were done in 100 ml medium in 500 ml Erlenmeyer flasks and incubated at 28 $\pm$1 °C on a rotary shaker at 250 rpm for 72 h.

Factorial design

Factorial experiments were designed using the approach described earlier (Davies 1993, Box *et al.* 1978, Paul *et al.* 1998). The effects of yeast extract (y), glucose (g) and threonine (t) were studied using two-level factorial design presented in Table 3.1. For quantitative variables, a "minus" sign represents the low level and a "plus" sign, the high level. Determination of volumetric production of carotenoids (mg/l) assessed the response.

Data obtained from the factorial experiments were fitted into Yates algorithm for evaluating the relative importance of each variable according to Box *et al.* (1978). New treatment combinations of different variables were determined so as to search for optimum combination, using the method of steepest ascent, as recommended by Davies (1993). Second factorial (two-level) was performed above the best point in the steepest ascent experiment. This was repeated till no further increase in carotenoid production was noted. All the experiments were done in duplicates.

Growth and carotenoid production in optimized medium

Growth and carotenoid production by mutant 32 were studied using optimized medium in shake flasks (in quadruplets), at 28 °C, on a rotary shaker at 250 rpm. Samples were removed periodically and analyzed for cell growth, residual glucose as well as carotenoid content.

Table 3.1 Factorial design matrix.

Flask No.	Yeast extract	Glucose	Threonine
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

Glucose estimation

The residual glucose in the sample broth was estimated by DNSA method (Miller 1959). A standard curve was prepared using different concentrations of glucose.

Effect of cultural conditions

Effect of various cultural conditions was studied using optimized medium. For shake flask experiments, mutant 32 was grown in 100 ml medium in 500 ml Erlenmeyer flasks (Borosil, India), at 28 °C, on a rotary shaker at 240 rpm for 72-96 h.

Inoculum

The effect of inoculum was studied using varying inoculum size between 1 and 20% (v/v). The culture used was in early exponential phase (24 h). The cell density (mg) in the inoculum is mentioned in Table 3.11.

pH

Effect of initial medium pH was studied using buffered medium (Clark-Lubs) as described by Komemushi *et al.* (1993).

Agitation-aeration

Effect of agitation and aeration on carotenoid production was studied by varying shaker speed (between 100 and 300 rpm) and type of flasks (Erlenmeyer, baffled and indented). Effect of shifts in shaker speed was studied by shifting the flask at different time intervals to the desired speed.

Temperature

Effect of temperature on growth and carotenoids production was initially studied in 100 ml medium in 500 ml Erlenmeyer flasks, incubated at different temperatures between 10 and 40 °C on a constant temperature rotary shaker (NBS, Edison, N.J. USA), at 240 rpm, for 96 h.

Effect of temperature shift in shake flasks was studied by transferring the flasks at different time interval to desired temperature.

Growth and carotenoid production pattern at optimum temperatures were studied in a 1 L bottom driven, magnetically stirred fermenter (Gallankamp, U.K.), at pH 7.0, using 10% inoculum (v/v) grown for 18 h in shake flask. The fermentor was operated at 28 °C, with 0.7 litre per minute airflow rate and 400 rpm agitator speed. Samples were withdrawn at regular intervals to measure pH, growth (determined as absorbance at 500 nm after 100 fold dilution), dry weight (g/l) and analysis of carotenoid content (mg/l, mg/g) as described in chapter 2.

The effect of temperature shift on growth and carotenoid production by mutant 32 was investigated by growing it in the fermenter, operated at 30 °C initially and then cooling down the coolant bath rapidly, by addition of ice, to attain 15 °C in the fermenter. It was observed that the temperature of the fermenter was attained within 15 minutes of the change over.

Effect of light

Effect of light on growth and carotenoid production was studied in 1 L stirred fermenter. The fermenter was illuminated by six 40 watt white fluorescent tubes held at 20 cm from the fermenter surface (1000 Lx). The fermenter was run at 30 and 20 °C in two separate experiments. Other fermentation conditions were as described earlier.

Effect of complex nitrogen sources as replacement of threonine

Soyapeptone was used as nitrogen supplement replacing threonine from the “optimized medium”. It was selected after studying various complex nitrogen sources as replacements for threonine. Batch, fed-batch, continuous and membrane cell recycle fermentation were performed using soyapeptone-supplemented medium.

Batch fermentation

Carotenoids production was studied in a 1 L stirred fermenter (working volume 700 ml, Gallenkamp, UK) and in a 14 L stirred fermenter (working volume 10 L, Labroferm, New

Brunswick Scientific, USA). The New Brunswick bioreactor was equipped with three Rushton disk impellers having six-blades fitted on a top-driven shaft. The inoculum was grown in basal medium as described in chapter 2 and added at 10% (v/v) to the fermenter. Sigma 260 antifoam was used at 0.2 mg/l concentration in the fermenter medium. A 10% (v/v) suspension of antifoam was made in water, sterilized by autoclaving and added drop-wise to the fermenter, whenever necessary. All fermentations were carried out at 28 ±2 °C and 500 rpm. Airflow was maintained at 0.7 volume/volume of reactor/minute (VVM). Samples were withdrawn from the fermenters using a sampling device described by Jogdand and Karanth (1983). The sample sizes for 1 L and 14 L fermenters were 2 and 50 ml, respectively.

Fed-batch experiments

The fed-batch experiments to achieve high cell densities were performed in 14 L fermenter (New Brunswick Scientific, USA). The batch run was started with 6 L medium. The feed (4 L) contained double or triple the quantities of medium constituents used for batch fermentation. The stirrer speed used was between 300 and 600 rpm. The aeration rates were between 0.7 and 1.0 (VVM). The feed rate was controlled using a feed-back control based on dissolved oxygen (DO level, which was maintained between 10-40%), and feeding rate (between 120 ml/h to 180 ml/h). Samples were drawn from the fermenter aseptically, at regular interval, to monitor pH, dry weight and carotenoid content. The fermentation was continued till DO increased finally to almost 100% of saturation.

Continuous culture experiments

Continuous fermentation was performed in 1 L fermenter. The fermenter vessel was provided with an outlet (I.D. 4mm) for liquid to overflow, which enabled fermenter volume to be maintained at 700 ml. The dilution rate was varied from 0.05 to 0.6 h^{-1} with a calibrated peristaltic pump. At each dilution rate, the feed was added for at least five reactor volumes, to ensure steady-state. Samples were withdrawn at every steady-state and analyzed for cell growth, dry weight, residual glucose and carotenoid content as well as their proportion.

Cell recycle reactor

Mutant 32 was grown in a 1 L Gallenkamp fermenter coupled to a microfiltration module (Figure 3.11). The microfiltration unit contained a 0.45μ tubular ceramic-based

membrane element with an area of 80 cm^2 and hold-up volume of 25 ml. The cells were recycled using a gear pump (Micropump Watson Marlow, UK) having flow rates between 0.5 and 5 ml/min at a pressure of 2 kg/cm^2. The fermenter and membrane assembly was sterilized by autoclaving at 121 °C for 30 minutes.

Initially, mutant 32 was allowed to grow in batch mode for 60 h. Later, sterile medium was fed to the fermenter using calibrated peristaltic pump, at desired flow rates. Samples of retentate were removed to estimate dry cell weight and carotenoid content. Glucose content in retentate and permeate samples was analyzed after removal of cells by centrifugation.

Results and discussion

Effect of the C: N ratio

It was observed that using the basal medium, a lower C: N ratio resulted in better volumetric production (mg/l) of carotenoid due to relatively higher cell mass yield (g/l) (Table 3.2).

C: N ratio between 10 and 70, however, had no significant effect on carotenoid accumulation (mg/g) in the cells. A medium with C: N of 10 was selected for further studies as it resulted in better β-carotene production and accumulation (mg/l, mg/g) in the C: N range studied. These results corroborated the findings of Somashekar and Joseph (2000) wherein low C: N ratio had resulted in high carotenoid production (mg/l). Mutant 32 produces very low amount of torularhodin (4 ±3%), which remained unaltered with the decrease in C: N ratio, as against that reported by Nam and Rhee (1991).

Table 3.2 Effect of C: N ratio on carotenoid production from *Rhodotorula glutinis* mutant 32.

C:N ratio	Dry cell Weight g/l	Carotenoids Content mg/l	mg/g	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin, %, w/w)
10	11.3	33.3	2.90	22.9	69:27:4
20	8.0	16.4	2.05	11.2	68:27:5
30	7.1	12.2	1.70	8.2	67:26:7
40	5.6	9.2	1.64	6.3	68:28:4
50	4.2	7.4	1.76	5.2	70:29:1
60	4.37	7.5	1.71	5.3	70:29:1
70	4.6	7.7	1.91	5.7	74:23:3

Effect of carbon source

Variation in the carbon sources employed in the medium affected growth and carotenoid production of mutant 32 (Table 3.3). Glucose yielded proportionately higher percentage of β-carotene, whereas the torulene content was considerably higher when fructose was used. This is an interesting result, which needs further investigation. Although cellular accumulation of carotenoids (mg/g) was high with arabinose, rhamnose and xylose, their use in the medium resulted in less growth and relatively low β-carotene proportion.

Sucrose yielded a comparable cell mass (g/l), volumetric production (mg/l) and cellular carotenoid accumulation (mg/g), but torulene was the major carotenoid.

Glucose was reported to be a better carbon source for carotenoid production and accumulation in earlier studies done with *Rhodotorula glutinis* (Nam and Rhee 1991) and *phaffia rhodozyma* (Fang and Cheng 1993, Xu *et al.* 2000). Easily utilizable sugars, like glucose, promote rapid cell growth and since the pigment production is directly proportional to the growth, presumably results into the higher production of carotenoids.

Table 3.3 Effect of carbon source on carotenoid production from mutant 32.

Carbon source	Dry cell weight	Total carotenoids		β-Carotene	Proportion (β-carotene : torulene : torularhodin %, w/w)
	g/l	mg/l	mg/g	mg/l	
D-Arabinose	5.7	42.3	7.4	19.0	45:53:3
Fructose	9.3	19.0	2.0	6.9	36:63:1
Glucose	**13.1**	**33.0**	**2.9**	**22.8**	**69:27:4**
Glycerol	8.6	9.8	1.1	5.6	57:41:2
Mannitol	7.6	11.7	1.5	6.9	59:38:3
Rhamnose	8.2	37.1	4.5	19.3	52:45:3
Sorbitol	9.2	8.4	0.9	3.9	46:52:2
Starch	6.1	13.3	2.2	5.2	39:59:2
Sucrose	11.6	23.3	2.0	8.9	38:60:2
Xylose	3.8	23.9	6.3	11.9	50:48:2

Effect of nitrogen source

Influence of organic and inorganic nitrogen sources on carotenoid production is presented in Table 3.4. Organic nitrogen sources resulted in higher carotenoid production in contrast to observations by Paroja *et al.* (1998), wherein, inorganic nitrogen sources were reported to result in higher growth and carotenoid accumulation. Hydrolyzed nitrogen sources, except casein hydrolysate, resulted in higher β-carotene proportions in mutant 32. Yeast extract, supported maximum carotenogenesis in the present mutant was, on the contrary, reported to inhibit carotenogenesis in *Rhodotorula glutinis* (Ozawa and Takahashi 1994). Positive results were obtained with other

carotenoid producing yeasts like *Phaffia rhodozyma,* recently (Fang and Cheng 1993), by using yeast extract in the growth medium. Yeast extract was selected as the nitrogen source for further studies because of the favourable results.

The different inorganic nitrogen sources studied, resulted in poor growth of mutant 32. Ammonium nitrate resulted in distinctly higher β-carotene content in contrast to ammonium sulphate and ammonium chloride. Nitrate salts are reported to stimulate carotenoid production (Bobkova 1965) in yeast but the poor cell yield that was obtained in present studies, limited its further utility.

Table 3.4 Effect of nitrogen sources on carotenoid production from mutant 32

Nitrogen source	Dry cell weight g/l	Total carotenoids		β-Carotene	Proportion (β-carotene : torulene : torularhodin, %, w/w)
		mg/l	mg/g	mg/l	
Ammonium chloride	4.5	6.1	1.4	1.1	18:70:12
Ammonium nitrate	4.1	13.1	3.0	9.6	73:22:5
Ammonium suphate	5.5	6.8	1.2	1.4	21:67:12
Casein hydrolysate	10.8	11.7	1.0	3.4	29:67:4
Cotton seed meal	10.4	20.2	1.9	8.9	44:52:4
Peptone	12.5	23.0	1.8	14.5	63:34:3
Soya peptone	11.8	25.0	2.0	17.5	70:28:2
Soybean meal	7.6	16.0	2.5	5.2	32:66:2
Tryptone	12.5	26.0	2.0	18.9	73:25:2
Urea	4.0	17.0	4.2	7.8	46:51:3
Yeast extract	**13.1**	**33.0**	**2.9**	**22.8**	**69:27:4**

Effect of amino acids

Growth of the mutant 32 was poor with all amino acids, except lysine. Threonine resulted in relatively highest volumetric (mg/l) and cellular carotenoid accumulation (mg/g), however, the proportion of β-carotene was low (Table 3.5). Threonine, along with tyrosine and serine, is known to be a precursor of pyruvic acid, which was reported to enhance biosynthesis of carotenoid in *Blakeslea trispora* by several folds (Vasilchenko *et al.* 1993). This might be the reason for increase in accumulation (mg/g) of carotenoids by mutant 32.

Table 3.5 Effect of amino acids on carotenoid production by mutant 32.

Amino acid	Dry cell weight g/l	Total carotenoids mg/ l	Total carotenoids mg/g	β-Carotene mg/l	Proportion (β-carotene : torulene : torularhodin, %, w/w)
DL-Threonine	**6.1**	**30.0**	**4.9**	**13.5**	**45:52:3**
Glycine	8.1	20.6	2.5	11.4	55:41:4
L-Aspartic acid	4.8	21.2	4.4	9.8	46:52:2
L-Histidine	3.9	18.9	4.7	8.5	45:52:3
L-Lysine	9.3	18.2	2.0	8.9	49:46:5
L-Serine	7.3	20.4	2.8	10.4	51:45:4
L-Tryptophan	3.9	19.4	4.8	9.5	49:48:3
L-Tyrosine	7.7	20.4	2.6	8.4	41:56:3

Effect of metal salts

Table 3.6 shows the effect of metal salts on the growth and carotenoid production by mutant 32. All divalent cation salts studied resulted in a higher volumetric production of carotenoids. Magnesium sulphate increased the proportion of β-carotene up to 77% (w/w), unlike other metal salts that resulted in higher total carotenoid production but mainly because of increased torulene formation. Calcium, zinc and ferrous salts had maximum stimulatory effect on total carotenoid production. Torularhodin content remained almost unaltered in studies with all the investigated salts. There was no major pH change observed in culture broths due to the presence of any of the salts investigated.

There are only a few reports on enhancement of microbial carotenoid production due to metal ions and salts (Atamanyuk and Razumorskii 1974, Daushvili and Elisashvili 1990). Earlier, Gammal and Rizk (1989) reported *Rhodotorula rubra* to be tolerant to metal ions Cu^{+2}, Co^{+2}, Ca^{+2} and Ba^{+2}. In a recent report, the effect of metal salts on growth of *Rhodotorula glutinis* demonstrated that divalent cations act as stimulants for growth (Komemushi *et al.* 1994). In the present study, it was observed that divalent cations have stimulatory effect on volumetric production (mg/l) as well as cellular accumulation (mg/g) of carotenoids. It can be assumed that this positive effect was due to stimulatory effect of cations on carotenoid synthesizing enzymes, as reported earlier in the case of manganese (Goodwin 1980a).

Table 3.6 Effect of metal salts on carotenoid production from mutant 32.

Salts	Dry cell weight	Total carotenoids		β-Carotene	Proportion (β-carotene : torulene : torularhodin %, w/w)
	g/l	mg/l	mg/g	mg/l	
Control	**11.1**	**33.0**	**2.9**	**22.8**	**69:27:4**
$AlNO_3.9H_2O$	11.5	53.7	4.7	31.7	59:38:3
As_2O_3	9.3	36.3	3.9	21.4	59:40:1
$BaCl_2.2H_2O$	11.0	46.0	4.2	27.1	59:39:2
$CaCl_2.2H_2O$	11.4	52.0	4.6	29.7	57:42:1
$Ca(OH)_2$	**11.3**	**67.0**	**5.9**	**38.9**	**58:41:1**
$CoCl_2.5H_2O$	7.2	38.0	5.3	24.7	65:32:3
$CuSO_4.5H_2O$	8.4	53.0	6.3	28.1	53:44:3
$FeSO_4.7H_2O$	12.8	66.4	5.2	37.2	56:41:3
$MgSO_4.7H_2O$	6.9	22.5	3.3	17.4	77:21:2
$MnSO_4.7H_2O$	13.2	57.0	4.4	31.9	56:42:2
$Na_2B_4O_710H_2O$	8.4	52.0	6.2	28.1	54:35:1
$(NH_4)_2C_2OP_4.H_2O$	11.4	48.0	4.2	27.8	58:40:2
Na_2SO_4	11.4	28.1	2.5	17.9	64:35:1
$ZnSO_4$	10.9	68.8	6.3	37.8	55:44:1

Medium optimization by factorial design

Based on the results obtained from the studies on carbon, nitrogen, amino acids and metal ion sources, studies were performed to check the interactive effects between yeast extract, glucose, threonine, Ca $(OH)_2$ and $MgSO_4.7H_2O$, on carotenoid production and growth of mutant 32. A slight decrease in the volumetric production was observed when (g/l) glucose 25, yeast extract 10 and threonine 10 were used, along with the said micronutrients, as compared to when they were used independently in basal medium.

Multiple experiments at these concentrations indicated that total carotenoid content was 39.5 ±0.7 mg/l (standard error 1.5) with cellular accumulation of 2.7 ±0.2 mg/g. Under these conditions, β-carotene was the major carotenoid produced (32.8 ±0.9 mg/l), with minor quantity of torulene (5 ±0.3 mg/l) followed by torularhodin (3 ±0.4 mg/l). Concentrations of metal salts in the medium were kept constant for further optimization studies because increase in their concentration had no effect on carotenoid production.

When the effect of above-mentioned factors on carotenoid production was studied using factorial design experiment, the first factorial indicated that effects of glucose and threonine were much higher than yeast extract (Table 3.7). Interactive effect between glucose and threonine was also significant.

Table 3.7 Results of first factorial design.

Flask No	Dry cell weight	Carotenoids		β-Carotene	Effect (E)
	±1 g/l	±2mg/l	±0.1mg/g	±1mg/l	
1	14	28	2.0	22.8	E_0: 49.75
2	13	30	2.3	23.9	E_y: 5.00
3	10	39	3.9	28.9	E_g: 16.50
4	16	44	2.8	34.0	E_{yg}: -1.00
5	15	44	2.9	33.5	E_t: 29.00
6	10	50	5.0	37.8	E_{yt}: -1.50
7	13	78	6.0	59.7	E_{gt}: 11.00
8	16	85	5.3	59.8	E_{ygt}: -1.00

Levels (g/l) First factorial: Yeast extract +15, -5, Glucose +37.5, -12.5; Threonine +13.5, -4.5

Experiments along the direction of steepest ascent yielded best results at yeast extract, glucose and threonine concentration of 11.74, 46 and 18 (g/l), respectively (Figure 3.1, Flask 6).

When the second factorial was performed with the above point at the center of the cube, the effect of yeast extract and glucose was found to be more pronounced than threonine (Table 3.8).

Table 3.8 Results of second factorial design.

Flask No	Dry Cell weight ±1g/l	Carotenoids ±2mg/l	±0.1 mg/g	β-Carotene ±2 mg/l	Effect (E)	
1	18	73	3.96	55.9	E_0:	94
2	16	92	5.75	70.9	E_y:	21
3	20	81	4.00	57.9	E_g:	15.6
4	24	117	4.88	93.9	E_{yg}:	5.5
5	20	84	4.20	63.4	E_t:	6.5
6	15	96	6.40	75.8	E_{yt}:	-1.0
7	13	96	7.40	72.0	E_{gt}:	-6.5
8	22	113	5.14	87.5	E_{ygt}:	-3.0

Levels (g/l) Second factorial: Yeast extract +16.74, -6.74, Glucose +51, - 41; Threonine +23, -13.

Experiments performed along the direction of steepest ascent resulting from second factorial design showed no further improvement in volumetric carotenoid production (mg/l), although, increase in dry cell mass (g/l) was evident. Further increase in the medium components led to decrease in carotenoid content, which might be because of oxygen limitation in the culture broth as a result of considerably increased cell concentration (Figure 3.1) and prolonged incubation in such cases did not increase carotenoid content further.

At the end of optimization studies, the maximum volumetric carotenoid content increased to 129 ±2 mg/l, which was four fold of that in the basal medium at the beginning. In the optimized medium, cellular accumulation was found to be 5.4 ±0.1 mg/g that was almost two fold higher than that in the basal medium. The factorial approach for optimization of medium, used in the present studies, provides an estimate of the effect of a single variable at selected, fixed conditions of other variables. This technique also reduces the labour of using several flasks and time, required during “one-factor-at-a-time” method.

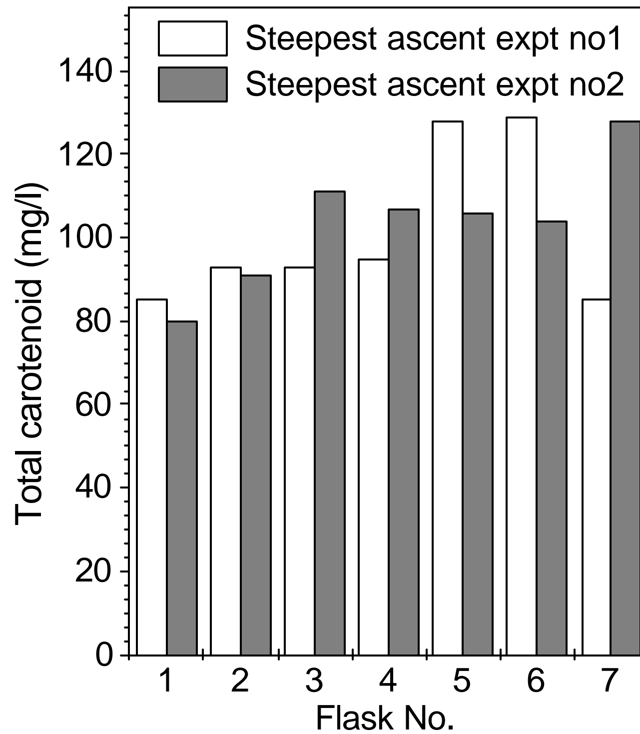


Figure 3.1 Direction of steepest ascent (Flask No 7 denotes the centroid of the experiment.

Buzzini and Martini (1999) had studied several strains of *Rhodotorula* for carotenoid production on grape juice and later optimized the conditions but even under optimized conditions yield of β-carotene was only 1.1 mg/l (Buzzini 2000). In the present investigation, the final volumetric yield (mg/l) and cellular accumulation mg/g) of β-carotene obtained was higher than even the alga, *Dunaliella salina* (Yamaguchi 1997) and all other yeasts reported (Table 3.9).

Table 3.9 Carotenoid production by *Rhodotorula spp.* reported earlier.

S No.	Organism	Carotenoid content mg/l	Carotenoid content mg/g	β-Carotene# (%)	Reference
1	*Rhodotorula glutinis* 48-23T	NG	0.24	63	Simpson *et al.* 1964
2	*Rhodotorula glutinis var glutinis*	7.37	0.63	100	Costa *et al.* 1987
3	*Rhodotorula glutinis var glutinis*	37.5	2.50	100	Matelli *et al.*. 1990
4	*Rhodotorula rubra*	6.03	1.26	NG	Martin *et al.* 1993
5	*Rhodotorula glutinis* 22P	2.26	NG	NG	Frengova *et al.* 1994
7	*Rhodotorula aurantiaca* CBS 317	NG	0.10	60	Perrier *et al.* 1995
8	*Rhodotorula glutinis* 22P	8.4	0.26	15	Frengova *et al.* 1995
9	*Rhodotorula rubra* NRRL Y-15596	1.04	0.13	NG	Shih and Hang 1996
10	*Rhodotorula gracilis* ATCC 90950	NG	0.54	NG	Govindaswamy *et al.* 1999
11	*Rhodotorula glutinis* DBVPG 3853	6.90	NG	16	Buzzini 2000
12	***Rhodotorula glutinis* mutant 32**	**129 ±2**	**5.4 ±0.2**	**80**	**Present studies**

NG: Data not given
#β-Carotene represents percentage of total carotenoid content.

Factorial approach used for optimization of medium components for carotenoid production from mutant 32 resulted in far better improvement than statistical methods reported earlier for medium optimization (Table 3.10).

Table 3.10 Improvement of carotenoid production using different techniques of medium optimization.

Organisms	Optimization approach	Increase (%)	Total carotenoid mg/l	Reference
Rhodotorula glutinis	Experimental factor method	15.6	6.8	Stabnikova *et al.* 1979
Phaffia rhodozyma	Factorial approach	23	2.0	Florencio *et al.* 1998
Phaffia rhodozyma	Empirical modeling and experimentation	16	5.8	Parajo *et al.* 1998
Rhodotorula sp. COS- 5	Orthogonal test	N.G.	13.5	Yueying *et al.* 1999
Rhodotorula glutinis DBVPG 3853	Multivariable factorial design	11.6	6.9	Buzzini 2000
***Rhodotorula glutinis* mutant 32**	**2 level 3 variable factorial design**	**226**	**129.5**	**Present studies**

N.G. denotes data not given

Cell growth and carotenoid production in the optimized medium

In the optimized medium, mutant 32 grew well and the maximum specific growth rate observed was 0.31 ±0.02 h^{-1}.

The carotenoid formation was primarily growth associated and directly proportional to the biomass concentration (Figure 3.2). However, considerable carotenoid production was observed during late logarithmic phase when the cell mass was 26 ±1 g/l. In this phase, the maximum carotenoid production rate observed was 7.25 mg/l/h. Results were in contrast to those of Frengova *et al.* (1994) wherein maximum cell mass and carotenoid production did not occur concurrently. Maintaining cells in stationary condition for more than 6 h did not result in further increase in carotenoid content.

At the end of the fermentation, β-carotene was the major carotenoid produced (102 ±2 mg/l) that accounted for 80% (w/w) of the total carotenoids. The cellular accumulation of β-carotene was observed to be 3.94 ±0.2 mg/g dry cell weight. Maximum specific β-carotene production was observed to be 7.25 mg/g/h between 44 and 48 h.

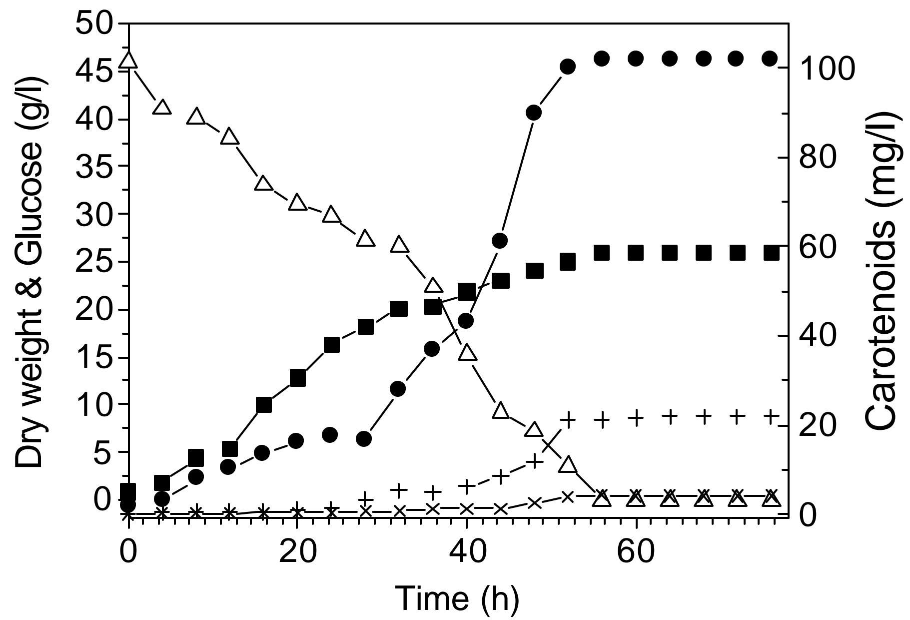


Figure 3.2 Carotenoid production in the optimized medium grown in shake flask. Dry cell weight (■, g/l), Glucose (Δ, g/l), β-carotene (•, mg/l), torulene (+, mg/l) and torularhodin (×, mg/l).

Effect of other cultural conditions

Effect of inoculum size

Inoculum size plays an important role in all fermentation industries. Recently, Govindaswamy *et al.* (1999) reported inoculum percentage to be an important parameter for growth and carotenoid production from *Rhodotorula gracilis*.

In the present studies, it was observed that inoculum size had no effect on the final dry mass (after 72 h). However, as expected, slower specific growth rate (μ) and higher lag phase was obtained when lower inoculum sizes were used. Slow growth is not a desirable trait for carotenoid production as it is growth associated and its yield is directly proportional to dry cell mass (g/l) obtained. After 72 h, maximum β-carotene production was obtained when 10% (v/v) inoculum was used. Hence, this was used as optimum inoculum size for further experiments.

Table 3.11 Effect of inoculum size on carotenoid production after 72 h incubation.

Inoculum % (Cell dry weight added, mg)	Lag phase h	Dry cell weight g/l	μ_{max} h^{-1}	Total carotenoids mg/l	mg/g	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin, %, w/w)
1 (10.2)	16	27	0.17	68	2.5	55.1	81:14:5
3 (30.6)	10	28	0.09	100	3.6	80.0	80:14:6
5 (51)	8	26	0.17	103	3.9	84.5	82:15:3
10 (102)	**4**	**26**	**0.30**	**128**	**4.9**	**102.4**	**80:17:3**
15 (153)	2	27	0.33	121	4.5	94.4	78:16:6
20 (204)	1	27	0.31	99	3.7	79.2	80:16:4

Effect of pH

The principal effect of different pH of growth medium was seen on growth profile. At extreme pH, delayed lag phase and low specific growth rate were observed. Maximum carotenoid content was observed at pH 7.0 (128 ±2 mg/l), while decline in cellular (mg/g) and volumetric (mg/l) production was observed at both sides of neutral pH.

Maximum β-carotene production by mutant 32 was observed at pH 7.0. At alkaline pH, decrease in β-carotene proportion was observed which was in contrast to earlier studies of Kim *et al.* (1996), who reported increase in β-carotene content at alkaline pH in

Blakeslea trispora. Controlling pH with buffers resulted in several fold increase in cellular carotenoid content and dry cell mass earlier (Endang *et al.* 1998) in *Phaffia rhodozyma*. However, no such effect was observed with mutant 32.

Table 3.12 Effect of medium pH on the growth and carotenoid production by mutant 32.

Initial pH	Dry cell weight	μ_{max}	***Total carotenoids***		***β-Carotene***	Proportion (β-carotene: torulene: torularhodin %, w/w)
	g/l	h^{-1}	mg/l	mg/g	mg/l	
2	12	0.29	19	1.6	16.6	87:9:4
4	18	0.33	53	2.9	45.1	85:10:5
6	26	0.29	97	3.7	79.6	82:13:5
7	**26**	**0.30**	**128**	**4.9**	**102.4**	**80:15:5**
8	23	0.72	102	4.4	85.7	84:12:4
9	16	0.51	80	5.0	64.8	81:16:3
10	13	0.32	59	4.5	42.5	72:26:2
11	7	0.10	47	6.7	29.7	63:36:1
12	4	0.10	24	6.0	15.2	63:35:2

Effect of shaker speed

Rhodotorula glutinis is an obligatory aerobic yeast (Choi *et al.* 1982) and is dependent on oxygen for energy and biosynthesis. Presumably, the better oxygen transfer resulted in higher growth rate as well as cell mass (g/l) at higher shaker speed (rpm). However, maximum β-carotene production was observed at the shaker speed of 250 rpm (Table 3.13).

Better oxygen transfer is normally achieved by using indented flasks or using stainless steel coils in the Erlenmeyer flasks. The increased oxygen transfer exerted a positive influence on growth rate and but beyond a particular limit, it caused decrease in β-carotene content, which is not desirable. Previously, Sakaki *et al.* (1999) also had reported similar results.

Table 3.13 Effect of aeration and agitation in shake flasks.

Agitation Shaker speed (rpm)	Dry cell weight g/l	μ_{max} h^{-1}	***Total carotenoids*** mg/l	mg/g	β-Carotene mg/l	***Proportion*** (β-carotene: torulene: torularhodin, %,w/w)
A. Erlenmeyer flask						
100	14.6	0.08	38.3	2.6	34.9	91:8:1
150	16.5	0.19	35.2	2.2	32.1	91:8:1
200	21	0.26	79.2	3.8	69.7	88:10:2
250	26	0.29	128	4.9	102.4	80:15:5
300	26	0.34	90.1	3.5	67.6	75:23:2
B. Indented flask						
100	10.4	0.25	65.5	6.3	60.3	92:7:1
150	27	0.27	121	4.5	101.7	84:14:2
200	26	0.29	121	4.6	99.3	82:16:2
250	26	0.29	124	4.7	89.3	72:23:4
300	3.08	0.40	89.0	1.8	70.3	79:20:1
C. Stainless steel coil in Erlenmeyer flask						
100	19.2	0.30	104	5.4	89.5	86:12:2
150	26	0.32	100	3.8	85.0	85:13:2
200	27	0.40	103	3.8	83.4	81:18:1
250	31.7	0.40	98.2	3.1	76.6	78:20:2
300	26	0.43	81	3.1	63.2	78:21:1

As better β-carotene proportion was observed at low shaker speed, an attempt was also done to shift yeast culture, after growth at 250 rpm, to lower agitation (Table 3.14). It was observed that shifting the flask in the exponential phase (24 h) resulted in 7 to 8% increase in the proportion of β-carotene (%) however, the volumetric β-carotene content (mg/l) was less than that in control, run at 250 rpm. Shifting the flask to low agitation in the late exponential phase (36 h onwards) did not increase β-carotene content.

Table 3.14 Effect of shifts in shaker speed on carotenoid production and growth.

Shift in shaker speed (rpm)	Time of shift h	Dry cell weight g/l	Total Carotenoids mg/l	mg/g	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin %, w/w)
Control (250 rpm)	**--**	**26.0**	**128**	**4.9**	**102.4**	**80:15:5**
250 to 100	**24**	**13.0**	**60**	**4.6**	**50.4**	**84:13:3**
	36	16.6	72	4.4	63.4	88:16:6
	48	24.5	124	5.1	94.3	76:19:4
250 to 150	**24**	**14.2**	**85**	**5.9**	**79.9**	**94:5:1**
	36	21.5	98	4.5	87.3	89:9:2
	48	24.5	125	5.1	97.5	78:20:2
250 to 200	24	21	82	3.9	64.8	79:17:4
	36	24	121	5.1	99.3	82:17:1
	48	25	117	4.7	97.2	83:15:2
250 to 300	24	25	133	5.3	99.8	75:21:4
	36	26	95	3.7	72.2	76:23:1
	48	27.3	87	3.2	66.9	77:22:1

Effect of temperature:

Optimum temperature for carotenoid production (mg/l, mg/g) was found to be lower than the optimum temperature for the growth of mutant 32. At and above 35 °C, growth was hampered, probably because of disruption of normal metabolic regulation.

Maximum volumetric β-carotene production (mg/l) and cellular accumulation (mg/g) were observed at 20 °C (Table 315). Proportion of β-carotene was also higher at lower temperature, whereas, more torulene was produced at higher temperature. A biosynthetic study showed that γ-carotene acts as the branch point of the carotenoid synthesis. Subsequent dehydrogenation and decarboxylation leading to torulene synthesis is known to be temperature dependent and is less active at lower temperature as compared to activity of β-carotene synthase (Simpson *et al.* 1964). This might be the reason for increase in β-carotene proportion at lower temperature. Maintaining cells at 5 °C for 21 days was found to block the production of torulene and torularhodin and result

in higher β-carotene accumulation (Nakayama *et al.* 1954, Frengova *et al.* 1995) but maintaining such conditions will not be practicable.

Table 3.15: Effect of temperature on carotenoid production.

Incubation Temperature °C	Dry cell weight g/l ± 1	μ_{max} h^{-1}	Total carotenoids mg/l ±3	Total carotenoids mg/g ±0.05	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin, %, w/w)
10	18	0.05	99	5.5	87.2	88:09:3
15	21	0.08	99	4.7	86.2	87:12:1
20	**23**	**0.12**	**164**	**7.0**	**136.1**	**83:15:1**
25	26	0.29	134	4.7	101.9	76:19:4
30	**26**	**0.32**	**128**	**4.9**	**97.3**	**76:20:4**
35	15	0.24	76	5.0	53.9	71:26:3
40	6.7	0.04	31	4.6	20.2	65:28:7

Growth and consumption of glucose by mutant 32 was studied at 20 as well as 30 °C (Figure 3.3, Figure 3.4). At 20 °C, during logarithmic phase, maximum specific growth and glucose utilization rate were 0.26 h^{-1} and 0.92 ±0.1 g/l/h, respectively, which were lesser than that at 30 °C (0.4 h^{-1}, 0.43 ±0.1 g/l/h) as expected. There was no major difference observed in the final dry cell mass (31.75 ±0.25 g/l) at both temperatures.

At 20 °C, maximum specific carotenoid production obtained was between 24-28 h (0.975 mg/g/h). β-Carotene accounted for more than 90% (w/w) of total carotenoids during fermentation run, maximum being 95 ±0.5% (w/w) between 36 to 40 h (Figure 3.5). At the end of the fermentation (90 h) β-carotene, torulene and torularhodin were in the proportion of 90, 8, and 2%, respectively, of the total carotenoid content (11.03 ±0.73 mg/g, 277 ±7 mg/l).

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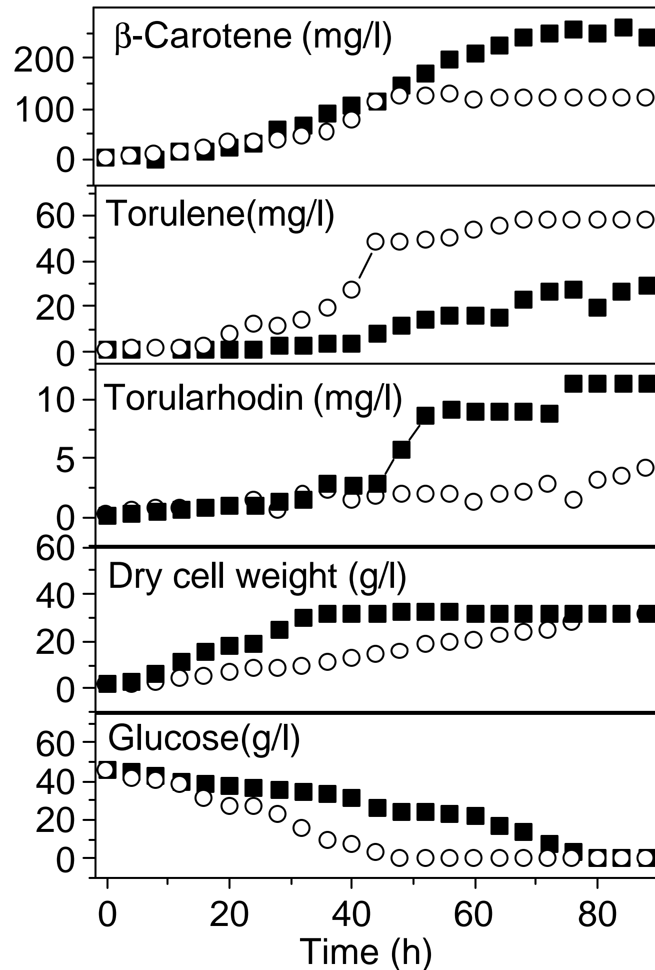

Figure 3.3 Effect of temperature on *Rhodotorula glutinis* mutant 32 during batch cultivation at 20 °C (O) and 30 °C (■).

Under similar conditions at 30 °C, maximum specific carotenoid production was found to be 0.46 mg/g/h during 40-44 h. β-Carotene was the major carotenoid produced but substantial amount of torulene was also produced, simultaneously. At the end of the fermentation (88 h), β-carotene, torulene and torularhodin were present in the

proportion of 63, 31 and 5 %, respectively, of the total carotenoid (178 ±6 mg/l and 5.57 mg/g).

Figure 3.4 Photograph of 1L fermenter.

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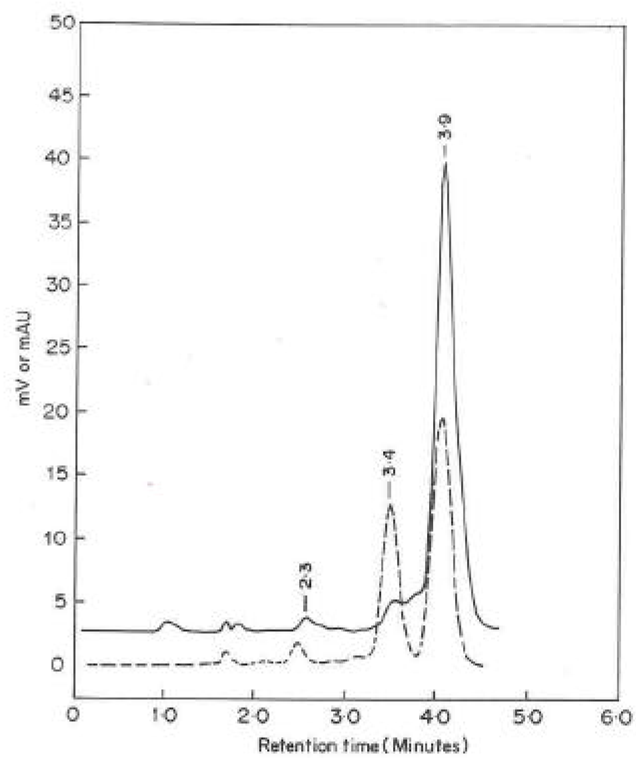


Figure 3.5 HPLC chromatogram showing carotenoid production at 20 (—) and 30 °C (---) at 36h. Torularhodin, torulene and β-carotene eluted at 2.3, 3.4 and 3.9 minutes, respectively.

Effect of shifts in incubation temperature

When the culture flasks were shifted from 30 to 15 °C at 36 h, volumetric β-carotene production increased from 98 to 140 mg/l along with 1.8 fold increase in cellular accumulation (mg/g) of β-carotene, as compared to the control run at 30°C.

Shifting temperature at 36 h to 20 °C also showed slight increase in total carotenoid content but no increase in β-carotene content was observed (Table 3.16). Torulene and torularhodin remained unaffected by the shift in the growth temperatures.

Table 3.16 Effect of temperature shift on carotenoid production and growth.

Temperature Shift °C	Time of shift h	Dry cell Weight g/l	Total Carotenoids mg/l	mg/g	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin %, w/w)
Control (30 °C)	_	**26**	**129**	**5.4**	**98.0**	**76:20:4**
30 to 10 °C	24	21	82	3.9	64.8	79:17:4
	36	21	121	5.8	99.3	82:17:1
	48	21	117	5.6	97.1	83:15:2
30 to 15 °C	24	22	134	5.6	112.6	84:13:3
	36	**21**	**160**	**7.6**	**140.8**	**88:16:6**
	48	26	133	4.9	103.7	78:18:6
30 to 20 °C	24	26	119	4.6	91.7	77:18:5
	36	**26**	**135**	**5.1**	**99.9**	**74:20:6**
	48	26	128	4.9	88.3	69:21:10

In the fermenter (Figure 3.6), shifting reactor temperature from 30 °C to 20 and 15 °C, after 40 h, resulted in increase in volumetric β-carotene production to 265 ±2 mg/l and 301 ±2 mg/l, respectively, as compared to control run at 30 °C (125 ±2 mg/l). This was a significant increase in productivity and can be used on large scale also.

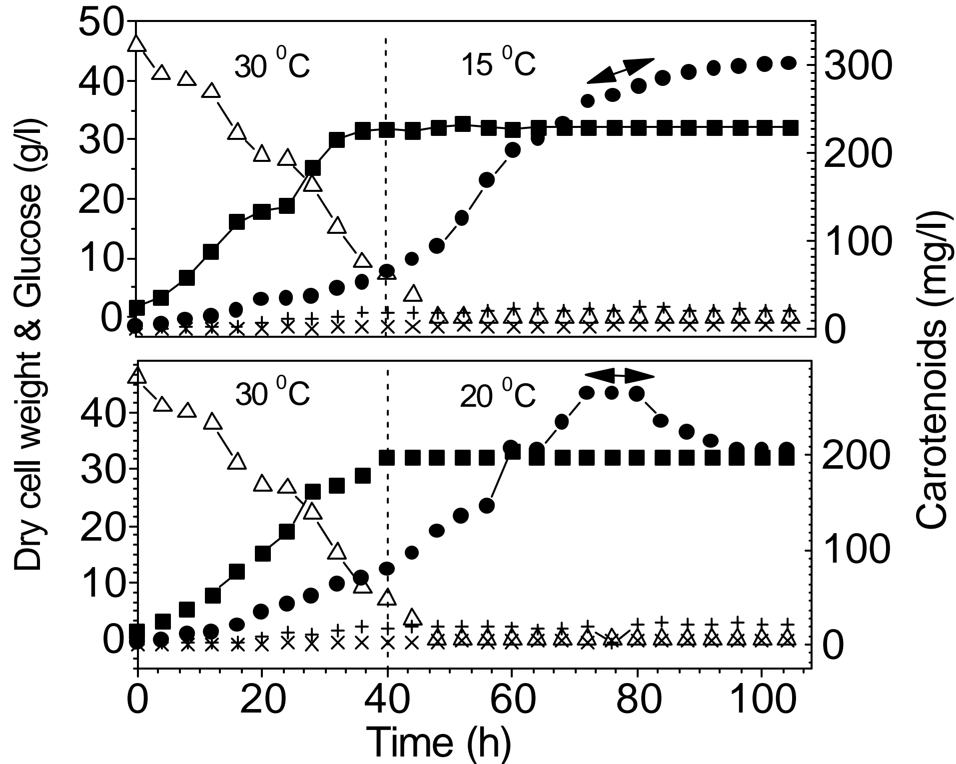


Figure 3.6 Effect of temperature shift from 30 to 15°C and 30 to 20°C on *Rhodotorula glutinis* mutant 32 during batch cultivation on production of Dry cell weight (■, g/l), glucose (Δ, g/l), β-carotene (•, mg/l), torulene (+, mg/l), torularhodin (×, mg/l), harvesting time (◂ ▸) 75 to 80 h.

Effect of illumination

Continuous exposure of the culture of mutant 32 in the fermenter to the white light hampered the growth and carotenoid production (Figure 3.7). The lag phase was very long and also the total cell mass generated was very low. Long lag phase must have been an expression of the time required to overcome the inhibitory effect of intense light on growth. Total carotenoid yield was also poor (83 ±4 mg/l).

Illuminating the fermenter, after late logarithmic growth phase, resulted in increase in the total carotenoids up to 249.9 mg/l as compared to control run at 30 °C (129 mg/l, Figure 3.3). Light is a well known inducer of enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, an essential enzyme for mevalonate accumulation which is a precursor for carotenoid synthesis in *Rhodotorula sp.* (Tada and Shiroishi 1982, Tada 1993). This might be the reason for increase in carotenoid content.

β-Carotene production was found to be enhanced in the presence of light. Maximum specific β-carotene production was observed to be 0.8 mg/g/h between 40 and 44 h. Although, β-carotene accounted for 91 ±2% (w/w) of total carotenoids between 20 and 40 h during growth phase, it later decreased to 80 ±3% (w/w) at 72 h (Figure 3.7).

A sharp decrease in torulene content in mutant 32 was observed upon exposure to light, which was in contrast to the earlier report by An (1996). Torularhodin content was found to increase drastically under illumination in a mutant of *Rhodotorula glutinis* reported recently (Sakaki *et al.* 2000) but no such effect was observed in mutant 32. Exposure to light in later stages of growth had no effect on volumetric carotenoid production (mg/l) or cellular accumulation (mg/g).

An and Johnson (1990) working with *Phaffia rhodozyma* reported a decrease in β-carotene content on exposure to white light, while Meyer and Du Preez (1994) found that there was no effect of light on pigment synthesis.

Present study suggests that β-carotene production by mutant 32 can be increased by proper manipulation of exposure to light.

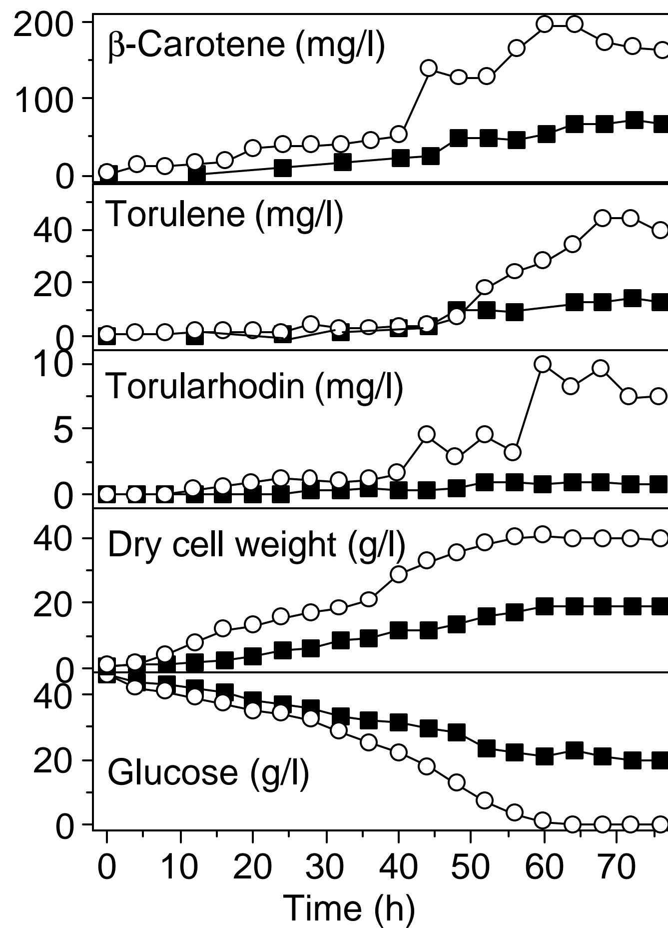


Figure 3.7 Effect of continuous illumination (■) and during growth phase (O) at 30°C on *Rhodotorula glutinis* mutant 32 on production of dry cell weight, β-carotene, torulene, torularhodin and utilization of glucose.

Effect of light was also studied at 20 °C wherein mutant 32 was exposed to white light during exponential phase. No distinct cumulative effect of light exposure and low temperature was observed. However, increase in total carotenoid production (304 ±9 mg/l) was noticed as compared to control run at 20 °C without illumination (Figure 3.3). β-Carotene accounted for 92 ±3% of the carotenoids during exponential phase, which eventually fell down to 86 ±3% at the end of fermentation run. (Figure 3.8).

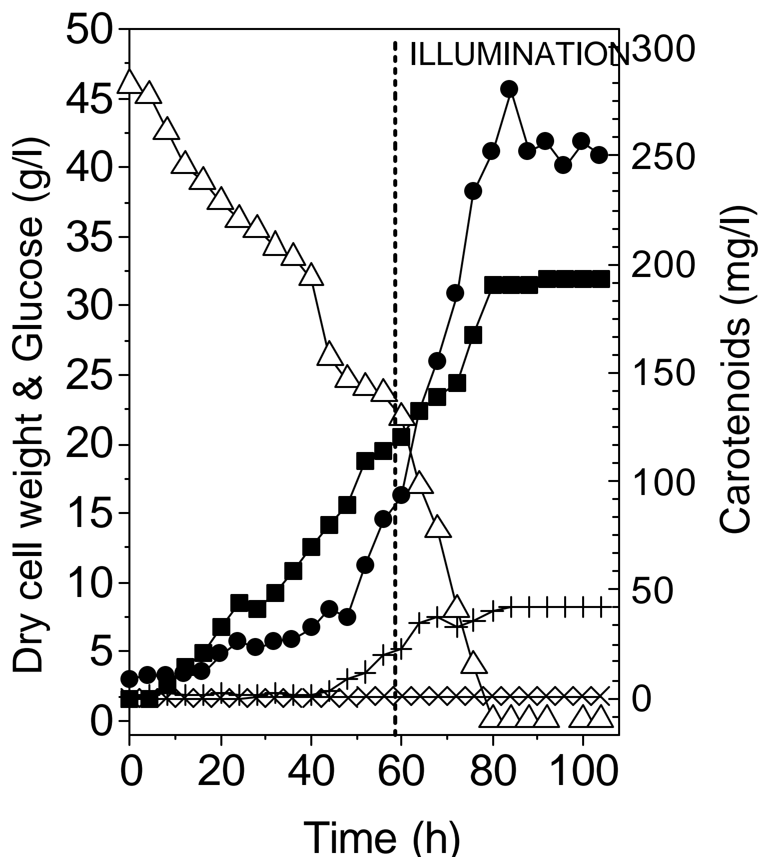


Figure 3.8 Effect of exposure of white light at 20°C during growth phase on *Rhodotorula glutinis* mutant 32 during batch cultivation on production of on Dry cell weight (■, g/l), Glucose (Δ, g/l), β-carotene (●, mg/l), torulene (+, mg/l) and torularhodin (×, mg/l).

Replacement of threonine with complex organic nitrogen sources

Mutant 32 was optimized for medium composition (quantitatively) using two-level three-variable factorial designs described in the earlier part of this chapter. Threonine was observed to be an important influencing component in the optimized medium. However, use of threonine on industrial scale will be uneconomic. Therefore, various organic complex nitrogen sources were studied as replacement for threonine in the optimized medium. They were selected based on their typical compositions, which also includes threonine (0.3-1%) along with other aminoacids and vitamins. During these studies, soyapeptone resulted into a cellular accumulation (mg/g) and volumetric production (mg/l) of carotenoids comparable to threonine (Table 3.17).

Table 3.17 Effect of replacement of threonine with other complex nitrogen sources in optimized medium.

Nitrogen sources	Dry cell weight	Total carotenoid		β-Carotene
	g/l	mg/l	mg/g	mg/l
Threonine (control)	**26**	**138**	**5.3**	**102**
Casein acid hydrolysates	24	93	3.9	73
Cotton seed meal	28	115	4.1	95
Pharmamedia*	16	80	5.0	65
Proflo*	21	80	3.8	61
Soyapeptone	**24**	**121**	**5.4**	**103**
Soybean meal	24	99	4.1	75
Yeast extract	20	88	4.1	70

* Cotton seed meals from Traders Proteins, USA.

Soyapeptone concentration (g/l) in the medium was further optimized using different concentrations in the medium and it was observed that increasing the concentration above 10 g/l had no further effect either on cellular accumulation or volumetric production (Table 3.18). Proportion of β-carotene, torulene and torularhodin remained unaffected and were found to be 83, 15 and 2%, respectively. An optimum medium containing soyapeptone was used for subsequent fermentation studies.

Table 3.18 Effect of soyapeptone concentration on carotenoid production.

Soyapeptone Concentration	Dry cell weight	Total carotenoid		β-Carotene
(g/l)	g/l	mg/l	mg/g	mg/l
2.5	22.5	75	3.4	63
5	23.0	82	3.5	64
10	**23.0**	**119**	**5.2**	**95**
15	24.5	123	5.0	103
20	26.0	109	4.2	72
25	24.0	107	4.4	66

Fermentation studies

Batch fermentation

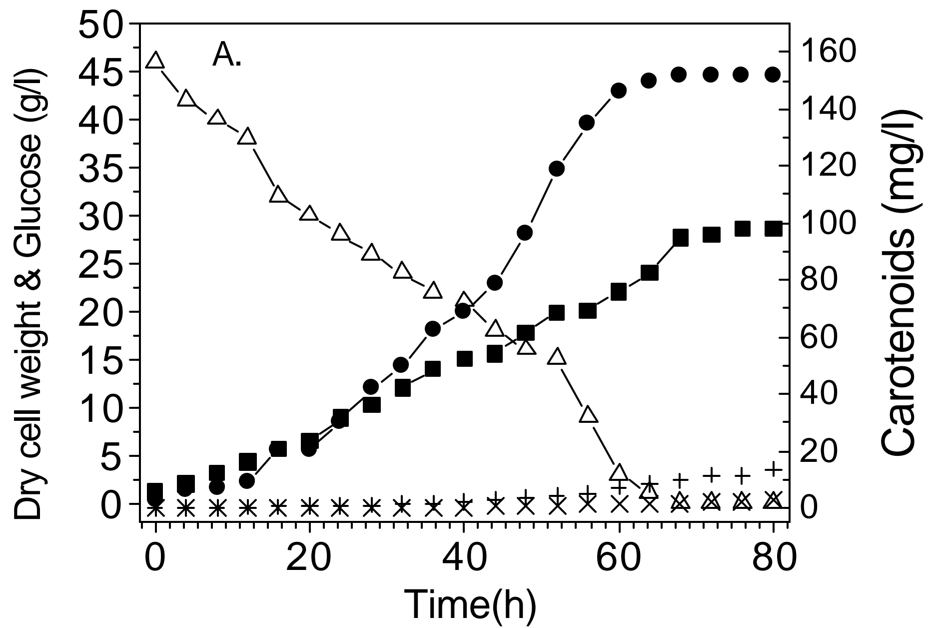
In 1 L fermenter, in a medium containing soyapeptone, mutant 32 had a maximum specific growth rate of 0.45 ±0.02 h^{-1} during exponential phase of growth. The maximum dry cell weight achieved was 28.6 ±2 g/l. β-Carotene was the major carotenoid produced and maximum specific β-carotene productivity was observed between 24-28 h (2.2 mg/g/h). At the end of the fermentation, β-carotene, torulene and torularhodin accounted for 92, 7 and 1% of the total carotenoid content (166 ±5 mg/l, 5.8 ±0.2 mg/g), respectively.

In 14 L fermenter, the carotenoid production was higher than that in the 1 L fermenter and in addition to that, the batch was shortened by 20 ±4 h as compared to 1 L fermenter. This may be because of the better design of the fermenter, leading to better mixing and mass transfer. Mutant 32 grew very well and exhibited a maximum specific growth rate of 0.83 h^{-1} during exponential phase. Maximum cell mass achieved was 46 ±2 g/l.

Carotenoid production profile indicated that, specific β-carotene productivity during exponential phase was 1 mg/g/h during 28-32 h. During stationary phase (between 44-60 h), specific β-carotene productivity was observed to be 15 ±2 mg/g/h. These production profiles were similar to those reported by Costa *et al.* (1987), who reported that 70% (w/w) of the total pigment was accumulated (mg/g) in the stationary phase by *Rhodotorula glutinis* but were in contrast with the results obtained earlier with *Phaffia rhodozyma* (Johnson and Lewis 1979, Meyer and Du Preez 1994).

At the end of fermentation, in the present mutant, β-carotene, torulene and torularhodin were 87, 11 and 2%, respectively, of the total carotenoid content (218 ±5 mg/l, 4.7 mg/g) (Figure 3.8).

It was also observed that cell growth of mutant 32 stopped much before the total depletion of sugar and during stationary phase, the residual sugar was utilized slowly (mg/l/h). The slow utilization of glucose probably can be correlated with the increase in the carotenoid accumulation (mg/g) and thus, there was probably a biochemical shift from growth to carotenoid accumulation.



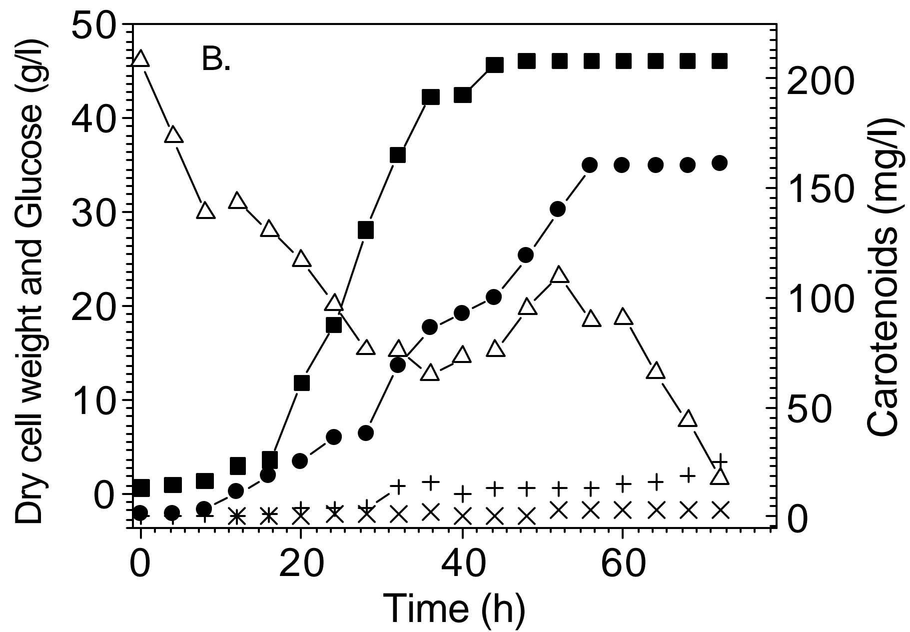


Figure 3.8 Carotenoid production during batch fermentation in 1L (A) and 14 L (B) fermenter using soyapeptone supplemented in optimized medium. Dry cell weight (■, g/l), Glucose (∇, g/l) β- Carotene (●, mg/l), Torulene (+, mg/l) and torularhodin (×, mg/l).

Batch fermentations in shake flasks and fermenters, with optimized media, using *Blakeslea trispora* are reported to yield β-carotene with volumetric specific productivity of 4.2 mg/l/h but high cost of processing of cell mass was reported to limit its commercialization (Ciegler *et al.* 1963, Nelis and Deleenheer1991).

In case of the exhaustively studied alga, *Dunaliella,* slow growth and high costs involved in maintenance of stress conditions for growth and carotenoid production were reported to be the limiting factors (Nelis and Deleenheer1991). Under such conditions, *Rhodotorula glutinis* mutant 32 may have a scope for commercialization, mainly as a carotenoid-rich feed.

Fed-batch fermentation

Major objective of the fermentation in research and industries is to maximize the volumetric productivity i.e. *to obtain the highest possible quantity of the product, in a given volume, within a certain time. High cell densities are a prerequisite for such high-rate productions.*

Fed-batch fermentation is a production technique in between batch and continuous fermentation. During fed-batch fermentation, under controlled environmental conditions for a well characterized microorganism involved in fermentation, a continuous feed of an essential nutrient can be programmed in such a way that the nutrient is not depleted and also the nutritional environment is maintained approximately uniform during the course of the batch. It has an advantage over batch fermentation in that it avoids presence of excessive nutrients in the fermenter and thus, minimizes the production of unwanted by-products and also avoids possible catabolite repression effects. Various parameters are used to control fed-batch fermentation. Controlling specific growth rate and dissolved oxygen (DO) level are two major controls practiced during such fermentation.

Earlier, DO-based fermentation were reported to increase β-carotene production from *Blakeslea trispora* (Kim Seon Won *et al.* 1999). Fed-batch fermentations were also reported for carotenoid production from *Dunaliella* (Yukiho *et al.* 1997), *Phaffia rhodozyma* (Reynders *et al.* 1996), *Euglena gracilis* Z (Haruko *et al.* 1997) and *Monascus* (Somchai *et al.* 2000).

Preliminary fed-batch studies in shake flasks with mutant 32 (data not shown) revealed that maximum carotenoid accumulation was achieved when feeding was started during early stationary phase. Initiation of feeding at later stages had no significant effect on carotenoid content.

In 14 L fermenter, during fed-batch run, mutant 32 produced carotenoid in three segments. Initially, during growth phase, carotenoids were actively synthesized (2.5 ±0.5 mg/g/h). During early stationary phase, when feeding was started and DO level was maintained between 10 and 40% saturation, decrease in specific carotenoid productivity (0.24 ±0.05 mg/g/h) was noticed which may be because of dilution of the broth by continuous addition of feed solution. Significant increase in carotenoid productivity was observed after feeding was over.

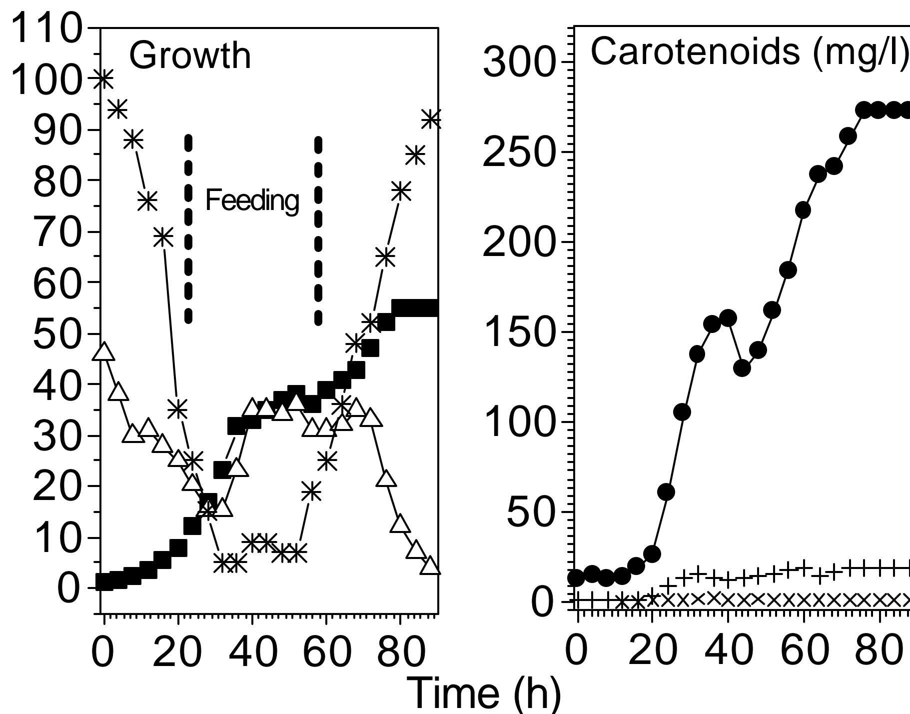


Figure 3.9 Growth and carotenoid production during fed-batch fermentation in optimized (X) soyapeptone supplemented medium using double strength (2X) feed concentrate. Dry cell weight (■, g/l), glucose (∇, g/l), β-carotene (• , mg/l), Torulene (+, mg/l), Torularhodin (×, mg/l) and Dissolved oxygen (∗, % saturation).

In fed-batch fermentation where double strength medium was employed, mutant 32 was allowed to grow in the batch mode for 28 h. During initial batch fermentation, maximum specific growth rate μ_{max} was 0.5 h^{-1}. Feeding was started when 70% (w/v) of initial glucose was consumed. During feeding, exponential increase in cell mass (g/l) was observed. Maximum cell mass achieved was 55 g/l at 76 h.

β-Carotene was found to be the major carotenoid (≥ 90% of the total carotenoids). The maximum specific production rate was found to be 8.2 ±2 mg/l/h between 64 and 68 h. At the end of fermentation (76h) the proportion of carotenoids was found to be 93, 6.5 and 0.5 % of the total carotenoid content (292 mg/l, 5.3 mg/g).

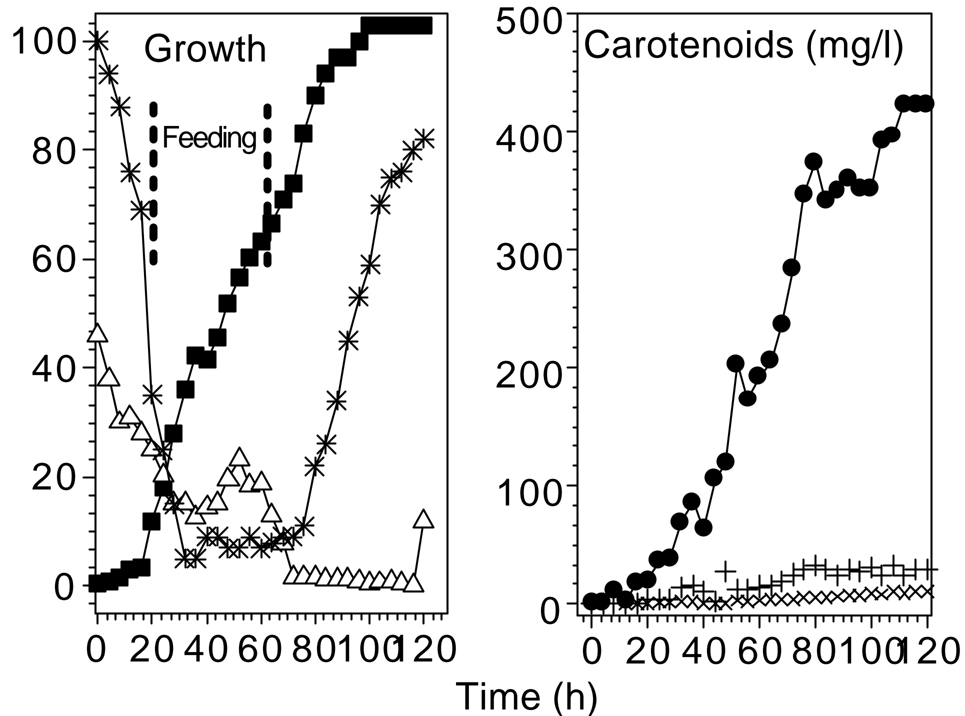


Figure 3.10 Growth and carotenoid production during fed-batch fermentation in (X) soyapeptone supplemented with optimized medium (X) using Triple strength (3X) feed. Dry cell weight (■, g/l), glucose (∇, g/l), β-carotene (●, mg/l), Torulene (+, mg/l), Torularhodin (×, mg/l) and Dissolved oxygen (*, % saturation).

In the fed-batch fermentation using triple-strength feed, mutant 32 was allowed to grow in batch mode for 24 h. During batch fermentation, maximum specific growth rate μ_{max} was 0.46 h^{-1}. Feeding was started when 70% glucose was consumed and DO started dropping. During feeding, exponential increase in cell mass (g/l) was observed. Maximum cell mass achieved was 100 ±3 g/l at 100 h.

Carotenoid production profile indicated that β-carotene was the major carotenoid throughout the run (≥ 90% of the total carotenoids). The maximum β-carotene production rate was found to be 7.8 ±2 mg/l/h. At the end of fermentation (104 h), the respective

proportion of carotenoids was found to be 92, 6 and 2% of the total carotenoid content (461 mg/l, 4.47 mg/g).

Thus, the yield of carotenoids obtained by fed-batch fermentation using double strength and triple strength feed was 1.3 and 2 fold more, respectively, than that obtained during batch fermentation.

Membrane cell-recycle reactor

Rhodotorula glutinis mutant 32 was cultivated in 1 L gallankamp fermenter with 700 ml working volume (Figure 3.11). After a batch run of 60 h, a microfiltration membrane module was coupled to the fermenter and the batch was continued. The fresh medium containing soyapeptone was pumped to the fermenter at a flow rate of 0.25 ml/min and equivalent amount of cell-free permeate was removed through the microfiltration module. The dry cell weight and glucose concentration just before the beginning of recycle run were 22 and 13 g/l, respectively. As the culture was in exponential phase, excess glucose accumulation was not observed during initial feeding as against reported by earlier researchers (Lee *et al.* 1994). Medium feed rate was adjusted according to the permeate flux during the experiment.

Cell mass began to increase linearly with glucose consumption, with a cell mass yield of 0.56 g dry weight per gram of glucose and a stable specific growth rate (μ_{max} 0.52 h^{-1}) between 84 h to 100 h. Maximum cell concentration attained in this experiment was 84 ±2 g/l in 144 h and further recycling did not increase the cell mass (Figure 3.12). This could be because of nutrient, probably oxygen, or mass transfer limitation as described by Qureshi and Cherian (1989).

Carotenoid production profile during the membrane cell-recycle fermentation also showed that carotenoid production was growth associated. A decrease in cellular accumulation was observed during feeding. Carotenoid production rate was low initially during feeding (60 to 112 h). However, it rose later, as the culture became stationary at 128 h (12.58 ± 0.5 mg/l/h). Maximum carotenoid content was achieved at 152 h. (466 ± 8 mg/l, 5.68 mg/g). β-Carotene, torulene and torularhodin were found to be in the proportion of 94, 5 and 1%, respectively, of the total carotenoid content. Membrane cell recycle technique is widely reported to achieve high productivity (Chang *et al.* 1993).

Membrane cell recycle bioreactors were reported to pose problems for product recovery, especially for enzymes (Nipkow *et al.* 1989). This mode of fermentation will however, be ideal for carotenoid production from mutant 32 because the product is intracellular and

yeasts are reportedly known to have tough cell wall, which can resist the high shear in the recycle loop (Shimuzu *et al.* 1992).

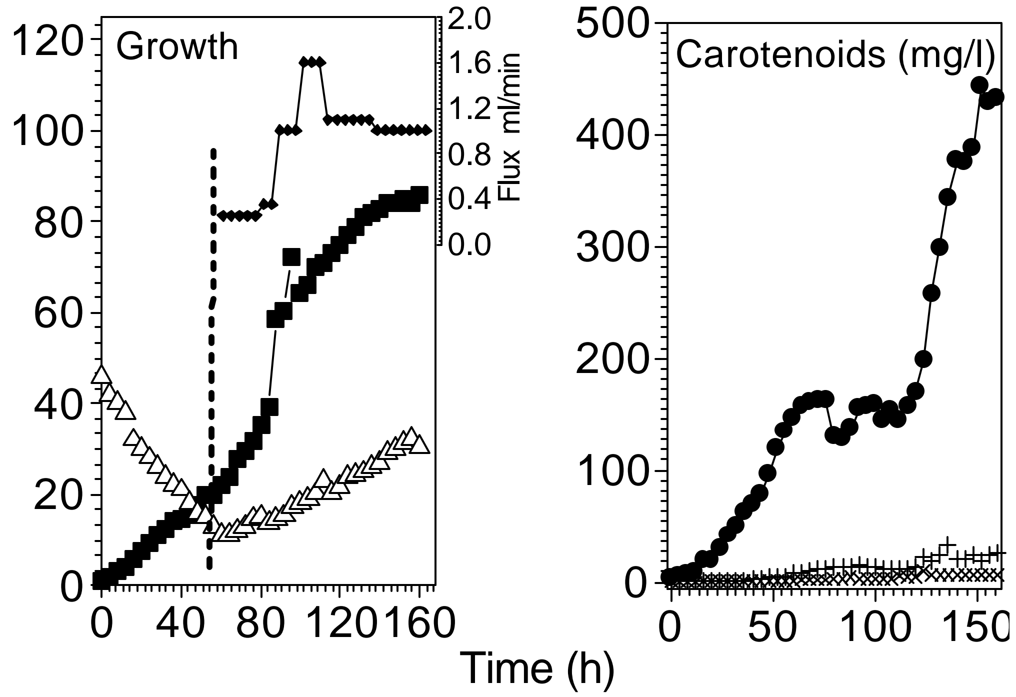


Figure 3.12 Growth and Carotenoid production during membrane cell recycle fermentation in 1L fermenter using soyapeptone based medium (10 g/l). Dry cell weight (■, g/l), Glucose (∇, g/l), Flow rate (♦, ml/min), β- Carotene (●, mg/l), Torulene (+, mg/l) and torularhodin (×, mg/l).

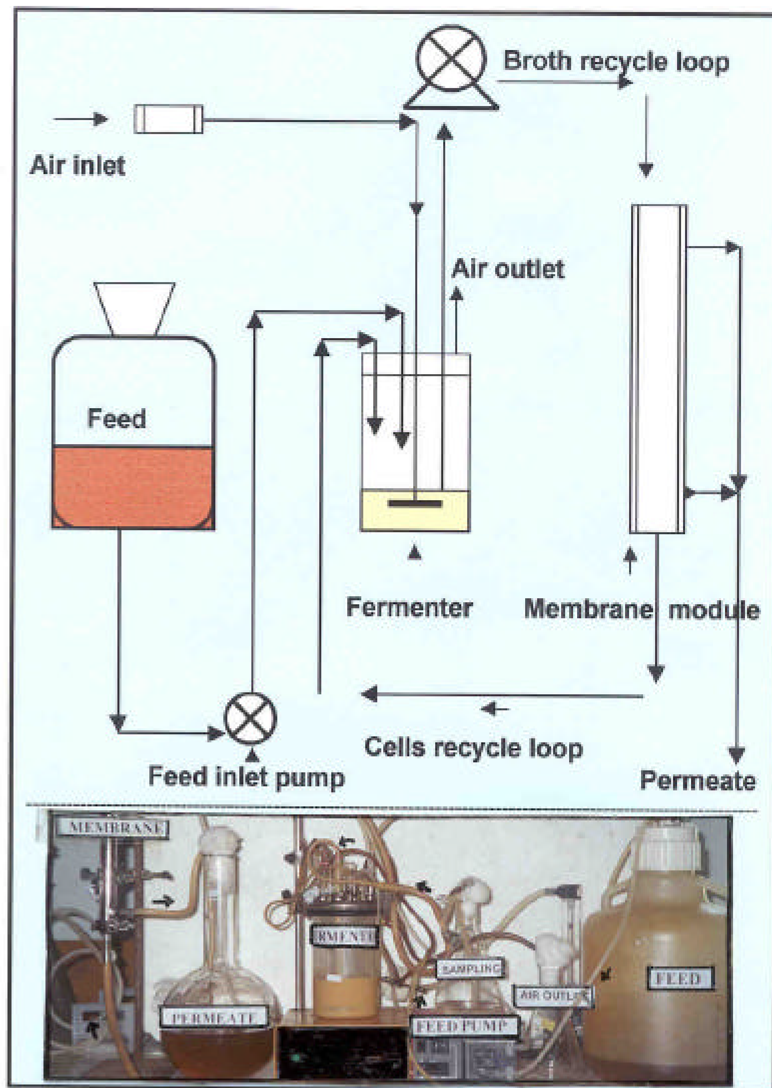


Figure 3.11 Schematic diagram and assembly of a membrane recycle bioreactor.

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The process will be beneficial for obtaining high bioreactor productivity as well as it will be advantageous, as it can integrate downstream processing step for cell recovery with fermentation.

Continuous fermentation

The continuous culture experiments were performed at dilution rates (D) between 0.05 and 0.6 h^{-1}. The steady-state values of dry cell mass, carotenoid content, reducing sugars at different dilution rates are shown in Table 3.19. Maximum carotenoid content was observed at 0.05 h^{-1} dilution and the yield was comparable with that of batch fermentation. The cell mass and carotenoid content decreased with the increase in dilution rate. Increase in residual sugar was observed with the increase in dilution rate indicating that substrate was not utilized completely which resulted in low cell mass and carotenoid production at the higher dilution rates.

Table 3.18 Cell growth, carotenoids and glucose content in the continuous fermentation with feed of optimized medium in 1 L fermenter during steady state.

Dilution rate	Dry cell weight	Residual glucose	Carotenoids		
			β-Carotene	torulene	torularhodin
h^{-1}	±2 g/l	g/l		mg/l	
0.05	32	16	89	7.8	1.6
0.1	35	23	64	3.9	1.74
0.2	23	32	33	1.6	0.44
0.3	16.4	32	25	2.7	0.30
0.4	13.6	35	18	1.8	0.16
0.5	8.9	39	15	0.87	0.13
0.6	6.0	49	8	0.80	0.19

Conclusion

Rhodotorula glutinis mutant 32 was studied for the effect of different media components on carotenoid production. Selected components were systematically optimized in the shake flask using two-level, three-variables factorial method. The optimized medium resulted in 4-fold increase in volumetric production and 2-fold cellular accumulation of β-carotene as compared to the initial basal medium. The yield was further improved by optimization of other cultural conditions like pH, temperature and illumination. Temperature and light played important role in enhancing the accumulation of

carotenoids. This study demonstrates that two-step approach consisting of media optimization and manipulation of cultural conditions can improve carotenoid production by microorganisms by several folds. Fermentation studies were performed using batch, fed-batch and continuous culture, followed by membrane cell-recycle reactor to obtain higher yield of carotenoids, which can help to define process parameters for carotenoid production, especially β-carotene, from yeasts.

References

References are listed in the **chapter 10.**

Chapter 4

Carotenoid production from mutant 32 using industrial by-products.

Summary

Rhodotorula glutinis mutant 32 was studied for growth performance, carotenoid production and β-carotene proportion in a medium composed of sugar cane molasses and corn steep liquor.

In the plain molasses medium, maximum carotenoid production was observed at 40 g/l TRS (total reducing sugars) and pH 6.0, wherein, β-carotene accounted for 70 ±5% (w/w) of total carotenoids. Supplementation of additional nitrogen sources further increased β-carotene proportion by 12 ±4% (w/w) with simultaneous decrease in torulene proportion.

In stirred fermenters, during batch fermentation, increase in torulene content by 20 ±5% was observed in plain molasses medium as compared to that in the shake flask. However, by addition of yeast extract and CSL, the effect was reversed and 31 ±5% increase in β-carotene content was observed.

DO-stat fed-batch cultivation of mutant 32 using double strength and triple strength feed of optimized medium yielded high volumetric content in plain molasses medium (71 and 185 mg/l, respectively) whereas in yeast extract supplemented plain molasses medium, it was 97 and 183 mg/l, respectively.

Introduction

The industries producing carotenoids are facing tough challenge to meet the heavy demand of carotenoids required in food and medicinal industries. Biological sources of carotenoids are receiving major attention because of stringent rules and regulations imposed on chemically synthesized carotenoids (Chen 1999, Nelis and Deleenheer 1991). However, high cost of carotenoid production is the major limiting factor in their supply from biological sources.

Production of carotenoids by yeast can become economically feasible by using cheap industrial by-products as nutrient sources. Growth of *Rhodotorula* in sauerkraut brine, sugar cane juice, grape must and ultrafiltered whey, for carotenoid production, was reported by earlier researchers (Martelli *et al.* 1990, Frengova *et al.* 1994, Shih and Hang 1996, Buzzini and Martini 1999). The strains used in these studies, however, were not of industrial significance because of very low carotenoid yield.

Sugar manufacture from cane is a very large agro-industry in India. The sugar cane molasses, which is a by-product of this industry, accounts for 3.5% (w/w) of the total cane crushed. The molasses has a high sugar and mineral content and thus, can be used as medium for fermentation. Other industrial by-products, like corn steep liquor (CSL), also have potential as nutrient source and they can be used as nitrogen sources although there is yet much to be done to exploit their potential as nutrient source for carotenoid production.

The objective of the work presented in the following chapter was to determine the utility of sugar cane molasses and corn steep liquor as nutrient sources for carotenoid production by *Rhodotorula glutinis* mutant 32.

Materials and methods

Microorganism

Rhodotorula glutinis mutant 32 was investigated in the studies.

Growth of the yeast

Ten ml inoculum in logarithmic phase (9.8 g/l dry cell weight), grown in a basal medium described in chapter 2, was added to 100 ml medium as described in individual subsections below, in 500 ml Erlenmeyer flasks and incubated at 28 °C ±1 on a rotary shaker at 240 rpm for 72 h. Samples were removed periodically for determination of growth and carotenoid content as described earlier.

Sugarcane molasses

Pretreatment

Sugarcane molasses having 85% (w/w) solid content was diluted two-fold (w/v) using distilled water. pH of this solution was adjusted to 2.0 with 10N HCl and was held in boiling water bath for 40 minutes for sucrose hydrolysis. After hydrolysis, the solution was cooled to room temperature and pH was adjusted to 6.0 by 1 N NaOH. Precipitate formed on standing for 1 h was removed by centrifugation.

Reducing sugar estimation

Total reducing sugar (TRS) concentration in the molasses after hydrolysis was assayed spectroscopically using DNSA reagent (Miller 1959). One-gram molasses was found to

contain 0.56 ±0.05g total reducing sugar. The molasses had a C: N ratio of 80 as estimated using automated elemental analyzer (Model CHNS-O EA 1108 – Elemental analyzer, Carlo Erba).

Optimization of total reducing sugar concentration in plain molasses medium

Mutant 32 was grown in hydrolyzed molasses containing total reducing sugar (TRS) between 10 and 100 g/l (17.86 and 178.6 g/l molasses), achieved by suitable dilution of hydrolyzed molasses with distilled water. After addition of 2 g/l each of K_2HPO_4 and KH_2PO_4, the supernatant was sterilized for 30 min at 121 °C and used as nutrient source as "plain molasses medium".

Effect of pH

The mutant was studied for growth and carotenoid production in molasses medium having 40 g/l TRS (selected during above mentioned experiment) but different initial pH between 2 and 10, adjusted by HCl or NaOH.

Effect of nitrogen supplementation

Effect of various inorganic and organic nitrogen sources supplementation in molasses medium on carotenoid production was studied at C: N ratio 30. All inorganic nitrogen sources were procured from S.D fine Chemical Limited (Boiser, Mumbai). Complex organic nitrogen sources were obtained from Hi Media (Mumbai, India). Corn steep liquor was obtained from Anil Starch Products (Ahemdabad, India).

Corn steep liquor

Pretreatment and optimization

Eighty g corn steep liquor (CSL) and 62.5 ml of 2N NaOH were mixed and diluted to 300 ml with distilled water. The solution was centrifuged and supernatant was used for medium preparation. After addition of 2 g/l KH_2PO_4, pH was adjusted to 6.0 with HCl.

Pretreated CSL was used as the nitrogen source, while molasses was used as carbon source, at optimized concentration of total reducing sugar (TRS). Different concentrations of CSL were used in the medium initially (keeping other media components constant) for determination of best supporting concentration of CSL, for carotenoid production.

In some experiments, CSL was used in the medium with different sugars (glucose, fructose and sucrose) in individual flasks, at a final C: N ratio of 40.

All flasks were incubated at 28 °C for 72 h.

Fermentation studies

Medium

Batch and fed-batch fermentation studies were done using medium containing molasses and CSL. Composition of the medium was decided on the basis of shake flask experiments described earlier.
Medium 1: Plain molasses medium containing (g/l) molasses 71.6, (equivalent to 40 g/l TRS), K_2HPO_4 2, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.1 at pH 6.0.

Medium 2: Molasses medium supplemented with yeast extract, containing (g/l) molasses 71.6, (equivalent to 40 g/l TRS), yeast extract 10, K_2HPO_4 2, KH_2PO_4 2, and $MgSO4.7H_2O$ 0.1 at pH 6.0.

Medium 3: Molasses medium supplemented with CSL, containing (g/l) molasses (equivalent to 40 g/l TRS), CSL 10, K_2HPO_4 2, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.1 at pH 6.0.

Batch fermentation and fed-batch fermentation

Batch and fed-batch fermentation in 14 L fermenter were carried out as described in chapter 3 During fed-batch fermentation, four liter feed concentrate was fed to the fermenter. The feed concentrate had double (80 g/l TRS) or triple (120 g/l TRS) the amounts of nutrients, of those used in batch fermentation (40 g/l TRS) in plain molasses medium. In case of molasses supplemented with yeast extract in the feed, C: N ration of 30 was maintained in the feed concentrate.

Results and discussion

Shake Flask studies

Use of sugarcane molasses for carotenoid production

Effect of reducing sugar concentration

Using different concentrations of plain molasses and thereby having different total reducing sugar concentrations between 10 g/l and 100 g/l, it was observed that β-carotene was the major carotenoid produced comprising 73 ±6% of the total carotenoids (mg/l) in all sugar concentrations studied. Torulene (22 ±6%) and torularhodin (4 ±2%) were the other carotenoids produced.

Although higher carotenoid content (17.3 ± 1.3 mg/l) was observed when initial TRS concentration was between 40 g/l and 70 g/l, higher cell mass (10.8 g/l), maximum specific growth rate (0.12 h^{-1}) and β-carotene production (12.4 mg/l) was observed at 40 g/l (Table 4.1).

Table 4.1 Effect of total reducing sugar concentration on carotenoid production from mutant 32 grown for 96 h.

Initial TRS g/l ± 2	Residual TRS g/l ± 2	μ_{max} h^{-1} ± 0.03	Dry cell weight g/l ± 1	Total carotenoids mg/g/g[#] ± 0.2	mg/l ± 2	mg/g ± 0.2	β-Carotene (mg/l) ± 2	Proportion (β-Carotene: torulene: torularhodin, %, w/w)
10	0.3	0.05	4.5	0.2	10	2.2	7.5	75:19:6
20	2.3	0.06	8.0	0.17	14	3.1	9.8	70:23:7
30	1.3	0.04	8.0	0.06	14	1.8	9.4	67:28:5
40	2.3	0.12	10.8	0.05	18	1.7	12.4	69:27:4
50	9.1	0.05	8.1	0.45	16	1.9	10.6	66:31:3
60	37	0.05	6.3	0.12	18	2.8	12.9	72:25:3
70	42	0.03	5.3	0.12	18	3.4	13.7	76:22:2
80	43	0.03	5.4	0.05	11	2.0	8.3	75:232:
90	59	0.02	4.6	0.09	12	2.6	9.3	77:21:2
100	60	0.01	1.3	0.09	5	3.8	3.9	79:19:2

Mg (carotenoid) per gram of dry cell per gram reducing sugar.

Effect of initial pH of plain molasses medium

Mutant 32 when grown at 40 g/l sugar concentration maximum dry cell weight (g/l), specific growth rate (μ_{max}, h^{-1}) and volumetric β-carotene production (10.33 ±1.4 g/l, 0.32 ±0.01 h^{-1} and 9.8 ±2.0 mg/l, respectively) were observed at pH range between 5 and 7. Poor growth and low carotenoid yield (mg/l, mg/g) were observed at other pH values studied. However, carotenoid proportion remained rather unaltered (Table 4.2).

Comparison with refined sugars

The total carotenoids content (mg/l, mg/g), dry cell weight (g/l) and maximum specific growth rate (h^{-1}) achieved in plain molasses medium were comparable with those in media composed of yeast extract (10 g/l) with glucose, fructose and sucrose. The growth and carotenoid production was much less in the medium composed of untreated molasses (Table 4.3).

Table 4.2 Effect of initial pH of the plain molasses medium on carotenoid production from mutant 32 grown for 72 h.

Initial pH ± 0.2	Final pH ± 0.4	μ_{max} h^{-1} ± 0.03	Dry cell weight g/l ± 0.7	Total carotenoids		β-Carotene mg/l ±1	Proportion (β-carotene: torulene :torularhodin, %, w/w)
				mg/l ± 2	mg/g ± 0.2		
2	2.8	0.017	1.6	4	2.5	2.6	66:24:10
3	3.8	0.08	7.9	10	1.3	6.6	66:31:3
4	5.3	0.09	9.1	13	1.4	8.5	65:32:3
5	6.3	0.11	8.9	16	1.8	10.3	64:33:3
6	6.5	0.11	10.8	18	1.7	11.9	65:34:1
7	6.6	0.11	11.3	18	1.6	7.2	63:33:4
8	6.8	0.23	7.6	11	1.4	6.7	61:34:5
9	6.9	0.23	9.4	8	0.8	4.8	60:34:6
10	7.3	0.10	3.5	8	2.3	4.7	59:35:6

Table 4.3 Comparison between the effect of refined sugars in basal medium and plain molasses medium on carotenoid production from mutant 32 grown for 72 h.

Sugar 40 g/l ± 2	μ_{max} h^{-1} ± 0.01	Dry cell weight g/l ± 0.5	i. **Total Carotenoids**		β-Carotene mg/l ± 1.5	Proportion (β-carotene: torulene: torularhodin, %, w/w)
			mg/l ± 0.9	mg/g ± 0.1		
Glucose	0.24	12.2	22.0	1.8	15.4	70:26:4
Fructose	0.18	9.3	14.4	1.5	7.5	52:45:1
Sucrose	0.14	11.7	18.3	1.6	8.6	47:50:3
Untreated molasses	0.10	6.8	10.1	1.5	6.4	64:28:8
Hydrolyzed molasses	0.12	10.8	18.0	1.7	12.4	69:27:4
Heat treated# molasses	0.10	9.4	15.1	1.6	9.5	63:28:9

Heated for 40 min in boiling water bath without acid treatment.

Effect of addition of nitrogen sources

Various inorganic and organic nitrogen sources were added to the molasses to get C: N ratio 30. Addition of yeast extract and corn steep liquor (CSL) showed 2.7 (33.6 mg/l) and 2.0 (24.9 mg/l) fold increase in β-carotene content, respectively, as compared to the plain molasses medium (12.4 mg/l). Simultaneous decrease in the torulene content was also observed in both cases. Yeast extract was preferred over CSL for fermentation studies because of better yield (Table 4.4).

Table 4.4 Effect of addition of nitrogen sources to plain molasses medium (having 40 g/l TRS) on growth and carotenoid production from mutant 32 having incubated for 72 h.

Nitrogen sources	Dry cell weight g/l ± 0.5	ii. iii. mg/l ± 1.96	**Total Carotenoids** mg/g ± 0.19	β-Carotene mg/l ± 1.6	Proportion (β-carotene: torulene: torularhodin %, w/w)
Control	**10.8**	**18**	**1.7**	**12.4**	**69:27:4**
A. Organic nitrogen sources					
Casein acid hydrolysate	12.1	24.5	1.9	17.2	70:26:4
Cornsteep liquor	13.2	28.3	2.2	24.9	88:11:1
Cotton seed meal	13.6	16.4	1.3	10.7	65:39:6
Malt extract	10.1	24.9	2.5	15.4	62:36:2
Peptone	10.5	21.2	2.0	15.3	72:26:2
Soyabean meal	16.1	18.9	1.2	12.1	63:36:1
Soyapeptone	12.0	29.3	2.4	21.8	75:23:2
Tryptone	11.3	22.0	1.8	16.5	75:19:6
Yeast extract	**12.7**	**42.6**	**3.4**	**33.6**	**79:16:5**
B. Inorganic nitrogen sources					
Ammonium chloride	7.2	17.8	2.4	10.2	57:35:8
Ammonium nitrate	10.2	8.8	0.9	4.9	56:43:1
Ammonium sulfate	12.1	14.4	1.3	10.7	74:25:1
Potassium nitrate	10.2	12.1	1.19	7.26	60:35:5
Sodium nitrate	11.1	15.1	1.4	9.5	63:36:1
Urea	9.3	17.3	1.9	11.0	64:35:1

Sugarcane molasses, which is rich in sucrose and minerals, is favourite in fermentation industries as a cheap carbon source. It has been used earlier for production of astaxanthin from *Phaffia rhodozyma* (Haard 1987) and carotenoid-rich biomass from *Rhodotorula rubra* (Eugenia *et al.* 1996). In our studies, it was observed that plain molasses having high C: N ratio 80 supported satisfactory total carotenoid production. Decreasing the C: N ratio to 30, by addition of yeast extract and CSL, resulted in

improved β-carotene production (mg/l) and accumulation (mg/g). Composition of cane molasses can undergo seasonal variation (Vercellotti *et al.* 1996) and it may be necessary to fine-tune the quantities to be used frequently, for the best yields of carotenoids. In India, molasses is commonly used for production of ethanol. There are reports on process development for production of high quality yeast feed protein, using molasses as carbon source. The production of carotenoids from molasses can be a latest addition to the list of industrial uses of sugarcane molasses.

Batch fermentation

Production of carotenoids and cell mass was studied in batch mode in 14 L fermenter using selected media compositions as mentioned earlier.

In plain molasses medium, the maximum specific growth rate of mutant 32 was observed to be 0.32 h^{-1}. Culture became stationary after approximately 28 h. The maximum dry cell weight reached 12 ±2 g/l.

Maximum volumetric carotenoid productivity was observed during exponential phase between 20 and 24 h (1.14 mg/l/h). However, during stationary phase (between 44 and 60 h), it was observed to be 0.53 mg/l/h. The total carotenoid content remained constant after 64 h. The volumetric carotenoid concentration after fermentation was 47.96 ±1.5 mg/l.

Production profile of individual carotenoids indicated that both β-carotene and torulene were co-produced with almost similar rate (0.3 ±0.08 mg/l/h) between 20 and 40 h. After 40 h, β-carotene production rate remained the same (0.3 ±0.03 mg/l/h) but increase in torulene production rate was observed (0.65 ±0.09 mg/l/h) which was almost double of that during logarithmic phase.

This production pattern resulted in decrease in proportion of β-carotene with simultaneous increase in the torulene proportion. Interestingly, after 72 h, β-carotene content, which accounted for 69 ±5% (w/w) of total carotenoids in shake flask, was found to be only 44 ±2% (w/w) in 14 L stirred tank fermenter.

This might be due to following reasons:

a. The C: N ratio of the molasses is high (80) and presumably nitrogen is getting exhausted prior to complete utilization of sugars. Residual sugar at the beginning of

stationary phase, which mainly contains fructose because of slow utilizing capability by mutant 32 (0.1 g/l/h) as compared to that of glucose (0.5 g/l/h), favors the formation of torulene (Please refer to "effect of carbon sources" in chapter 3).

b. Higher oxygen transfer in the fermenters as compared to the shake flask. (Please refer to the "effect of shaker speed" in chapter 3).

However, further detailed study is required to reveal the actual cause of this shift in the carotenoid proportion. Addition of yeast extract (C: N 30) to plain molasses was observed to reverse the effect of this shift in the carotenoid proportion. Carotenoid production profile of mutant 32 indicated that β-carotene was the major pigment produced in the 14 L batch fermentation run. Maximum volumetric production rate between 24 and 28 h (2.78 mg/l/h) was achieved with virtually no torulene and torularhodin production during exponential growth phase. There was no change observed in carotenoid production and proportion profile during stationary phase.

When yeast extract was added to the molasses, available nitrogen increased, which was sufficient to consume all sugar (both glucose and fructose) for growth. β-Carotene being actively synthesized during growth, and because not much sugar was left over at the end, β-carotene was the main product and there was no accumulation of any carotenoid in stationary phase.

Supplementation of yeast extract had no major effect on the maximum specific growth rate ($\mu_{max:}$ 0.29 h^{-1}), final dry cell mass (12.2 ±0.2 g/l) and total carotenoid content (42.7 ±3 mg/l and 3.5 mg/g). In contrast to our present results, yeast extract supplementation in relatively inexpensive Yuca medium was previously reported to decrease carotenoid production (Ramirez *et al.* 2000) in *Phaffia rhodozyma*.

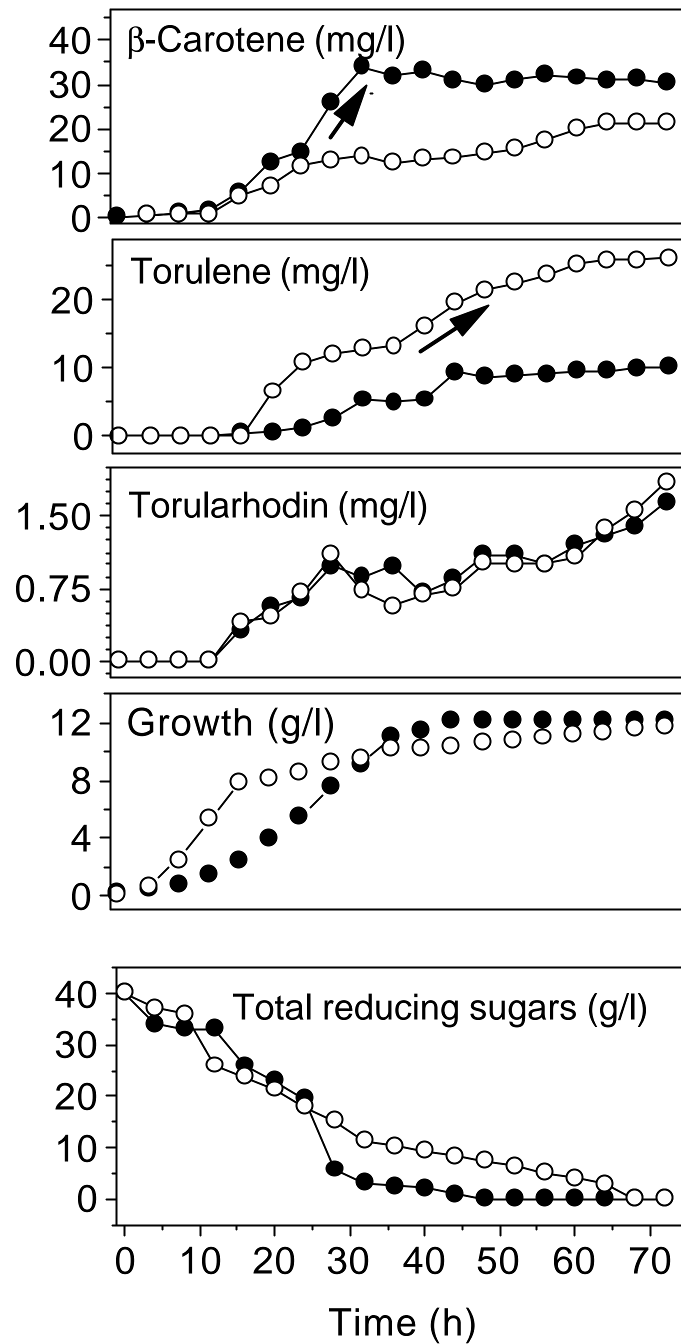

Figure 4.1 Carotenoid production during batch fermentation in 14 L fermenter using plain molasses medium (O) and plain molasses medium supplemented with yeast extract (●).

Fed-batch fermentation

Shake flask

Preliminary fed-batch experiments in shake flask indicated that feeding fresh medium to a growing culture in the late exponential phase (24 h) led to increase in carotenoid production (mg/l) and accumulation (mg/g). Addition of feed, during early stages and late stages of growth, had inhibitory effect on carotenoid production (Table 4.4). Thus, further experiments were planned accordingly.

Table 4.4 fed-batch fermentation from mutant 32 in shake flask.

Strategy ®ψ *Feeding time (h)* ⁻		*1#*	*2*	*3*	*4*	*5*
12h		**-**	***5g/l***	**-**	**-**	**-**
24h		**-**	***5g/l***	***5g/l***	**-**	**-**
36h		**-**	***5g/l***	***5g/l***	***5g/l***	**-**
48h		**-**	***5g/l***	***5g/l***	***5g/l***	***5g/l***
A. Plain molasses medium						
Carotenoid	(mg/l)	***18***	***21***	***34***	***28***	***17***
	(mg/g)	***3.0***	***2.2***	***3.8***	***3.0***	***2.5***
Dry cell mass	(g/l)	***12.3***	***19.4***	***18.0***	***18.2***	***13.8***
Residual sugar	***(g/l)***	***7.2***	***15.9***	***13.7***	***11.3***	***9.3***
B. Plain molasses supplemented with yeast extract ∞						
Carotenoid	(mg/l)	***42***	***33***	***48***	***30***	***39***
	(mg/g)	***3.4***	***1.7***	***3.0***	***2.3***	***3.3***
Dry cell mass	(g/l)	***12.7***	***20***	***16***	***13***	***12.0***
Residual sugar	***(g/l)***	***1.05***	***10.4***	***6.8***	***1.2***	***1.8***

ψ The feed concentrate was added (0.5 ml).
# Indicates the amount of sugar and equivalent amount of nitrogen.
∞ Indicates that the equivalent amount of yeast extract corresponding to 5 g/l TRS was added.

Fermenter studies

Plain molasses medium

During fed-batch fermentation with double strength of plain molasses medium as feed concentrate (molasses equivalent to 80 g/l TRS), mutant 32 was allowed to grow in batch mode for 20 h, initially. Feeding was started at 20 h. During feeding, maximum specific growth rate (μ_{max}) was observed to be 0.21 ±0.07 h^{-1}. Stationary phase was reached at 72 h. Maximum dry cell mass achieved was 31.49 ±0.5 g/l which was three fold more than that obtained with batch fermentation. DO level was maintained between 12 and 40% during feeding (Figure 4.2).

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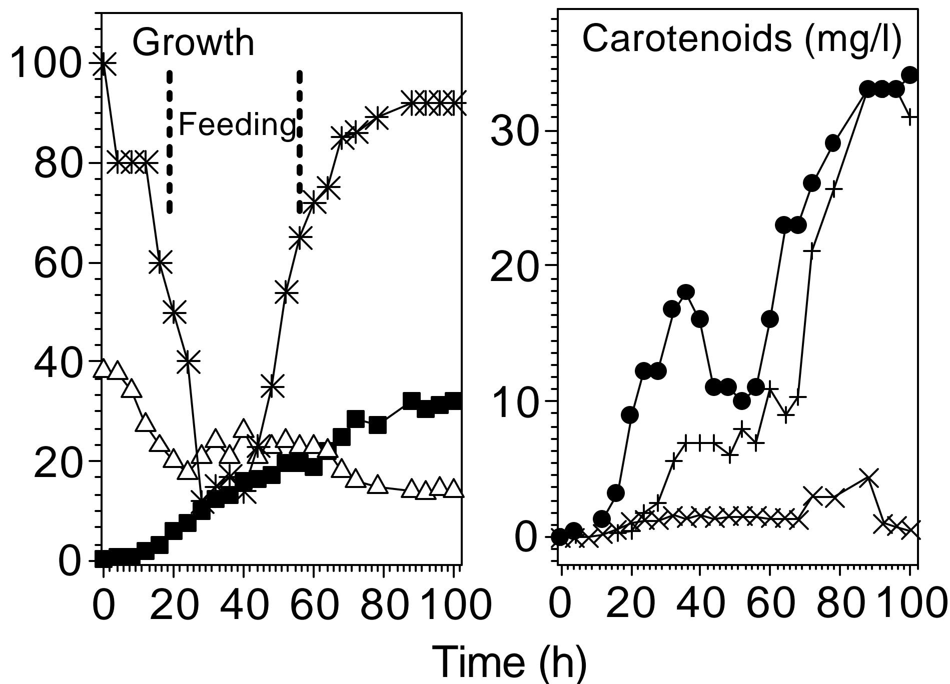

Figure 4.2 Growth and carotenoid production during fed-batch fermentation in plain molasses using double strength (2X) plain molasses as feed. Dry cell weight (■, g/l), Total reducing sugar, TRS (∇, g/l) β-carotene (●, mg/l), Torulene (+, mg/l), torularhodin (×, mg/l) and dissolved oxygen (∗, % saturation).

Carotenoid production profile indicated that initially, till 36 h, β-carotene was the major carotenoid produced at the rate of 1.1 ±0.02 mg/l/h (between 28 and 32 h). Slight decline in carotenoid production (mg/l) was observed during 36 and 60 h. This decline can be

attributed to the dilution of the fermentation broth due to feeding (between 20 and 52 h). However, after 60 h, both torulene and β-carotene were co-produced at almost similar rate and maximum volumetric production rate was found to be between 68 and 78 h (1.2 ±0.02 mg/l/h).

Final carotenoid content was observed to be 71 ±2 mg/l which was 1.5 fold more than that obtained in batch fermentation with plain molasses medium. At the end of fermentation run (100 h), β-carotene and torulene were produced at equal proportion (48 ±2%, w/w). Mutant 32 also produced very low concentration of torularhodin (3 ±1%) that remained unchanged during fermentation run.

During fed-batch fermentation with plain molasses medium and triple strength (120 g/l TRS) feed, mutant 32 exhibited exponential increase in cell mass (g/l) during feeding with a maximum specific growth rate of 0.16 h^{-1}. Culture achieved stationary at 64 h. Maximum dry cell mass achieved was 53 ±2 g/l.

Carotenoid production profile indicated that β-carotene and torulene were produced in equal proportion and production rate till 68 h, maximum being between 28 and 32 h (2.0 mg/l/h). After 68 h, β-carotene did not increase. A drop in β-carotene content (mg/l) was observed from 80 h onwards till the end of fermentation (100 h). On the other hand torulene production showed linear increase till 96 h. This led to decline in the β-carotene proportion (%) in the cell. This alteration in carotenoid proportion was probably because of excess accumulation of fructose in the fermented broth.

At the time of harvesting, β-carotene, torulene and torularhodin were found to be in the proportion of 39, 59 and 2%, respectively, of the total carotenoids (172 mg/l, 3.25 mg/g). The total carotenoid and β-carotene content was 3.6 and 4 fold more, respectively, than that obtained with batch fermentation using plain molasses medium.

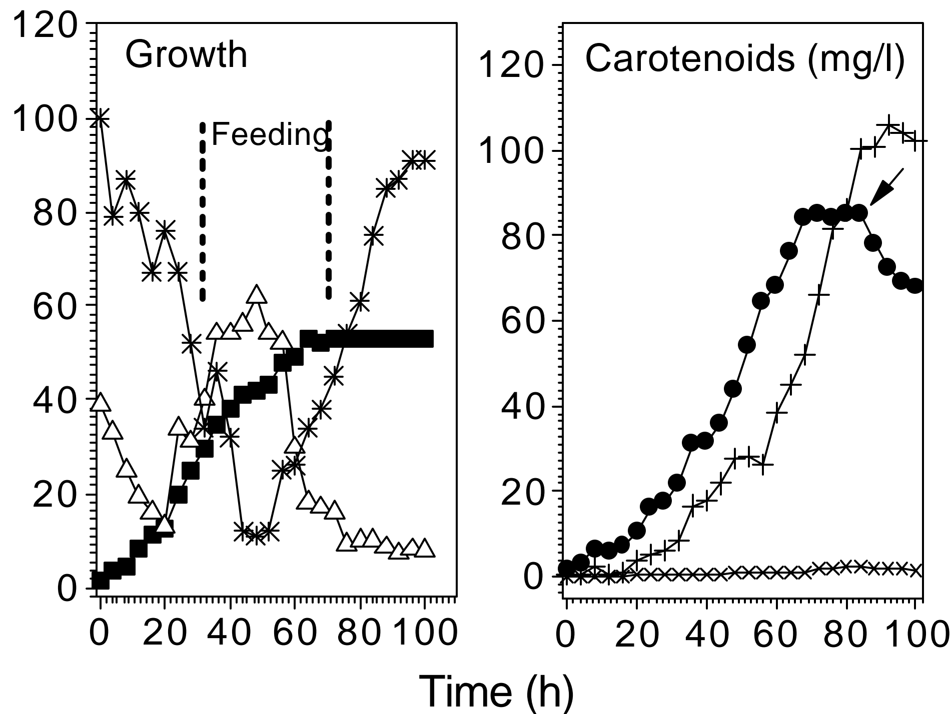

Figure 4.3 Growth and carotenoid production during fed-batch fermentation in plain molasses using triple strength (3X) plain molasses as feed. Dry cell weight (■, g/l), Total reducing sugar, TRS (∇, g/l) β-carotene (●, mg/l), Torulene (+, mg/l), torularhodin (×, mg/l) and dissolved oxygen (∗, % saturation).

Molasses supplemented with yeast extract

Double strength (80 g/l TRS) and triple strength (120 g/l TRS) fed-batch fermentation performed with plain molasses medium supplemented with yeast extract (C: N 30) supported good growth and resulted in much higher cell mass (g/l).

During double fed-batch fermentation with double strength feed, mutant 32 was allowed to grow in batch mode till 16 h. Feeding was started at 16 h. Maximum specific growth rate (μ_{max}) was observed to be 0.31 ±0.04 h^{-1}. Culture reached stationary at 60 h (15 h after feeding was over). Maximum dry cell mass achieved was 43.5 ±0.25 g/l which was 3.6 fold more than that obtained with batch fermentation with yeast extract supplemented plain molasses medium. DO level was maintained between 12 and 40 % during feeding (Figure 4.4).

Carotenoid production profile demonstrated that β-carotene was the major carotenoid produced during fermentation run. Volumetric β-carotene production rate was observed to be 2.75 ±0.25 mg/l/h between 20 to 52 h. However, during stationary phase no major change in the carotenoid production (mg/l) was observed.

At the end of fermentation run (80 h), β-carotene, torulene and torularhodin were found to be in the proportion of 79, 14 and 7%, respectively, of the total carotenoids (94 mg/l, 2.2 mg/g). The total carotenoid and β-carotene content was 2.2 and 2.18 fold more, respectively, than that obtained with batch fermentation using plain molasses medium supplemented with yeast extract.

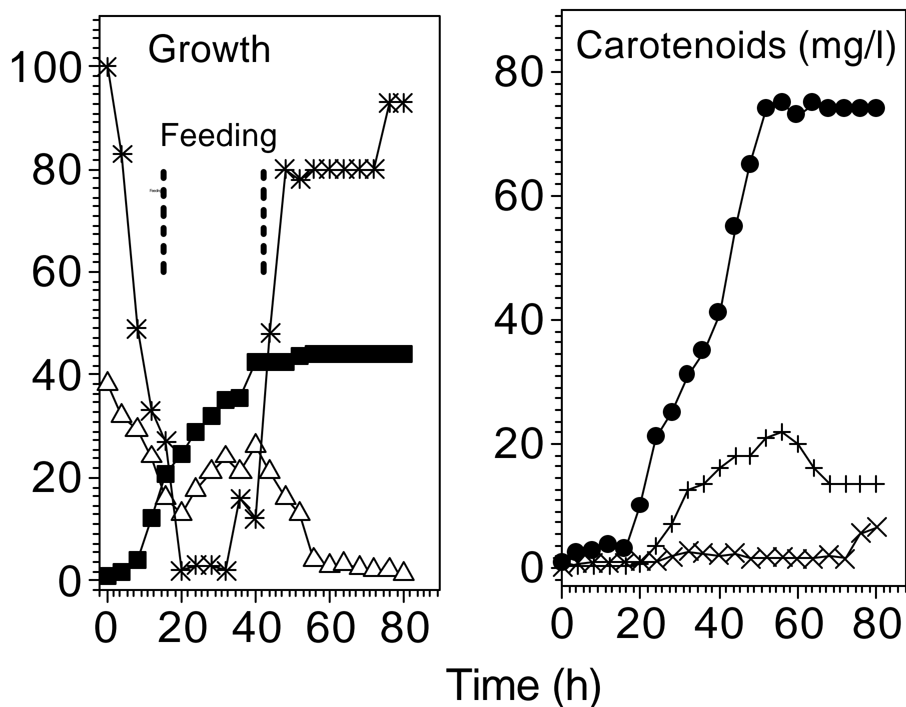

Figure 4.4 Growth and carotenoid production during fed-batch fermentation in plain molasses using double strength (2X) yeast extract supplemented plain molasses medium as feed concentrate. Dry cell weight (■, g/l), Total reducing sugar, TRS (∇, g/l) β-carotene (●, mg/l), Torulene (+, mg/l), torularhodin (×, mg/l) and dissolved oxygen (∗, % saturation).

During fed-batch fermentation using triple strength feed, mutant 32 was allowed to grow in batch mode till 12 h. After that, feeding was started. During feeding, maximum specific growth rate (μ_{max}) was observed to be 0.36 ±0.03 h^{-1}. Culture reached stationary at 72 h (24 h after feeding was over). Maximum dry cell mass achieved was 70.25 ±4 g/l, which was 6 fold more than that obtained during batch fermentation. DO level was maintained between 27 and 37% during feeding (Figure 4.5).

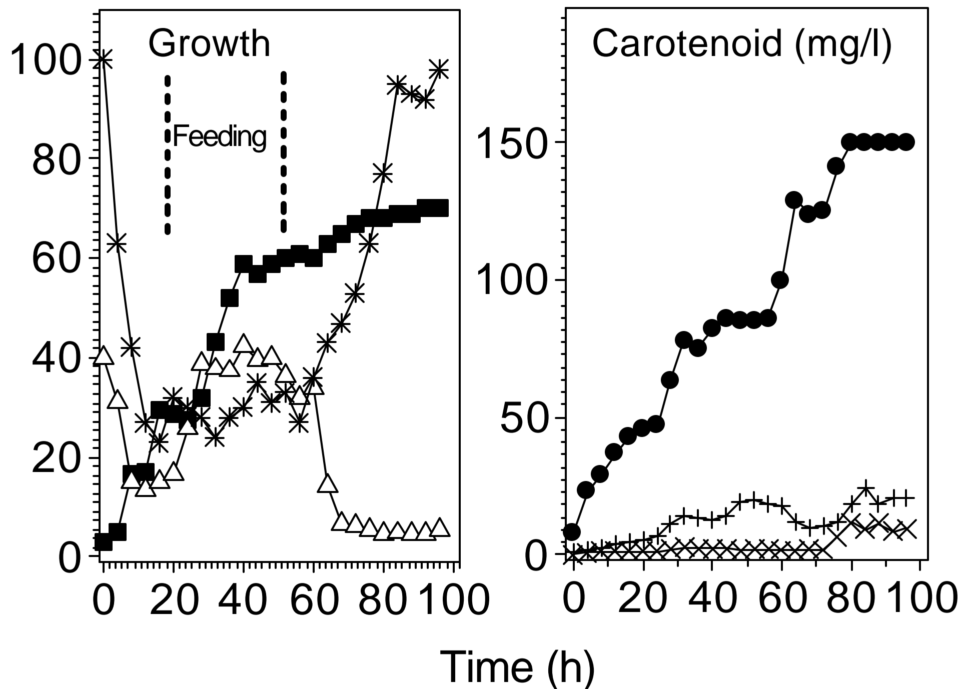

Figure 4.5 Growth and carotenoid production during fed-batch fermentation in optimized (X) molasses supplemented with yeast extract using Triple strength (3X) molasses and yeast extract as feed concentrate. Cell dry weight (■, g/l), Total reducing sugar, TRS (∇, g/l) β- Carotene (●, mg/l), Torulene (+, mg/l) and torularhodin (×, mg/l) and Dissolved oxygen (∗, % saturation).

Carotenoid production profile showed that β-carotene was distinctly the major carotenoid produced. Maximum volumetric β-carotene productivity observed was 4.25 mg/l/h during 28 and 32 h. At the end of fermentation, proportion of β-carotene, torulene and torularhodin was observed to be 87, 11and 2% (w/w), respectively, of the total carotenoids (181.1 mg/l, 2.6 mg/g). Thus, it was observed that the yeast extract

supplemented feed showed better production (mg/l) and accumulation (mg/g) of β-carotene in mutant 32. However, no major increase in total carotenoid production was observed.

Thus, it was observed that supplementation of additional nitrogen source to plain molasses medium lead to substantial increase in β-carotene content (mg/l, mg/g) and proportion (%).

Corn steep liquor

Corn steep liquor (CSL) is a by-product of corn wet milling and has is used as complex nitrogen source in fermentation. It was also used for carotenoid from microorganisms like *Rhodococcus* (Xiaodong *et al.* 1999) and *Phaffia rhodozyma* (Fontana *et al.* 1996, Kesava *et al.* 1998). In addition, it has been used for production of many other fermentation products like pullulan (West and Strohfus 1999), acetate (Shah and Cheryan 1995) and alcohol (Rainho *et al.* 1982).

In the present studies, CSL was observed to be suitable nitrogen source for carotenoid production from *Rhodotorula glutinis* mutant 32.

Shake flask

During use of corn steep liquor (CSL) as additional nitrogen source in the plain molasses medium, it was observed that β-carotene was the major carotenoid produced under all different C: N ratio (between 10 and 70) studied (Table 4.5).

However, β-carotene content (mg/l) was found to decrease with the increase in C: N ratio. The best yields of β-carotene were obtained between at C: N 40 (23.2 ±3 mg/l). Together, CSL and sugarcane molasses were also reported to increase yield of alcohol by *Saccharomyces cerevisiae* (Rainho *et al.* 1982). The present studies revealed that combination of CSL and molasses could be used as an industrial medium for carotenoid production from *Rhodotorula glutinis* mutant 32.

Table 4.5 Effect of different C: N ratio on carotenoid production and growth using CSL and molasses and nitrogen and carbon source respectively.

C: N	Dry cell weight ± 0.5 g/l	Total carotenoids ± 3 mg/l	± 0.2 mg/g	β-Carotene ±0.2 mg/l	Proportion (β-Carotene: torulene: torularhodin %, w/w)
10	9.1	5	0.5	4.5	90:10:0
20	11.7	12	1.1	10.7	89:9:2
30	13.2	23	1.7	19.8	86:14:0
40	16.2	28	1.7	23.2	83:16:1
50	15.4	24	1.2	19.7	82:16:2
60	15.2	22	1.5	18.0	82:18:0
70	11.6	21	1.8	16.1	77:22:1

CSL also supported good growth and carotenoid production when supplied along with refined sugars (Table 4.6).

Table 4.6 Comparison of effect of refined sugars and molasses added to CSL on carotenoid production from mutant 32.

Sugars (C:N 40)	Specific growth rate (μ_{max})	Dry cell weight g /l ±0.5	Total Carotenoids mg/l ± 0.9	mg/g ± 0.1	β-Carotene mg/l ± 2	Proportion (β-carotene: torulene: torularhodin %, w/w)
Glucose	0.21	12.2	31	2.5	22.3	72:25:2
Fructose	0.19	12.0	27	2.3	17.6	65:32:3
Sucrose	0.17	11.0	28	2.5	17.4	62:32:6
Hydrolyzed molasses	0.15	13.2	28.3	2.14	23.2	83:16.:1

Fermenter

In 14 L fermenter, mutant 32 grew well with maximum specific growth rate of 0.51 h^{-1} and the final dry cell weight achieved was 11.4 g/l. Stationary stage was reached at 40 h.

Carotenoid production profile indicated that β-carotene was the major carotenoid produced during the fermentation run. Maximum β-carotene production rate (1.0 mg/l/h) was observed during exponential phase (between 20 and 24 h). No carotenoid production was observed during stationary phase (after 40 h). At the time of harvesting (72 h), β-carotene, torulene and torularhodin accounted to 81, 14 and 5%, respectively, of total carotenoid (36.95 ±2 mg/l, 3.2 ±0.2 mg/g).

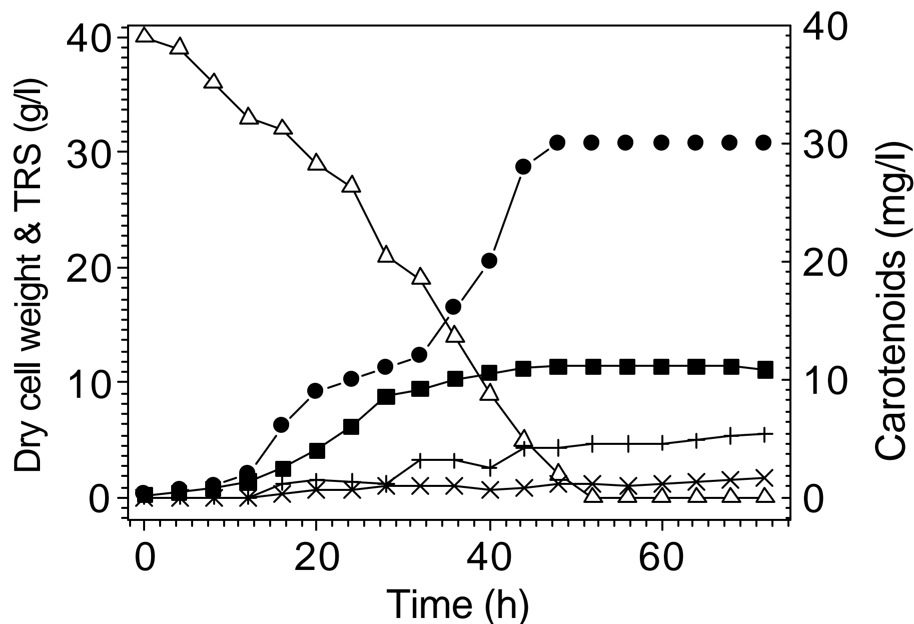

Figure 4.5 Growth and carotenoid production during batch fermentation in optimized plain molasses medium supplemented with corn steep liquor. Dry cell weight (■, g/l), Total reducing sugar (∇, g/l), β-carotene (●, mg/l), torulene (+, mg/l) and torularhodin (×, mg/l).

Thus, similar to yeast extract, supplementation of plain molasses medium with CSL supported β-carotene production and minimized torulene. Use of CSL in the

fermentation medium, instead of yeast extract, will definitely result in reduction of production cost.

However, removal of insoluble solids present in CSL (Lawford and Joyce 1997) along with washing of the cell mass prior to extraction of carotenoids can have negative impact on the cost structure associated with the carotenoid production while using CSL in large scale fermentation.

Conclusion

Mutant 32 was studied for growth and carotenoid production (mg/l, mg/g) in plain sugar cane molasses medium supplied as sole nutrient source. In shake flask, best carotenoid content was observed at pH 6.0, when molasses equivalent to 40 g/l TRS was used. When grown in 14 L fermenter, β-carotene production (mg/l, mg/g) was observed to be growth associated, whereas, better torulene production was observed during stationary phase. However, Supplementation by additional nitrogen sources like yeast extract and corn steep liquor (CSL) was observed to support β-carotene production (mg/l) leading to increased β-carotene proportion (%) in the cell. Batch and fed-batch fermentation runs performed with the optimized medium and fermentation conditions resulted in higher production of carotenoids, especially β-carotene.

Thus, cheaper substrates, like sugarcane molasses and CSL, can be employed for carotenoid production from *Rhodotorula glutinis* mutant 32 to make a commercially viable process of carotenoid production.

References

References are listed in **chapter 10.**

Chapter 5

Use of seawater as a water source in carotenoid production

Summary

Mutant 32 was grown in a medium prepared in artificial seawater. At the end of incubation period, the total carotenoid content and dry cell mass of the mutant was 86 ±4 mg/l and 16 ±1 mg/l, respectively. Two-fold increase in β-carotene (67 ±4 mg/l), simultaneous with 2.3 fold decrease in torulene content (15.3 ±2 mg/l) was observed when medium was prepared in seawater, instead of distilled water. When grown at pH 6.0 in seawater medium, 83 ±5% carotenoids could be extracted from the mutant 32 cells without any mechanical disintegration. The ease in extraction was observed with the parent strain (*Rhodotorula glutinis* NCIM 3353) also, but to a less extent.

Introduction

Yeasts like *Rhodotorula glutinis* and *Phaffia rhodozyma* have potential to replace conventional microbial carotenoid sources like algae (mainly *Dunaliella sp.*) because of the higher growth rate. But, tough cell wall of the yeasts is a major obstacle in development of a simple process for extraction of carotenoid from yeast biomass (Hari *et al.* 1992). Previously, various methods like thermolysis and enzymatic hydrolysis were investigated to break the cell wall for extraction of carotenoids from *Rhodotorula sp.* but they were not industrially feasible (Motoo and Sawao 1978, Stabikova 1981, JaeGu and Shick 1984, Sedmak *et al.* 1990, Bindu *et al.* 1998).

Rhodotorula strains can be isolated commonly from saline environments and seawater (Phaff and Ahearn 1970, Phaff and Fell 1970, Fell *et al.* 1984). Seawater is rich in minerals and has a potential as a water and nutrient source as it is abundant in nature.

In this chapter, an attempt was made to grow *Rhodotorula glutinis* mutant 32 in a medium prepared in seawater, instead of distilled water. The effect of this change on production (mg/g, mg/l) and proportions (%) of individual carotenoids by mutant 32 was studied. Along with this, the effect of pH of the medium, prepared in seawater, on extractability of the pigments was also studied.

Materials and methods

Media

Mutant 32 was grown in the basal medium containing (g/l) glucose 25, yeast extract 10, K_2HPO_4 2, KH_2PO_4 2 but prepared in artificial seawater, containing (g/l) NaCl 23.5, Na_2SO_4 4, KCl 0.68, H_3BO_4 0.026, $MgCl_2.2H_2O$ 10.7, $CaCl_2.2H_2O$ 1.47, $NaHCO_3$ 0.196, $Na_2SiO_3.9H_2O$ 0.030, Na_4EDTA 0.0003. The salinity was 34 $\pm$0.5% and initial pH was 8.0 $\pm$0.2. All chemicals were added and dissolved in the order given (Rand et al. 1976).

Growth of yeast

Mutant 32 was grown in 100 ml the seawater medium, at different pH, in 500 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 250 rpm, at 28 °C for 72 h. Growth and carotenoid production profile were studied in a 1L fermenter (Gallenkamp, U.K.) in the medium prepared in distilled water as well as in seawater, at pH 6.0. This pH was selected after above-mentioned experiment. Fermentation conditions were similar to those described in chapter 3.

Extraction of carotenoids

Effect of seawater on the extraction of carotenoids from cells grown at different pH was studied by growing mutant 32 in medium prepared in seawater, at different initial pH, which was adjusted, as desired, with NaOH or HCl. After 72 h of fermentation, one ml culture broth was centrifuged and the pellet was washed three times with distilled water by suspension and centrifugation.

Extraction without mechanical disintegration was studied by suspending the pellet in one ml of HPLC mobile phase containing acetonitrile, iso-propanol and ethylacetate (40:40:20) for 10 minutes. Cell debris was then centrifuged out and the supernatant was microfiltered for HPLC analysis. The extraction efficiency was compared with the control experiment by extraction of the carotenoids using mini bead beater, described earlier in chapter 2.

Effect of individual components of seawater on ease of extraction of carotenoids was also studied by using growth medium without the individual ingredient under study.

Results and discussion

Effect on growth

Mutant 32 grew well at different initial pH in shake flask and the cell mass achieved was 11 ±2 g/l. The pH of the medium, however, was found to change considerably, presumably because of the growth of the yeast. No effort was made to control the medium pH in the shake flasks. However, fermenter studies were done at pH 6.0 (Table 5.1).

Table 5.1 Effect of initial pH on growth and carotenoid production from mutant 32 grown in distilled and seawater medium for 72 h.

Initial pH	Final pH	Dry cell weight g/l	Total carotenoids mg/l	Total carotenoids mg/g	β-Carotene mg/l	Proportion (β-carotene: torulene: torularhodin, %, w/w)
A. Distilled water medium						
2	1.7	12.8	34	2.6	23.8	70:24:6
4	6.0	12.8	36	2.8	24.5	68:27:5
5	6.9	12.8	36	2.8	23.4	65:28:7
6	7.1	12.0	35	2.9	24.2	69:27:4
7	7.3	12.0	44	3.7	23.7	54:40:6
8	7.6	12.8	44	3.4	18.5	42:52:6
10	8.0	13.6	44	3.2	16.7	38:56:6
B. Seawater medium						
2	2.3	13.7	40	2.9	32.0	80:16:4
4	6.0	11.0	36	3.3	30.9	86:11:3
5	6.1	11.3	37	3.3	31.0	84:10:6
6	7.1	11.3	36	3.2	30.9	86:10:4
7	7.1	11.2	39	3.5	33.1	85:14:1
8	7.2	11.4	38	3.0	30.0	79:19:2
10	8.2	10.9	42	3.3	33.2	79:20:1

In 1L fermenter, *Rhodotorula glutinis* mutant 32 grew well in seawater medium. Its maximum specific growth rate during logarithmic phase was 0.35 h^{-1} and the final cell mass achieved was 16 ±1 g/l, which was higher than that in distilled water medium (0.17 h^{-1} and 12 ±1 g/l, respectively).

Salts added to the seawater medium have been reported to stimulate cell growth of *Rhodotorula glutinis* (Komemushi *et al.* 1994) and could have been the reason for better growth rate and higher final cell mass of the mutant 32 also.

Effect on carotenoid production

In shake flask, the total carotenoid content obtained with seawater and distilled water medium was almost similar (39 ±4 mg/l). However, in seawater medium substantial improvement in β-carotene proportion (%) was observed as compared to the distilled water medium (Table 5.1).

Carotenoid production profile indicated that in seawater medium, β-carotene was the major carotenoid produced. Maximum specific β-carotene productivity was observed to be 3.9 mg/g/h between 32 and 36 h, as compared to 3.17 mg/g/h, during 16 and 20 h in case of distilled water medium (Figure 5.2).

After 68 h, in seawater medium, total carotenoid content was found to be 86 ±4 mg/l wherein β-carotene, torulene and torularhodin were in 78, 18 and 2% (w/w) proportion, respectively. β-Carotene showed an increased proportion in seawater medium than the control run in distilled water medium, where, the proportion of β-carotene, torulene and torularhodin was observed to be 48, 43 and 9% (w/w), respectively. This might be because of active cyclization of γ-carotene to β-carotene promoted by metal salts as discussed earlier when the effect of metal salts was investigated.

Thus, mutant 32 can be grown in seawater as an alternative water source for production of carotenoids on industrial scale using mutant 32. In addition to that, use of seawater can lead to increase in β-carotene content as well as the proportion.

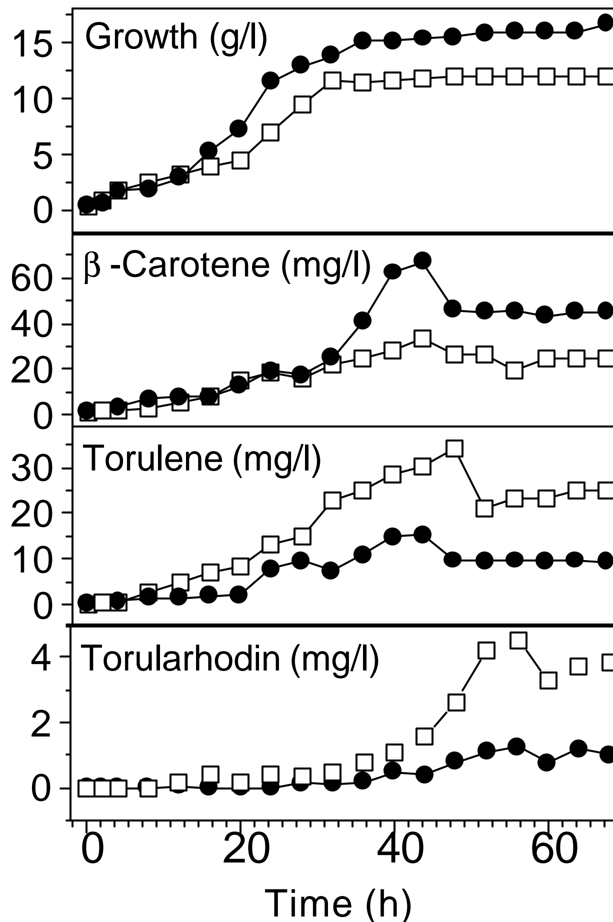

Figure 5.1 Growth and carotenoid production of *Rhodotorula glutinis* mutant 32 grown in seawater (•) and distilled water ( ) at pH 6.0 in a 1L fermenter.

Effect on extraction of carotenoids

It was observed that when mutant 32 was grown in seawater medium, the pigments could be easily extracted in the solvents without any mechanical disruption. However, such property was not observed when the yeast was grown in distilled water medium. Extraction of the pigments was found to be dependent on pH of growth medium also, because, maximum extraction (83 ±5%) was observed when the mutant was grown at pH 6.0 (Figure 5.2).

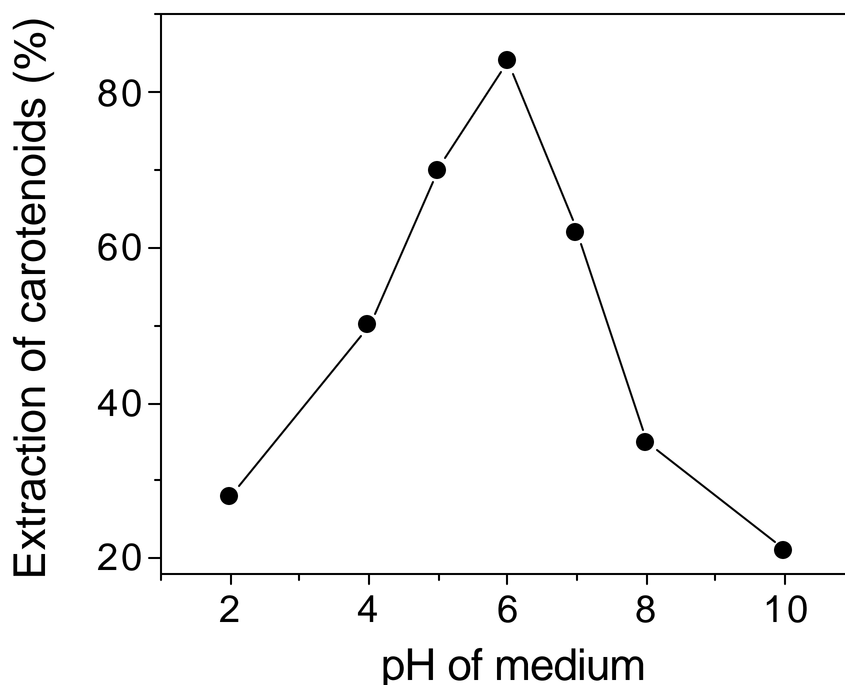

Figure 5.2 Effect of initial pH of growth medium on extraction of carotenoids from *Rhodotorula glutinis* mutant 32 in HPLC mobile phase. Percentage extraction is expressed as that compared to the pigments extracted from cells subjected to mechanical destruction as mentioned in the text.

There was no selectivity observed in extraction for a particular pigment (Figure 5.3). At pH 6.0, HPLC mobile phase (80 ±5%) and acetone (83 ±5%) were found to be the better solvents for extraction of pigments as compared to hexane (62 ±5%) and ethanol (59 ±5%).

Studies on effect of individual components on carotenoid extraction indicated that these metal salts probably had an effect on the cell composition and that led to easy extraction of carotenoids (Table 5.2).

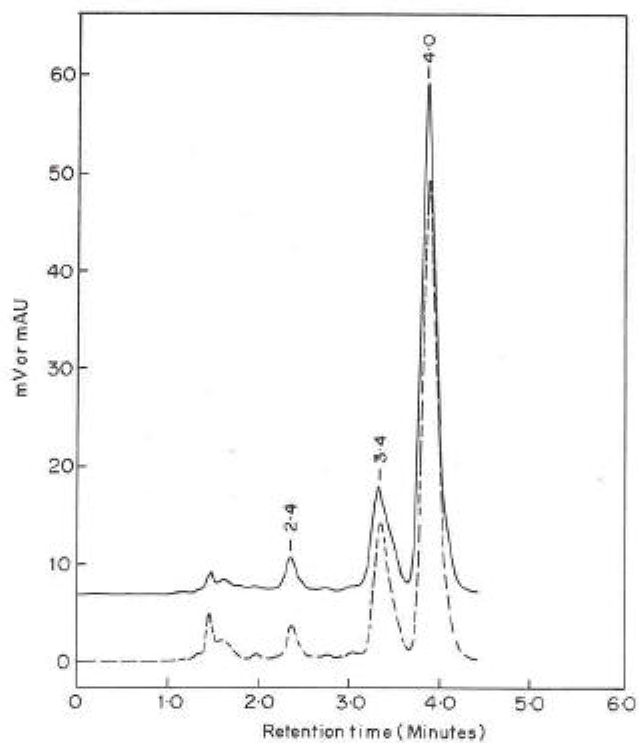

Figure 5.3. HPLC chromatogram of cell crushed with (—) and with out mechanical disintegration (---). Torularhodin, torulene and β-carotene eluted at 2.4,3.4 and 4.0 minutes, respectively.

Table 5.2 Effect of seawater components on extraction of carotenoids.

Salt	Dry cell weight ±0.5 g/l	Total carotenoids ±4 mg/l	Extraction # % ±4
$CaCl_2 . 2H_2O$	11.3	41	25
KCl	11.1	32	86
H_3BO_4	11.1	39	62
$MgCl_2 .2H_2O$	13.1	37	42
NaCl	11.2	38	8
Na_4EDTA	9.3	41	60
$NaHCO_3$	13.2	37	66
Na_2SO_4	12.1	33	89
Seawater control	11.4	40	75
Mineral control♣	3.2	7.9	86
Distilled water medium control∂	13.6	35	---

The percentage extraction is compared with that extracted by crushing in mini bead beater.
♣ Minerals present in seawater.
∂ Basal medium prepared in distilled water.

The parent culture, *Rhodotorula glutinis* NCIM 3353, also showed increase in β-carotene proportion by 30% (w/v) and ease of pigment extraction in seawater medium. Thus, the increase in β-carotene content and easy extractability was not a property of mutant 32 alone.

Conclusion

In any fermentation industry, use of pure, demineralized water becomes a prerequisite. Considerable inputs in the form of chemicals, equipment, energy and manpower are required for getting a source of good quality water, suitable for fermentation. Hyper producing *Rhodotorula glutinis* mutant 32 could be conveniently grown in the medium prepared in relatively cheap, seawater. Increase in β-carotene content and proportion can be desirable a trait from industrial point of view. In addition to that, the ease of carotenoid extraction due to cultivation in seawater at pH 6.0 can make extraction process “one-step” and cost effective. It will avoid number of unit operations involved in

the extraction of carotenoids from *Rhodotorula* in the scheme given by earlier workers (Hari *et al.* 1992). With these properties, mutant 32 of *Rhodotorula glutinis* can probably compete with carotenoid producing alga, *Dunaliella salina*.

Reference

References are listed in **chapter10.**

Chapter 6

Use of microfiltered broth as a water source for carotenoid production from *Rhodotorula glutinis* mutant 32

Summary

Mutant 32 was grown in the basal medium as described in chapter 2, for 96 h, at 28 °C. The culture broth was microfiltered using ceramic membrane of 0.45 μm pore diameter. 80% (v/v) of the microfiltered broth containing unused nutrients and extracellular products was replenished with carbon and nitrogen source and used for growth of mutant 32. This process of recycling was performed 15 times, consecutively. It was found that the reuse of microfiltered broth gradually enhanced β-carotene content (mg/l, mg/g) and proportion (%) with simultaneous decrease in torulene content and its proportion.

Introduction

Over the years, single cell production (SCP) was a favourite topic among fermentation researchers and industries. Asporogenous yeast, *Rhodotorula glutinis*, is a recent addition to that. *Rhodotorula* has drawn interest as feed and fodder additive in livestock, poultry and fish industries because of its well-known, characteristic carotenoids (Eugenia *et al.* 1996, Eugenia *et al.* 1997, Naidu *et al.* 1999). However, during such fermentation process, varying amounts of waste materials are produced. The liquid effluent, which contains unconsumed inorganic and organic media components, as well as extracellular products, can have large pollution load. Disposal of such wastewater becomes a challenge to the biotechnology industries.

Innovative techniques, like membrane microfiltration, are playing a major role in wastewater recycling that enable reuse of water. Microfiltration is also now proved to be cost effective and affordable (Fane 1996, Colin and Brian 1998). An attempt was done to reuse the fermented broth, after microfiltration, for carotenoid and cell mass production from mutant 32 and is described in the following chapter.

Materials and methods

Microorganism and medium

Rhodotorula glutinis mutant 32 *was* grown in the basal medium described in chapter 2. Flasks were incubated at 28 °C ±2 on a rotary shaker at 250 rpm for 96 h. All the experiments were done in triplicate.

Microfiltration

At the end of the fermentation, the culture broth was subjected to cross-flow microfiltration (Figure 6.1) using a ceramic microfiltration membrane with 0.45 µm pore diameter.

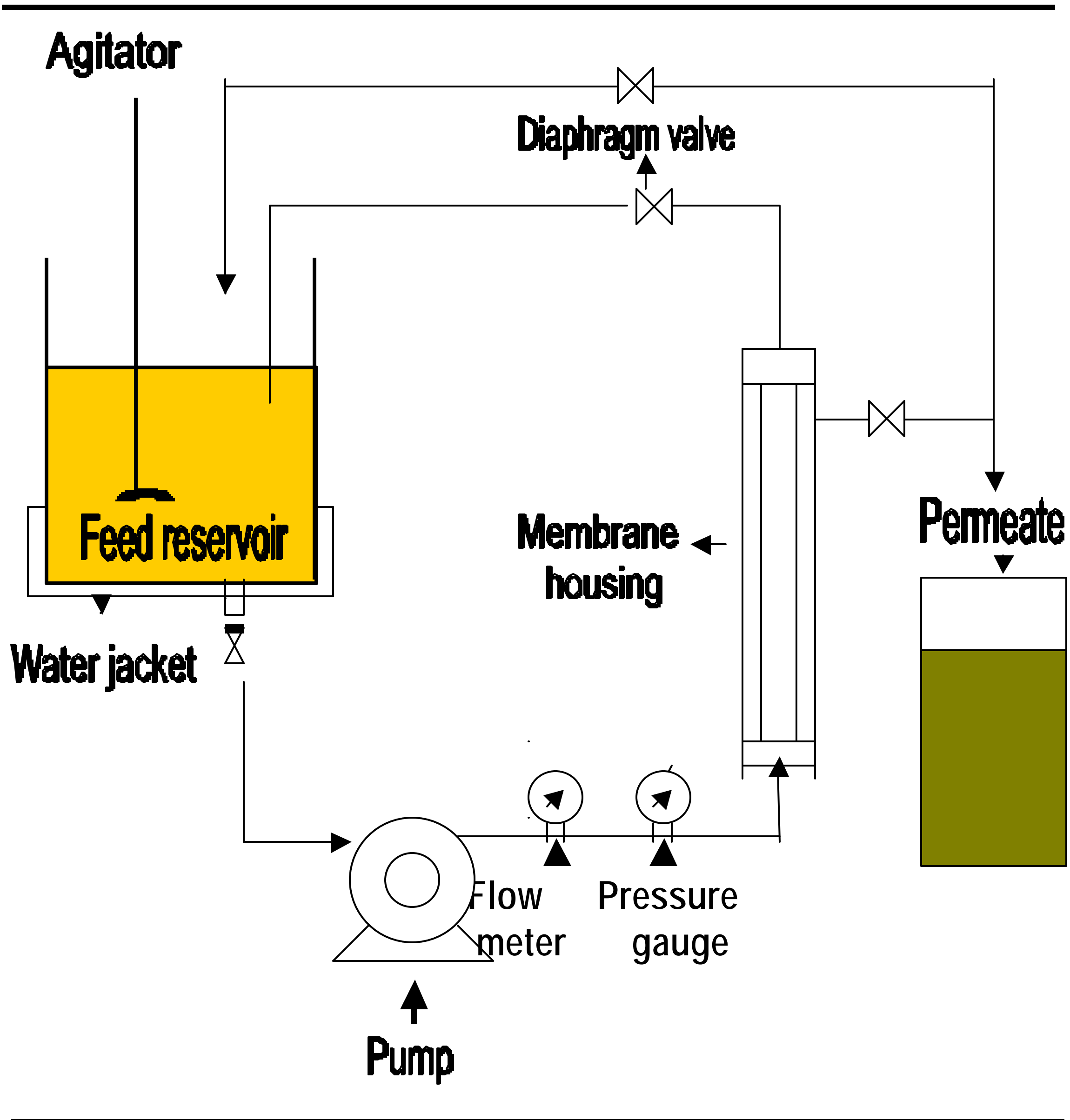

Figure 6.1 Microfiltration assembly used in the experimentation.

Trans-membrane pressure was maintained at 0.5 kg/cm^2. Flux measurements were made by collecting aliquots of permeate over a fixed time interval. Flux was monitored until a constant level was reached that defined the steady state. Cell mass concentrated

in the retentate was spray dried using Buchi's mini spray drier (as described in chapter 7).

Results and discussion

Microfiltration of fermented broth enabled recovery of 80% (v/v) of the broth, which was used as a water source in the subsequent growth cycles after supplementation of other nutrients (Figure 6.2). Mutant 32 when grown in the shake flask for 96 h, there was no residual glucose at the end of incubation.

Total carotenoid content (mg/l) and β-carotene proportion (%, w/w) was found to be increase with concurrent decrease in torulene proportion with each run, which might be the effect of extracellular products accumulating in the culture broth (Table 6.1).

Many fermentation processes become vulnerable to cost calculation because of relatively large water requirement per unit volume of the product and subsequent problem of treatment and safe disposal of the waste water. There is now widespread interest in reducing overall consumption of the precious fresh water. Recycling of wastewater was an integral stage of large-scale SCP processes developed during the 1970s to minimize water consumption and to reduce effluent treatment costs (Ashley and Rodgers 1986). There are a few reports on *Rhodotorula* being grown on wastewater (Fustier and Simard 1976, Shih and Hang 1996). Membrane microfiltration is proved to be cost effective and affordable and enabled reuse of wastewater (Fane 1996, Colin and Brian 1998). Thus, It is worth using membrane technology as a tool for reuse of fermented broth for carotenoid production by using mutant 32.

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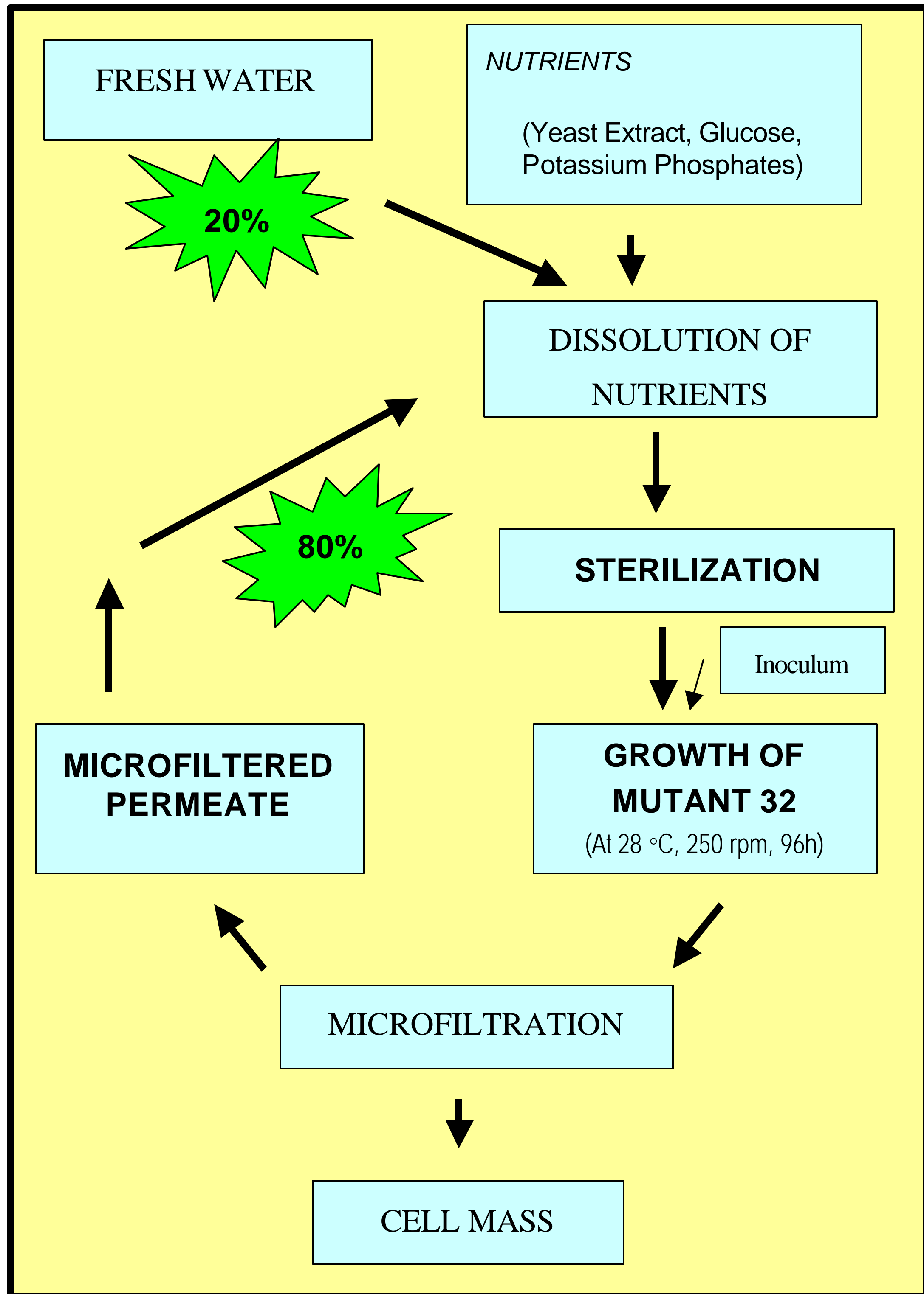

Figure 6.2 Flow sheet showing reuse of microfiltered broth.

Table 6.1. Reuse of microfiltered broth for carotenoid production.

Use no	Dry cell weight ± 0.5 g/L	***Total carotenoids*** ±0.8 mg/l	±0.1mg/g	Proportion β-carotene:torulene:torularhodin (%, w/w)		
1	12	33.0	2.9	69	27	4
2	12.3	36.6	2.9	68	27	5
3	13.7	39.0	2.8	69	25	6
4	17.2	35.7	2.1	71	26	7
5	18.0	37.2	2.0	73	20	7
6	18.3	44.8	2.5	79	18	3
7	18.4	52.4	2.8	84	11	5
8	17.2	54.0	3.2	86	9	5
9	17.1	58.1	3.4	89	8	3
10	18.3	63.5	3.5	89	9	2
11	17.7	67.0	3.8	89	10	1
12	17.8	76.6	4.3	89	10	1
13	17.3	81.7	4.7	90	9	1
14	17.4	78.9	4.5	90	8	2
15	18.0	77.9	4.3	90	8	2
16	17.8	78.9	4.4	90	7	3

Conclusion

Rhodotorula glutinis mutant 32 was studied for carotenoid production in a medium prepared in 80% (v/v) microfiltered culture broth. Based on this study, the incorporation of microfiltration unit to the carotenoid production unit, employing mutant 32, can be suggested for effective reuse of fermented broth, which otherwise is not only a waste but also source of pollution. The enhanced carotenoid production and the cell mass from mutant 32 are additional benefits from the industrial point of view.

Reference

References are listed in **chapter 10.**

Chapter 7

Carotenoid containing dry cell preparation from *Rhodotorula glutinis* mutant 32

Summary

Rhodotorula glutinis mutant 32 was grown in 14 L stirred tank (ST) fermenter in the basal medium. The volumetric carotenoid production, cellular accumulation and dry cell mass of the yeast mutant was found to be 74 ±4 mg/l, 5.8 ±0.05 mg/g and 12.8 ±0.2 g/l, respectively. The cell mass from ST fermenter was concentrated ten-fold by cross-flow microfiltration using tubular ceramic membrane and then spray dried at different inlet nozzle temperature between 40 and 200 °C in the presence of antioxidants like butylated hydroxyl toluene (BHT) and D-tocopherol. In the presence of BHT, 87 ±7% carotenoids were recovered at all the inlet temperatures studied. A two level, three variable, factorial experiment was performed using inlet nozzle temperature (°C), feed concentration (%, w/v) and antioxidant concentration (mg/l) as three different variables. Residual β-carotene content in the spray dried powder (µg/g) was used for calculation of the effect of each factor. Best recoveries were obtained when 160 °C, 11.6% cell mass and 1000 mg/l BHT concentration were used for spray drying. The dried product obtained by optimized method was stable in dark and cold condition over 10 weeks. In presence of BHT, under light conditions, the rate of degradation was much slower than the control.

Introduction

Carotenoid containing preparations from microbial sources (Desobry *et al.* 1997, Yamaguchi 1997, Leach 1998, Leach 1998a, Orset *et al.* 1999) and non-microbial sources (Gavez and Fernandez 1997, Chen and Tang 1998) are increasingly becoming popular because of their widespread utilities. *Rhodotorula spp.* are also receiving attention after being investigated for their nutritional, chemopreventive and radiopreventive properties (Bhuyan *et al.* 1985, Ershov *et al.* 1992, Eugenia *et al.* 1996, Eugenia *et al.* 1997, Kassim *et al.* 1997, Zalaskho *et al.* 1997, Naidu *et al.* 1999). *Rhodotorula* owes these properties to its characteristics carotenoids.

Carotenoids, in general, are chemically very sensitive to heat, light and oxygen (Scita 1992). Their biochemical activity and physical stability is important for the commercial utility of products derived from them. Drying and encapsulation are two common practices followed to increase the shelf life of carotenoid-containing products (Wagner and Warthesen 1995, Desobry *et al.* 1997, Chen and Tang 1998, Leach *et al.* 1998, Xuan *et al.* 1998).

However, encapsulation is reported to pose problems in extraction of carotenoids and results in oxidative destruction during processing (Orset *et al.* 1999). Spray drying and freeze drying are commonly employed drying methods. But, freeze drying results in a highly hygroscopic powder and is also reported to be non economic as compared to spray drying (Orset *et al.* 1999, Desobry *et al.* 1997).

Spray drying of carotenoids normally results in free flowing powders and the use of antioxidants during drying of carotenoids is reported to reduce the oxidative degradation caused by heat during spray drying (Barimalaa and Gordon 1988, Spencer 1989). The protective effects of antioxidants on natural carotenoid preparation from *Rhodotorula glutinis* have not yet been reported. Results of experiments done with the following objectives are described in this chapter.

a) Suitability of membrane microfiltration for concentration of cell mass grown in 14 L stirred tank fermenter,

b) Spray drying of concentrated cell mass in presence of exogenous antioxidants and

c) Stability of dried preparation under different light and temperature.

Materials and methods

Microorganism and media

Rhodotorula glutinis Mutant 32 was grown in basal medium as described earlier in chapter 2.

Fermentation

Inoculum for Stirred Tank (ST) fermenter (Labroferm, New Brunswick Scientific Co. USA) was prepared as detailed earlier in chapter 3. Fermenter was run for 96 h at 28 ^oC.

Microfiltration

At the end of the fermentation, the cell mass was concentrated by cross-flow microfiltration using a tubular ceramic microfiltration membrane (Orelis, France), 0.45 micron pore size and effective filtration area of 0.125 m^2. Trans-membrane pressure was maintained 0.5 kg/cm^2. Length of the membrane was 120 cm (diameter 20 mm). Flux measurements were made by collecting aliquots of permeate over a fixed time interval. Flux was monitored until a constant level was reached that defined the steady-state. The broth volume (9.5 l) was reduced to one tenth of original.

Spray drying

Concentration of cell mass in feed suspension was determined by drying yeast cell suspension washed with distilled water, centrifuged and dried to constant weight at 80 °C, in duplicates. Spray drying was performed using a Buchi mini spray dryer (Model 190). Concentrated cell suspensions were atomized using a nozzle (0.5 mm) with the help of compressed air. The flow rate of air was controlled at 600 l/h. The cell suspension was pumped into the nozzle using a peristaltic pump at a suitable rate (4 ±2 ml/min) in order to maintain the desired outlet temperature.

Initial studies were done using different concentrations of feed (1-20%, w/v) along with varying nozzle temperatures (40 to 200 °C) in the presence of synthetic (BHT) and natural antioxidant (D-tocopherol). Studies on protective effects of antioxidants during spray drying were done by mixing antioxidants (dissolved in 1ml ethanol) butylated hydroxyl toluene (BHT, S.D fine Chemicals) and alpha D-tocopherol (Loba chemicals, India), at 1000 mg/l concentration with cell suspension prior to spray drying.

Factorial design

A two-level, three variable factorial experiment for optimization of conditions for spray drying with respect to maximum pigment (β-carotene) retention was performed using nozzle temperature (160 °C), feed concentration (10%, w/v) and antioxidant concentration (1000 mg/l) as three variables at centroid (Box *et al.* 1978). The effects and further steps were determined as described in chapter 3.

Moisture estimation

For estimation of residual moisture in the spray dried cell mass, accurately weighed samples of spray dried cell preparations were taken to constant weight at 80 °C.

Elemental microanalysis

The dried and moisture-free cell mass was subjected to elemental microanalysis (Carbon, hydrogen, nitrogen) using automated elemental analyzer (Model CHNS-O EA 1108 –Elemental analyzer, Carlo Erba).

Viability determination

For determination of cell viability, spray dried samples were immediately transferred aseptically to sterile sample tubes. Ten mg dry cell mass was uniformly suspended, serially diluted in sterile physiological saline and plated on yeast extract malt extract

agar. A sample of culture broth before microfiltration was also serially diluted and plated. Plates were incubated at 28 °C for 48 h and colony forming units (CFU) were calculated.

Carotenoid stability studies

Five hundred mg aliquots of dry cell preparation, spray dried at optimized conditions, were placed in 50 ml screw capped glass tubes (diameter 1.5 cm, thickness 1mm) to check the effect of exposure of lght. The cell mass was spread inside the tubes uniformly and then continuously exposed to 40 W white fluorescent light from a distance of 20 cm, at 30 °C. Intensity of light at the surface of the glass tube was 1000 Lx.

For comparison, identical sets were stored in dark at 30 °C and at 4 °C. Fifteen mg aliquots of samples were withdrawn from each tube at 7 day interval over a period of ten weeks, carotenoids were extracted and quantified. The residual carotenoid content of dried powders was expressed as a percentage of the original carotenoids in the powder, analyzed immediately after the spray drying.

Determination of storage stability of the purified β-carotene (30 mg) was done under identical conditions, in glass tubes, in triplicates. Samples were withdrawn at every 4 h, Measurements were continued till complete degradation of the β-carotene was observed and is expressed in terms of percentage.

Carotenoid content was analysed using HPLC. Carotenoid content in dry cell preparation was expressed in μg/g because at many places, the torulene and torularhodin content were too less to express accurately in mg/g.

Results and discussion

Cell growth and microfiltration

When mutant 32 was grown in stirred tank (ST) fermenter for 96 h, maximum specific growth rate, sugar utilization rate and maximum biomass achieved was μ_{max} 1.0 h^{-1}, 0.67 ±0.1g/l/h and 12.8 ±0.2 g/l, respectively. Total volumetric carotenoid production was 74.52 ±2 mg/l. Cellular accumulation of carotenoids was found to be 5821 ±50 µg/g.

β-Carotene content was found to be 53.65 mg/l (4191 ±50 µg/g). β-Carotene content in the yeast cell mass was much higher than the recently reported dried preparations from carrot pulp (Chen and Tang 1998) and was comparable with algal preparation (Leach *et al.* 1998). β-Carotene, torulene and torularhodin proportions were found to be 72 ±2%, 20 ±2% and 8 ±2% (w/w), respectively.

Microfiltration was found to be very effective for concentration of *Rhodotorula glutinis* mutant 32 cell mass. Initial permeate flux observed was 300 liters per square meter, per hour (LMH) which eventually fell down to 145 LMH. The decline in the flux was presumably due to the formation of a layer of the rejected yeast cells on the membrane surface. The overall processing time required for 10 l broth was 27 minutes with the selected ceramic membrane cartridge to obtain a ten-fold concentration of cells.

There was no cell inactivation observed during membrane microfiltration of mutant *32* (observed microscopically by methylene blue staining). The entire cell mass could be concentrated into the retentate. This was an advantage over earlier reports on microfiltration of carotenoid producing algae, *Dunaliella,* which got inactivated owing to their fragile nature (Leach *et al.* 1998, Orset *et al.* 1999).

The membrane concentration is superior to the conventional techniques like centrifugation in terms of power requirement and ease of the operation (Sethi and Wiesner 1999). It also can minimize the time and energy required for subsequent processing of the cell suspensions. Fouling of the membranes was minimal and the membranes could be cleaned easily with routine, recommended washing procedures (Redker and Davies 1993).

Spray drying

It has been well established that solids content of the feed and inlet temperature have to be carefully controlled during spray drying for better recoveries of the product (Bhandari et al. 1992). In the present studies the emphasis was paid to these two factors along with the use of antioxidants.

Effect of solids content in cell suspension on spray drying

Recoveries of carotenoids and yeast powder were studied during spray drying at various cell concentrations (between 1 and 20% (w/v) at 160°C).

Lower cell concentration (1 and 5%, w/v) resulted in moist powder, while on the other hand, higher cell concentration (≥10%) resulted in loss of yeast powder quantity (Table 7.1). Both of which are not suitable from industrial point of view. The results were similar to Maa *et al.* (1998) wherein spray dried powder recovery was stated to be inversely proportional to the solid content of the feed solution used for spray drying.

Table 7.1 Effect of cell mass concentration in feed suspension (%, w/v) on yeast powder and carotenoid recovery at 140 °C from mutant 32.

Feed concentration %, (w/v)	Moisture % (w/w)	Viability (%)	Yeast Powder recovery (%)	Total Carotenoid recovery (%)	Total carotenoids (µg/g) ± 25		
					β-Carotene	Torulene	Torularhodin
Control	---	100*	100	100	4191	1164	466
1	10 ±1	21	96 ±1	86 ±1	3763	789	445
5	3.0 ±0.2	5	89 ±2	95 ±1	3925	1169	406
10	0.1 ±0.1	0.4	86 ±2	98 ±1	4125	1140	440
15	0.1 ±0.1	0.1	72 ±4	94 ±1	4000	1045	439
20	-	0.2	65 ±3	95 ±1	3965	1100	440

∗ 100% viability is equivalent to 73×10^5 CFU/g

Effect of spray drier nozzle temperature

With the increase in the nozzle temperature, a decrease in carotenoid recovery was observed. At 200 °C, carotenoid recovery was less than 50% of the original. However, yeast powder recovery was only marginally affected.

As expected, the viability and carotenoid content of the cells decreased with increase in the nozzle temperature (Table 7.2). Lower nozzle temperature resulted in a product with higher moisture content. Viability and moisture can alter the residual carotenoid proportion and further decrease the shelf life of the product (Maa *et al.* 1998).

Elemental microanalysis of the spray dried yeast powder showed that carbon, hydrogen and nitrogen content was 51.8, 4.7 and 7.8 % (w/w) of dry mass, respectively. The spray drying temperature did not affect the CHN content in the temperature range studied and thus, there was no gross modification in chemical composition of the yeast cells during spray drying.

Table 7.2 Effect of spray dryer nozzle temperatures on carotenoid recovery from mutant 32.

Temperature (°C) ±2		Total carotenoid Recovery (%)	Yeast Powder recovery (%) ±2	Moisture (%) ±2	Viability (%) ±2	Total carotenoids (µg/g)		
Inlet	Outlet					β-carotene	torulene	torularhodin
CONTROL		**100**	**100**	**100**	**100***	**4191**	**1164**	**466**
40	33	69	93	20	80	3604	594	327
60	43	65	92	18	50	3436	509	236
80	54	65	94	10	32	3352	456	314
100	61	62	97	9	13	3101	442	252
120	71	53	98	-	5.0	2640	435	190
140	72	54	98	-	0.9	2682	423	85
160	73	53	94	-	0.3	2473	385	54
180	82	54	93	-	0.3	2514	384	52
200	84	44	89	-	-	1970	323	40

∗ 100% viability is equivalent to 94×10^5 CFU/g

Effect of antioxidants

At higher nozzle temperature (≥ 120°C), BHT was found to be effective in prevention of carotenoid degradation (Table 7.3). It was observed that torulene and torularhodin were mainly affected. The mechanism for this degradation is not understood but probably can be related to the location of the carotenoids inside the cell. D-tocopherol also offered

protection but the degree of protection was lesser than BHT. This was in contrast to earlier studies on algal dried mass where both D-tocopherol and BHT failed to offer protection owing to alteration in characteristics in feed solution (Orset *et al.* 1999).

Table 7.3 Effect of antioxidants on carotenoid recoveries from *Rhodotorula glutinis* mutant 32.

Temperature (°C) ±2 Inlet	Temperature (°C) ±2 Outlet	Total carotenoid Recovery (%) ±2	Yeast Powder recovery (%) ±2	Moisture (%,w/w) ±2	Viability (%) ±2	Total carotenoids (µg/g) β-carotene	torulene	torularhodin
A. Butylated hydroxy toluene								
CONTROL		**100**	**100**	**100**	**100***	**4216**	**1126**	**459**
40	33	95	92	21	73	4047	1000	450
60	43	96	90	16	62	4132	1000	444
80	54	94	93	11	35	4089	913	432
100	61	91	94	8	8	4047	813	421
120	71	90	96	3	3	4213	754	259
140	72	81	97	-	0.2	3963	623	127
160	73	81	94	-	0.2	4047	508	109
180	82	80	91	-	0.1	4047	483	67
200	84	80	88	-	-	4047	463	55
B. D-Tocopherol								
40	33	90	91	17	78	3794	1000	452
60	43	90	90	15	47	3836	988	387
80	54	88	90	9	36	3878	785	359
100	61	82	87	8	16	3836	684	254
120	71	82	85	5	7	3921	652	213
140	72	75	78	-	0.8	3542	647	195
160	73	69	74	-	0.3	3288	632	123
180	82	66	73	-	0.3	3120	634	47
200	84	61	72	-	-	2951	541	45

∗ 100% viability is equivalent to 1×10^{6} CFU/g.

Factorial design

Factorial experiment was designed using feed concentration (%, w/v), nozzle temperature (°C) and antioxidant concentration (mg/l) as three variables to achieve moisture free, non-viable preparation. Results indicated that Interactive effect between three selected factors (E_{fna}) was considerable (Table 7.4). It was observed that feed concentration is the major factor determining recovery of β-carotene (µg/g) and yeast powder during spray drying (Table 7.4). Antioxidant concentration and nozzle temperature had negative effect on β-carotene recovery. Subsequent experiments were done by increasing the feed concentration and keeping other two factors constant. The step size for increment was determined using the method described earlier (Davies 1993).

Best β-carotene recovery was obtained when nozzle temperature, feed concentration and antioxidant concentration was 160 °C, 11.6% and 1000 mg/l, respectively. The optimized conditions were effective in quick drying with minimal possible damage to the delicate carotenoids (98% recovery) within the cells and cell components of yeast with maximum dry yeast cell mass (97%) recovery. The spray drying of membrane concentrated suspension of *Rhodotorula glutinis* mutant 32 under optimized conditions resulted in a free flowing, dry, light yellow coloured powder.

Table 7.4 Results of factorial experiment.

Experiment No	Feed (%)	Inlet temperature (°C)	BHT mg/l	β-Carotene (µg/g) ± 20	Effect (E)
CENTROID	**10**	**160**	**1000**	**4400**	**--**
1	5	120	500	3520	E_0: 3.6
2	15	120	500	4060	E_f: 0.16
3	5	200	500	3930	E_n: -0.15
4	15	200	500	3190	E_{fn}: 0.05
5	5	120	1500	3600	E_a: -1.11
6	15	120	1500	3280	E_{fa}: 0.26
7	5	200	1500	2780	E_{na}: 0.07
8	15	200	1500	3930	E_{fna}: 0.69

Storage stability studies

Stability studies performed with purified β-carotene and spray dried yeast indicated that the carotenoids in dried yeast preparation were more stable than the purified β-carotene under illuminated conditions.

Purified β-carotene

Visibly, β-carotene is light orange in colour. Upon exposure to light, its color disappeared with the advancement of time. Under illuminated condition at 30°C, β-carotene was completely degraded within 64 h as compared to 128 h in the presence of BHT (Figure 7.1).

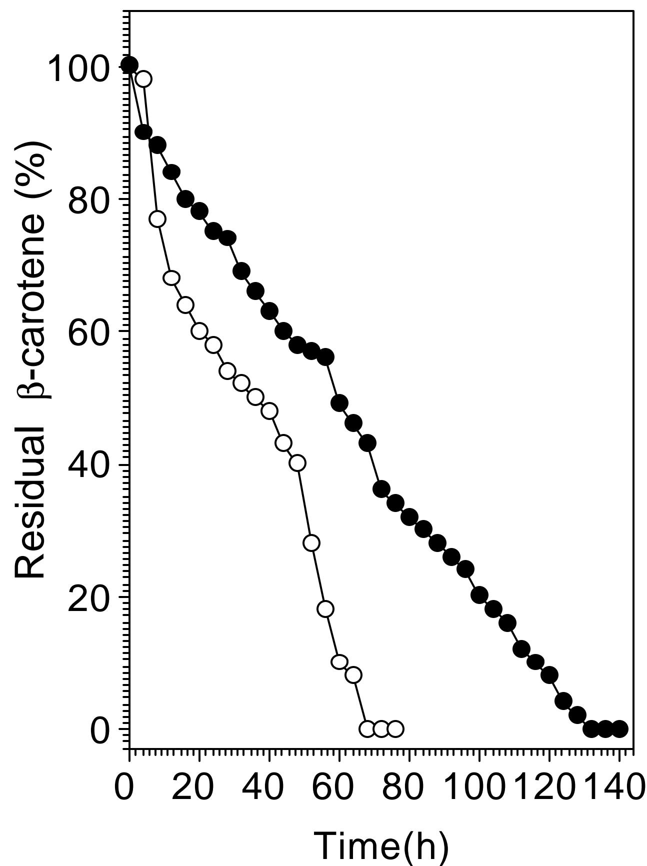

Figure 7.1 Degradation profile of β-carotene with (•) and with out (O) BHT under illumination (1000Lx).

An attempt was also made to qualitatively analyze the products from degraded carotenoids. The spectral scanning showed the appearance of UV-detectable, colourless products with wavelength maxima between 255 and 275 with, concomitant decrease in the β-carotene peak areas in the visible range.

Dried yeast

Stability of carotenoids in spray dried yeast cell preparations obtained from optimized process with and without BHT was also investigated under various storage conditions of light and temperatures. As expected, the carotenoids in cell preparations stored under dark and cold were relatively more stable as compared to those in illuminated conditions (Figure 7.2).

In the presence of BHT, the initial degradation rate was low for 5 weeks. However, the degradation profiles were similar later (Figure 7.2). The observed stability of carotenoids in the dried yeast cell mass was much better than the non-encapsulated powder of *Dunaliella*, that reported 90% degradation of the original carotene level within seven days of storage (Leach *et al.* 1998). The results were comparable with another report wherein β-carotene retention was 30 ±2% after 12 weeks (Desobry *et al.* 1997).

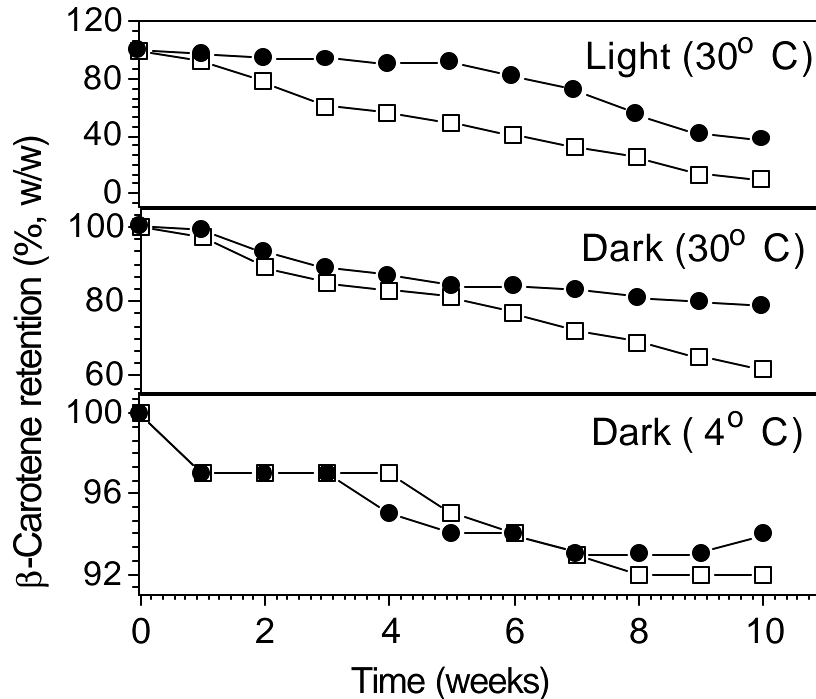

Figure 7.2 Stability of β-carotene in spray dried mutant 32 powder at different light and temperature conditions in the presence (●) and absence (ð) of BHT.

Conclusion

It is desirable that a specialized feed preparation for cattle, poultry, fish as well as other applications can be stored at room temperature. A preparation of nonviable, completely dry yeast cell mass, prepared by optimized method of spray drying was found to be reasonably stable at room temperature even in the absence of antioxidants. The process of carotenoid-rich cell mass preparation would be affordable to the industry as it avoids costs involved in extraction, purification and further stabilization of the pigments by encapsulation. Carotenoid loss during spray drying and storage can be prevented by using appropriate conditions of storage, which can increase the chances for commercialization of the process. These studies can help to promote *Rhodotorula glutinis* mutant 32 as an alternative carotenoid source, especially as animal feed.

References

References are listed in **chapter 10**

<u>Chapter 8</u>

Protective effects of spray dried *Rhodotorula glutinis* NCIM 3353 on the development of hepatic preneoplastic lesions in rats.

This work was carried out in collaboration with Dr K.V.K. Rao, Head, Carcinogenesis Division, Cancer Research Institute, Tata Memorial Centre, Parel, Mumbai - 400012, INDIA.

Summary

Rhodotorula glutinis NCIM 3353 (RG) was grown in 200 L stirred tank fermenter. Fermented broth was subsequently concentrated by microfiltration and spray dried at 140 °C. Chemopreventive and anticarcinogenic effects of spray dried RG on the development of preneoplastic lesions during N-nitrosodiethylamine (DEN) induced hepatocarcinogenesis in female Wistar strain rats was studied. The chemopreventive and anticarcinogenic effects of RG were monitored on the basis of weight of the animals, morphological appearance of the livers, liver weight profile, histological pattern and PCNA immunohistochemistry using PC-10 antibody. RG showed significant effect on the prevention of liver tumour development in these protocols tested. However, RG effects were relatively more significant in groups where RG was administered after DEN treatment suggesting that RG is quite effective in the prevention of liver tumour development indicating possible action at the level of inducing differentiation.

Introduction

Anticarcinogenic and chemopreventive actions of carotenoid-rich food were reported initially by Peto *et al.* (1981). Extensive research was carried out on animal models (Lee *et al.* 1999) as well as on human beings (Henneckens 1997) to establish the utility of such diets, subsequently. However, only fruits and vegetables rich in carotenoids were used for such studies. There are scanty reports (Hiroshi *et al.* 1993) on use of microbial sources of carotenoids for anticancer studies.

N-nitrosodiethylamine (DEN), a known heptatocarcinogen, causes perturbations in the nuclear enzymes involved in DNA repair/replication (Pashupathy and Bhattacharya 1999) and is normally used as a carcinogen in animal models, especially rats. In the previous reports, various antioxidants and vitamins were shown to offer protection against DEN-induced carcinogenesis (Pierre *et al.* 1997, Rizzi *et al.* 1998, Shamoan *et al.* 1998, Chuang *et al.* 2000) in animal models.

In the present work, chemopreventive and anticarcinogenic effects of feed additive preparation of spray dried yeast *Rhodotorula glutinis* NCIM 3353 (RG) containing carotenoids was studied using rat liver as a model system. Spray dried *Rhodotorula* cells were supplied in diet of Wistar (WR) strain rats. N-nitrosodiethylamine (DEN) was used as a carcinogen to induce preneoplastic lesions in liver.

Materials and methods

Chemicals

N-nitrosodiethylamine (DEN) was from Sigma Chemical Co. (St.Louis, MO, USA). PCNA (PC10) mouse monoclonal antibody from SantaCruz Biotechnology (USA), Anti-Mouse Ig (whole Ab) Biotinylated and Streptavidin-Biotinylated HRPO (complex) were purchased from Amersham (UK).

Microorganism and media

Rhodotorula glutinis NCIM 3353 was used in the studies. It was maintained on MGYP slopes and grown in the basal medium described in chapter 2.

Feed additive preparation

The yeast was grown in 14 L stirred tank fermenter (New Brunswick, USA) for 18 h as described in chapter 2 and was then used to inoculate 200 L (working volume 150 L) stirred tank fermenter. The stirred fermenter had two Rushton impellers with six blades each. The dissolved oxygen and pH probes were from Ingold, Switzerland. The aeration and agitation in fermenter were adjusted to maintain DO level above 30% air saturation.

At the end of fermentation, the broth was subjected to cross-flow microfiltration using a ceramic microfiltration cartridge, 0.45 μm pore diameter. Trans-membrane pressure was maintained at 1 kg/cm^2. The broth volume was reduced ten-fold. The concentrated cell suspension was spray dried using Buchi-190 spray dryer at spray drier nozzle temperature of 140°C. Antioxidants were not added during spray drying. Flow rate of the cell suspension was adjusted in order to maintain the inlet temperature. Airflow was maintained at 500 l/h. Spray dried samples were immediately transferred to sterile sample tubes and viability studied.

Carotenoid extraction

Accurately weighed 500 mg spray dried cell mass was suspended in 20 ml acetone and disintegrated for 15 sec in a Braun MSK cell homogenizer (Braun, Melsungen, Germany, Model number 01822) cooled by intermittent flushes with dry ice. The suspension was then centrifuged and the supernatant collected. It was further processed and dried as described in chapter 2. Extracted carotenoids were dried under vaccum and dissolved in mobile phase for HPLC analysis as described earlier in chapter 2.

Animals

A total of 48 female Wistar rats weighing approximately 150 g each from the Rat Colony of the Cancer Research Institute, were used for this study. The animals were 45 to 60 days old at the beginning of the experiment and were randomized and housed four per cage with rice husks for bedding. Food and water were provided *ad libitum*.

Experimental Design

The experimental protocol that was followed is shown in Figure 8.1. The animals were divided into six groups (8 animals per group).

Group 1 served as the untreated control. Groups 2, 4 and 5 were administered 200 mg/l DEN for a period of four weeks in M/Q drinking water as described earlier (Fernandes *et al.* 1991, Rao and Fernandes 1996, Fernandes and Rao 1994). After a period of 4 weeks, for Group 2, DEN was discontinued and animals were given plain tap water without DEN for 3 months. After 4 weeks, groups 3 and 4 were administered *Rhodotorula glutinis* at 5% in diet. Group 5 received *Rhodotorula* diet two weeks prior to DEN treatment and in continuation together with DEN treatment and after DEN treatment for 3 months. Group 6 received *Rhodotorula* diet similar to Group 5 but without DEN.

Body weights of the animals were recorded, once a week, during the period of experiment. Animals were sacrificed at the end of the experiment and the livers were washed with phosphate-buffered saline by perfusion through the portal vein to eliminate blood cells. The livers were then removed, weighed, photographed for morphology and further utilized for histopathology and PCNA immunostaining.

Immunohistochemical staining

Immunohistochemical analysis for PCNA was performed on formalin-fixed, paraffin-embedded tissue sections using Streptavidin Biotin complex method. Paraffin embedded tissue sections were deparaffinized in xylene, taken through the grades of alcohol and rehydrated in phosphate buffer saline (PBS) pH 7.5. Thereafter, the sections were microwaved in 10 mM citrate buffer saline (pH 6.0) twice for 5 min. each to expose the antigen (Antigen Retrieval Step). For PCNA immunostaining, normal sheep serum (3%) was used to suppress nonspecific binding. Tissue sections were incubated overnight at 4 °C with 1:50 diluted PC-10 mouse IgG_{2a} monoclonal antibody. The sections were then washed in PBS. Endogenous peroxidase was quenched by adding 3% hydrogen

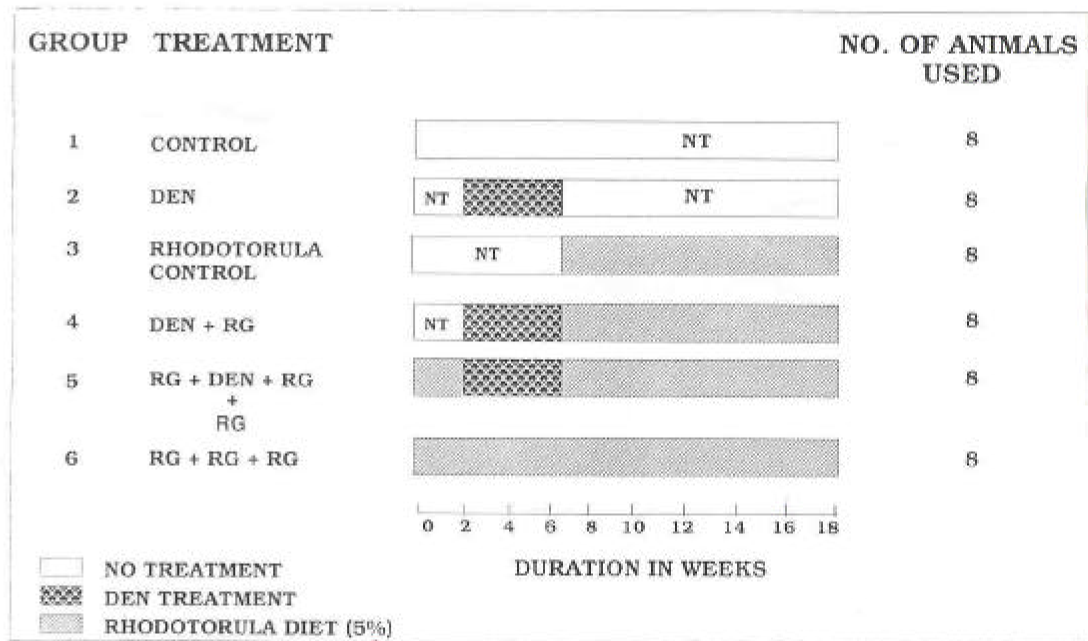

Figure 8.1 Experimental plan for studying the anticarcinogenic properties of spray dried *Rhodotorula glutinis* NCIM 3353.

peroxide in methanol to the sections for 30 min. followed by washing with PBS. A secondary antibody [Anti mouse Ig biotinylated species whole antibody (from sheep)] was applied at 1:50 dilution for 1 hour at 37 °C. This secondary antibody was biotinylated and recognized the primary mouse antibody. Excess secondary antibody was washed off with PBS, after which a 1:50 dilution of Streptavidin label conjugated with horse raddish peroxidase was applied to the sections for one hour at 37 °C. Excess label was rinsed off in PBS. 3,3'Diaminobenzidine was used as the chromogen and hematoxylin was used for counterstaining.

Statistical Analysis

Statistical analysis of the significance in the liver weight difference between different groups was performed using Student's 't' Test.

Results and discussion

Feed preparation

When the yeast *Rhodotorula glutinis* NCIM 3353 was grown in 200 L fermenter for feed preparation, it was observed that the carotenoid production reached its maximum in the stationary phase of growth. Yeast entered stationary phase at 18 h and beyond this major accumulation of carotenoids was observed. After 36 h there was no change seen in the volumetric production (mg/l) as well as cellular accumulation (mg/g) of carotenoids.

There was a linear increase in the carotenoid content and final volumetric production observed was 1.05 mg/l whereas cellular accumulation was 0.05 mg/g of dry cell weight. Fermented broth having a cell density of 20 ±1 g/l of cell dry weight was subjected to microfiltration. Initial permeate flow observed was 300 liters per square meter, per hour (LMH) which eventually fell down to 125 LMH when the cell mass was ten fold concentrated. The cell suspension was then spray dried at 140 °C. Two such fermenter sets yielded 4.7 kg dry cells powder, which was used for the experimentation. The stepwise photographs of complete process are shown in Figure 8.2.

Morphological appearance of the livers

The morphological appearance of the livers showed enhanced development of nodules and tumours in the animal group administered with plain DEN, as compared to the controls (Figure 8.3B). No visible changes were observed in the livers of the plain

Figure 8.2 Step wise feed preparation from *Rhodotorula glutinis* NCIM 3353 [A. Fermentation, B. Microfiltration and C. Spray drying].

controls (Figure 8.3A) and groups treated only with RG (Figure 8.3C, E) as well as in the group administered RG, following DEN treatment (Figure 8.3D). Continuous treatment with RG i.e. prior to and together with DEN also showed nodules and tumours but relatively less, compared to only DEN treated group (Figure 8.3F).

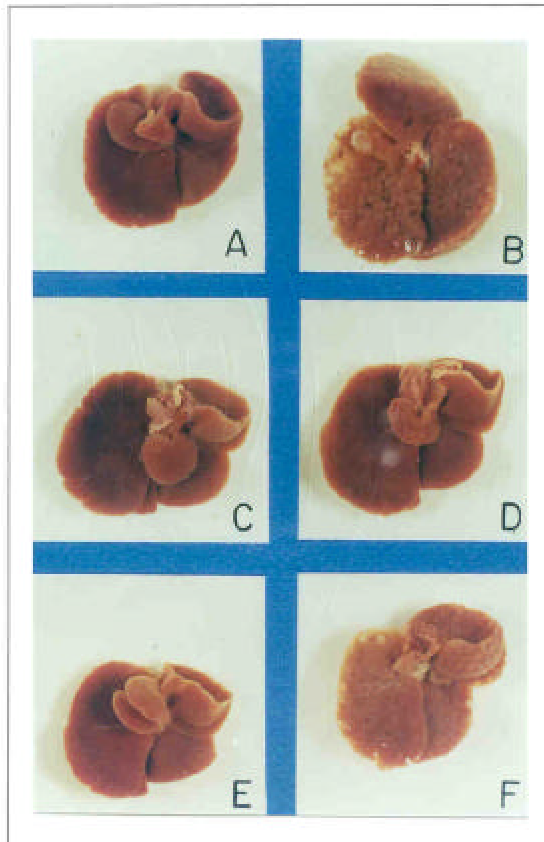

Figure 8.3 Morphological appearance of the livers of rat during tumour inhibition by *Rhodotorula glutinis* (RG) (A) Control, (B) DEN treated, (C) RG-Control, (D) DEN + RG (E) RG + RG + RG Control, (F) RG + (DEN+RG)+ RG treated.

The liver-to-body weight ratio was found to be increase in the group administered with only DEN. The increase in this ratio was relatively less in the group administered with RG prior to, together and after DEN treatment. In all other groups, either a decrease in the liver weight was noticed or there was no change in the liver weights (Table 8.1).

Table 8.1 Liver weight profile during modulation of DEN-induced hepatic preneoplasia by *Rhodotorula glutinis* NCIM 3353 (RG).

S No.	Group	Number of animals utilized	Average liver weight per 100 g body weight (mean ± SE)	Increase or decrease in liver wt (%).
1.	Control	8	2.74 ± 0.06	-
2.	DEN	6	3.40 ± 0.28	+ 24.08
3.	RG	8	2.35 ± 0.06	- 14.23
4.	DEN + RG	8	2.77 ± 0.15	+ 1.09
5.	RG + DEN + RG+RG	8	2.95 ± 0.23	+ 7.66
6.	RG + RG + RG	8	2.25 ± 0.04	- 17.88

Histological pattern

Histologically, all the control groups *viz*. plain controls, RG controls, RG continuous treatment controls and the group administered RG following DEN treatment, showed normal pattern (Figure 8.4 A, C, D, E).

The livers of DEN treated animals showed the development of large, eosinophilic focal areas of clear cell change with prominent, active nuclei and nucleoli together with mitotic cells (Figure 8.4B).

In the group treated with RG prior to, together with and after DEN treatment, focal eosinophilic changes with disturbed architecture were observed (Figure 8.4F). The results were similar to those obtained by Sinha *et al.* (1988) in Wistar albino rats.

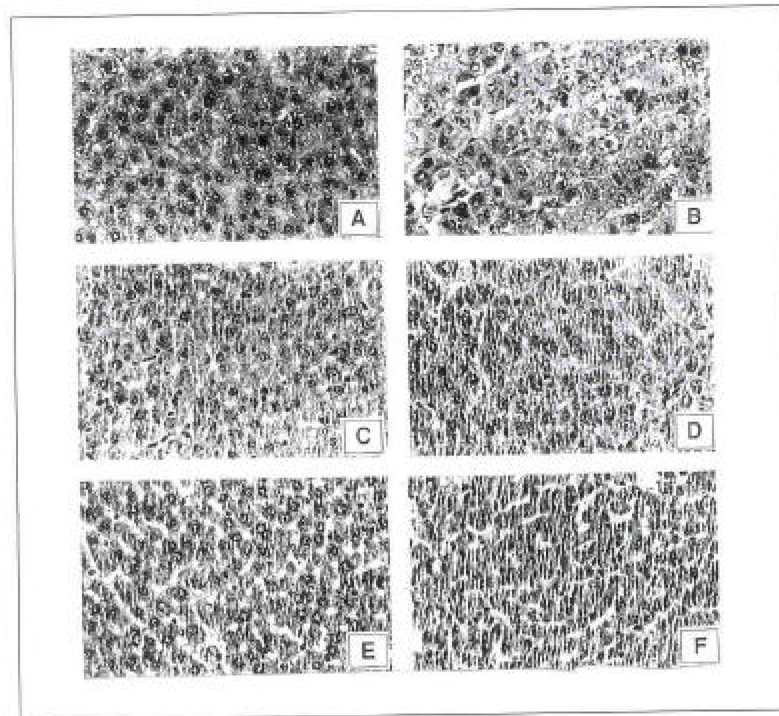

Figure 8.4 Histological pictures of the livers of rat during tumour inhibition by *Rhodotorula glutinis* (RG) (x 400). (A) Control, (B) DEN treated, (C) RG-Control, (D) DEN + RG, (E) RG + RG + RG Control, (F) RG + (DEN +RG)+ RG treated.

PCNA immunochemistry

The relationship between histopathological changes and cell proliferation pattern was studied using PCNA expression as a marker by immunohistochemistry in the adjoining sections. PCNA is a nonhistone nuclear protein, which enhances activity of DNA polymerase required for DNA replication and is expressed only in proliferating cells, but not in resting cells.

Previously Chuang *et al.* (2000) reported remarkable increase in PCNA proteins in DEN treated mice. In our studies, PCNA was only observed in the group treated with DEN (Figure 8.5B). Positive staining was not observed in control group as well as DEN plus

RG group, which indicates that RG is effectively inhibiting cell proliferation and there by DEN induced hepatocarcinogenesis.

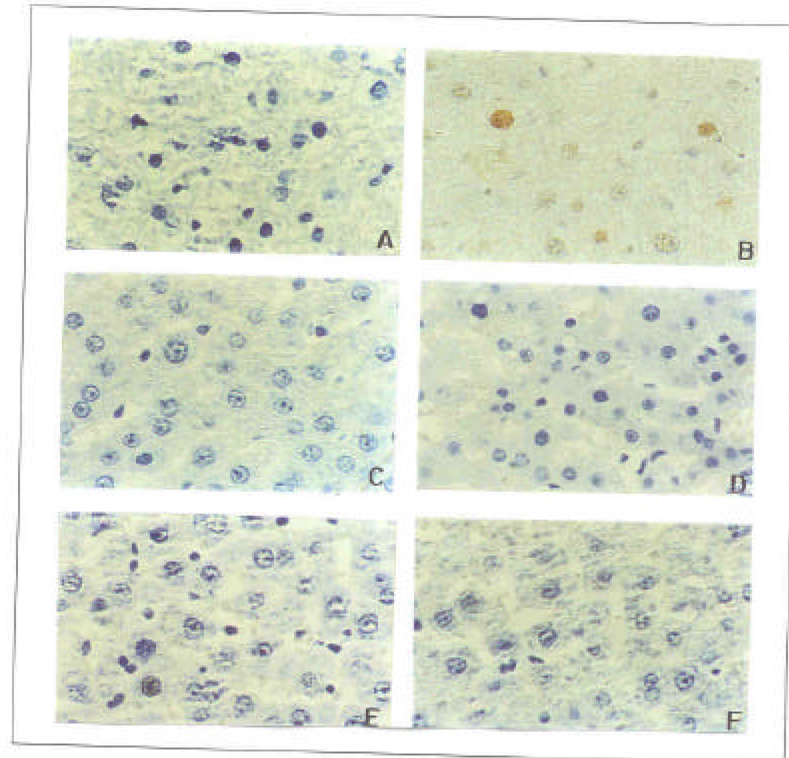

Figure 8.5. PCNA immunostaining of the livers of rat during tumour inhibition by *Rhodotorula glutinis* (RG) (x 400). (A) Control, (B) DEN treated, (C) RG-Control, (D) DEN + RG, (E) RG + RG + RG Control, (F) RG + (DEN +RG)+ RG treated.

DEN is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication (Pashupathy and Bhattacharya 1999). Carotenoids, like lycopene and β-carotene, along with vitaminA are known to exert anti-tumor effects by preventing chromosomal aberrations and DNA breaks during early preneoplastic stage of DEN induced hepatocarcinogenesis. (Sarkar *et al.* 1997, Pierre *et al.* 1997a, Rizzi *et al.* 1998, Shamann *et al.* 1998).

In this study, the preparation of *Rhodotorula glutinis* NCIM 3353 was found to be effective in the inhibition of tumour development following DEN treatment in female Wistar rats, when administered as compared to its administration prior to or together with DEN treatment. This suggests that inhibition of cell proliferation and prodifferentiation effects of RG were more predominant in the suppression of rat liver tumour development as compared to quenching and scavenging of reactive free radicals.

RG mainly accumulated torularhodin and torulene along with β-carotene. The antiproliferative property can be attributed to carotenoids or their provitaminA nature (Ershov *et al.* 1992). *Rhodotorula glutinis* is also reported to accumulate considerable amount of lipid (Perrier *et al.* 1995, Leman *et al.* 1997) but reports on lipids acting as anticarcinogen in DEN induced carcinogenesis are contradicting (Glauert *et al.* 1991, Ramesh and Das 1995). However, it will be important to investigate the role of individual components by using suitable controls.

The studies on the chemopreventive and anticarcinogenic effects of feed additive preparation of spray dried yeast *Rhodotorula glutinis* NCIM 3353 (RG) were performed before the development of mutant 32. Work with mutant 32 spray dried cell mass will be continued in the future

Conclusion

Anticancer property of dried *Rhodotorula glutinis* was studied using rat liver carcinogenesis model. *Rhodotorula spp* studied earlier in experimental animals was proved to be safe and non-toxic source of natural pigments and nutritional additives (Eugenia *et al.* 1997, Naidu *et al.* 1999). This is the first ever study on anti-proliferative property of dried *Rhodotorula.* Mechanisms associated with the chemopreventive efficacy of yeasts are, however, not clear. These studies can help to establish utility of carotenoids accumulating yeasts in the human health.

References

References are listed in **chapter 10.**

Chapter 9

General conclusion and future work

General conclusion

Carotenoids have proven their multiple uses mainly in aquacluture, medicinal and food industries. Despite the availability of various natural and synthetic sources, carotenoid production from microbial sources is getting attention owing to public awareness against the synthetic food additives.

Several microbes like algae, fungi, yeasts and bacteria are reported to produce carotenoids. Asporogenous and non-fermentative yeast, *Rhodotorula spp.* has drawn interest as feed additive in livestock and poultry, because of its well-known, characteristic carotenoids *viz.* torularhodin, torulene and β-carotene. However, low total carotenoid content and minute β-carotene proportion limits its industrial exploitation. Hence, there was a need to improve the total carotenoid and β-carotene proportion in *Rhodotorula.* Along with this, it was essential to develop an economic process by using industrial by-products, as nutritional sources, for the yeast growth.

In this thesis, wild strains of *Rhodotorula spp.* were studied for carotenoid production (mg/l), cellular carotenoid accumulation (mg/g), proportion of the carotenoids (%, w/w) and cell mass yield (g/l). *Rhodotorula glutinis* NCIM 3353, which produced 2.2 mg total carotenoid per liter of culture broth, in 72 h, was selected for further studies. Total carotenoid content in the cell mass of this strain was 0.122 mg/g and β-carotene accounted for 14% (w/w) of the carotenoids. *Rhodotorula glutinis* NCIM 3353 was subjected to UV mutagenesis for strain improvement by UV mutagenesis. UV light was selected as mutagen because of its relative harmlessness as compared to chemical mutagens.

Out of 2051 isolates screened, a yellow coloured "mutant 32" produced 15 fold (33 ±2 mg/l) more total carotenoids than the parent strain, in 72 h. β-Carotene was the major carotenoid produced and exhibited 120 ±2 fold (2.048 ±0.05 mg/g) more β-carotene than parent culture in 32 h, which was 88 ±5% (w/w) of total carotenoid content. Carotenoid production profile of mutant 32 indicated that the volumetric β-carotene production (mg/l) and cellular accumulation (mg/g) was distinctly growth associated. Selected mutant was also studied for its biochemical and nutritional properties. Mutant 32 was observed to be a stable mutant and showed favorable properties for its application in carotenoid production.

Carotenoid production from mutant 32 was increased systematically by optimizing the selected components of the growth medium in the shake flask using two-level, three-variables factorial method. The optimized medium resulted in four fold increase in volumetric production and two fold increase in cellular accumulation of β-carotene as compared to the initial basal medium. The factorial method resulted in 226% improvement in total carotenoid content, which was distinctly better than previously reported in the literature, till date.

The yield was further improved by optimization of other cultural conditions like pH of the medium, incubation temperature and exposure to illumination. Temperature and light played important role in enhancing the accumulation of carotenoids. Fermentation studies were performed in 1 L and 14 L stirred fermenter using batch, fed-batch, and continuous culture, followed by membrane cell-recycle reactor to obtain higher yield of carotenoids, which could help to define process parameters for carotenoid production, especially β-carotene, from the mutant yeast.

In order to make carotenoid production economically viable, *Rhodotorula glutinis* mutant 32 was studied for β-carotene production in a medium composed of sugar cane molasses and corn steep liquor. In plain molasses medium, maximum carotenoid production was observed at 40 g/l TRS (total reducing sugars) and pH 6.0, wherein, β-carotene accounted for 70 ±5% (w/w) of total carotenoids. Supplementation with additional nitrogen sources further increased β-carotene proportion by 12 ±4% (w/w) with simultaneous decrease in torulene proportion. In stirred fermenters, during batch fermentation, increase in torulene content by 20 ±5% was observed in plain molasses medium as compared to that in the shake flask. However, by addition of yeast extract and corn steep liquor, the effect could be reversed and 31 ±5% increase in β-carotene content could be achieved. DO-stat fed-batch cultivation of mutant 32 using double strength and triple strength optimized medium as feed yielded high volumetric β-carotene production in plain molasses (71 and 185 mg/l, respectively), whereas, in yeast extract supplemented molasses, it was 97 and 183 mg/l, respectively. Sugar cane molasses is the by-product of sugar manufacturing industries. It is being used for animal feed preparation and alcohol production but the present studies reveals its utility for production of carotenoids.

Mutant 32 was also grown in a basal medium prepared in artificial seawater as the water source. The total carotenoid content and dry cell mass were 86 ±4 mg/l and 16 ±1mg/l, respectively. Two fold increase in β-carotene (67 mg/l) simultaneous with 2.3 fold decrease in torulene content (15.3 mg/l) was observed as compared to that obtained in the medium prepared in distilled water. When grown in seawater medium at pH 6.0, 83 ± 5% carotenoids could be extracted from the cells without any mechanical disintegration. The ease of extraction was observed with the parent strain (*Rhodotorula glutinis* NCIM 3353) also, but to a lesser extent. The laboratory scale studies done using artificial sea in the present thesis can be employed in fields/pilot scale to examine the utility of seawater in the production of carotenoids.

Further, In order to minimize the cost of water during carotenoid production from mutant 32 and also minimize the wastewater generation, fermented broth was microfiltered using ceramic membrane of 0.45 micrometer pore size. 80% (v/v) of the microfiltered broth, containing unused nutrients and extracellular products, was replenished with carbon and nitrogen sources and used 15 times, consecutively. It was found that reuse of microfiltered broth enhanced β-carotene content (mg/l, mg/g) and proportion (%) with simultaneous decrease in torulene content and proportion. This part of the studies has opened up prospects of making an “integrated production unit” for carotenoid production, using mutant 32.

The cell mass of mutant 32 grown in a stirred tank fermenter was concentrated ten-fold by crossflow microfiltration using tubular ceramic membrane and spray dried at different inlet nozzle temperature between 40 and 200 °C in the presence of antioxidants like butylated hydroxyl toluene (BHT) and D-tocopherol. In the presence of BHT, 87 ±7% carotenoids were recovered at all nozzle temperatures studied. A two level, three variable, factorial experiment was performed using inlet nozzle temperature (°C), feed concentration (%, w/v) and antioxidant concentration (mg/l) as the three variables. β-Carotene content (µg/g) in the dry cell mass was used for calculation of the effect of each factor. Best recoveries were obtained when 160 °C, 11.6 % feed concentration and 1000 mg/l BHT concentration were used for spray drying. The dried product obtained by optimized method was stable in dark and cold conditions over 10 weeks. In the presence of a BHT, under light conditions, the rate of carotenoid degradation was much lower than the control. Present studies reveal that carotenoids in dried yeast preparation are fairly

stable and thus dried cell mass can be used as carotenoid source, mainly as animal feed.

Chemopreventive and anticarcinogenic effects of spray dried *Rhodotorula glutinis* NCIM 3353 (RG) on the development of preneoplastic lesions during N-nitrosodiethylamine (DEN) induced hepatocarcinogenesis in female Wistar strain rats were studied. The chemopreventive and anticarcinogenic effects of RG were monitored on the basis of weight of the animals, morphological appearance of the livers, liver weight profile, histological pattern and PCNA immunohistochemistry using PC-10 antibody. RG showed significant effect on the prevention of liver tumour development in these protocols tested. However, RG effects were relatively more significant in groups where RG was administered after DEN treatment, suggesting that RG is quite effective in the prevention of liver tumour development, especially when administered after DEN treatment, indicating possible action at the level of inducing differentiation. Anticancer property of dried *Rhodotorula glutinis* was studied using rat liver carcinogenesis model. This is the first ever study done on anti-proliferative property of dried *Rhodotorula.* These studies can help to establish utility of carotenoids accumulating yeasts in the human health.

Future work

The present work in the thesis is the part of the work ongoing in Biochemical Engineering Division, National Chemical Laboratory. There is much more to be done which is listed below.

- Mutant 32 obtained by UV mutagenesis produces torulene and torularhodin in minute quantities. Separation of these two carotenoids can add upon to the cost of β-carotene. Hence, it will be desirable to obtain a mutant, which lacks torulene and torularhodin completely. This can be achieved by further mutagenesis of mutant 32 or using modern approach like recombinant DNA technology.

- The parent culture, *Rhodotorula glutinis* NCIM 3353, showed antiproliferative and chemopreventive properties. The actual cause of these antiproliferative and chemopreventive properties is yet to be explored. Also, similar studied can be done using dried mutant 32.

- Use of artificial seawater lead to ease in extraction of carotenoids. Studies can be done further with natural seawater.

- Utility of dried Mutant 32 obtained in my studies can be studied as poultry and cattle feed.

- Mutant 32 can be cocultivated with “edible” algae like *Chlorella and Spirulina* to develop caroteno-protein product to meet the requirements of food industries.

Chapter 10

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Research Publications / patent filed

1. **Prakash Bhosale** and R.V.Gadre. Production of β-carotene by *Rhodotorula glutinis* mutant. *Applied Microbiology and Biotechnology (*2001) *In press.*

2. **Prakash Bhosale** and R.V.Gadre. Production of β-carotene in sea water medium. *Bioresource Technology* (2001) 76(3) 53-55.

3. **Prakash Bhosale** and R.V.Gadre. Production of β-carotene from *Rhodotorula glutinis mutant 32* from sugar cane molasses. Revised manuscript communicated to *"Journal of Industrial Microbiology and Biotechnology".*

4. **Prakash Bhosale** and R.V Gadre. A process for preparation of carotenoids, preferably β-carotene. *Indian Patent Filed* (communication number at NCL: 456.50/RPBD/99/734).

5. **Prakash Bhosale** and R.V.Gadre. Optimization of growth medium for carotenoid production from *Rhodotorula glutinis mutant* by factorial approach. *Manuscript communicated to "Letters in Applied Microbiology." (Communicated on 21st November' 2000.)*

6. **Prakash Bhosale** and R.V.Gadre. Manipulation of temperature and illumination conditions for enhanced β-carotene production by mutant 32 of *Rhodotorula glutinis*. *Manuscript communicated to "FEMS Microbiology Letters." (Communicated on 8th January 2001).*

7. **Prakash Bhosale**, Leena Motiwale, A.D. Ingle, R.V.Gadre and K.V.K. Rao. Antiproliferative properties of spray dried *Rhodotorula glutinis* NCIM 3353. *Manuscript communicated to "Current Science". (Communicated on 12th January 2001).*

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