

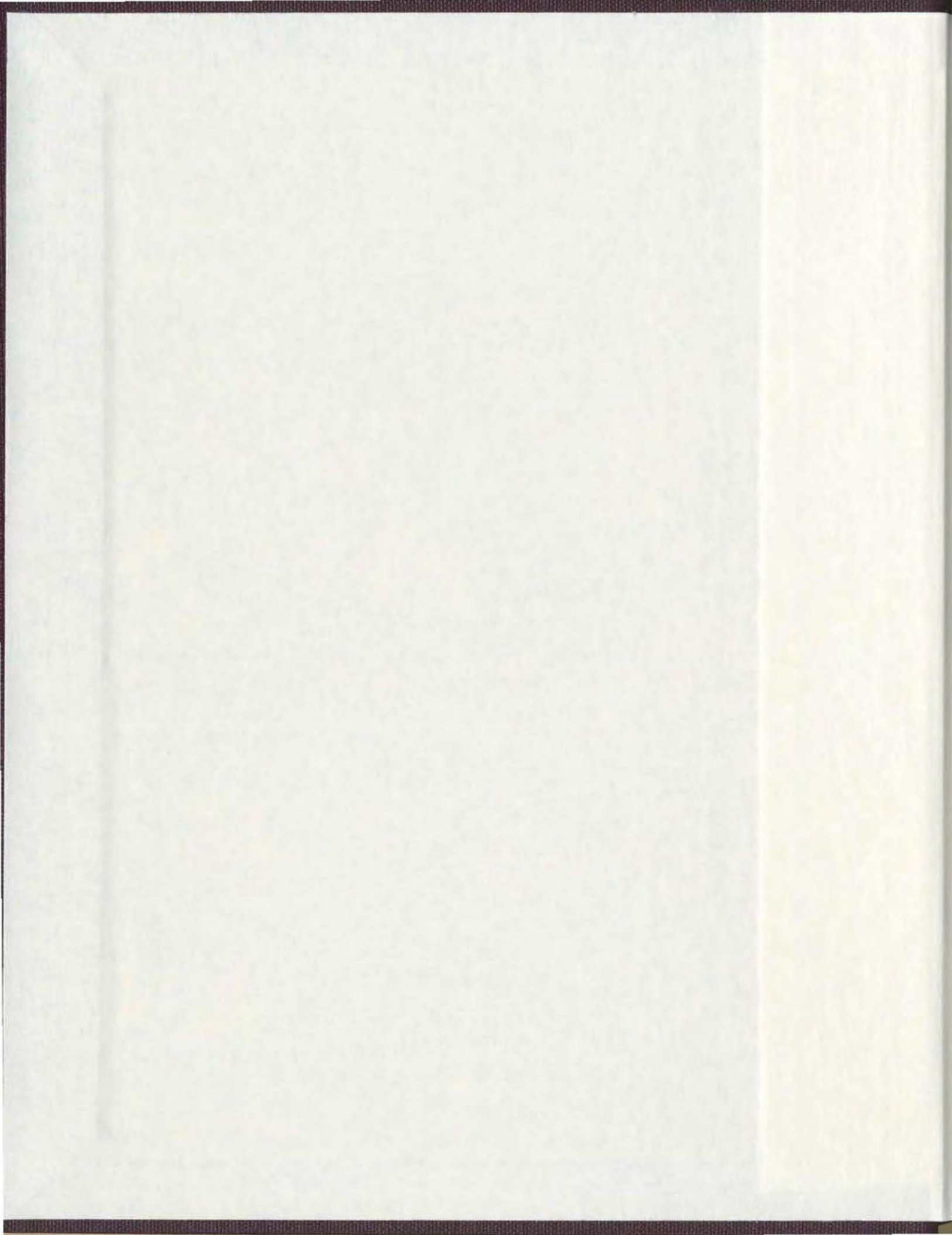
CHARACTERIZATION OF *Rhodotorula rubra*  
TP1 MUTANTS

CENTRE FOR NEWFOUNDLAND STUDIES

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**CHARACTERIZATION OF *Rhodotorula rubra* TP1 MUTANTS**

**by**

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**in partial fulfillment of the requirements**

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## ABSTRACT

Carotenoid pigments exist in nature and are widely distributed as colourants throughout the biological systems, such as microalgae, insects, birds, fish and crustaceans. They are responsible for interesting colours seen in various parts of these organisms, which play a great role in the biological functions like photoreception and photosynthesis. Carotenoids are mainly used as pigments for colouration of food products and pharmaceuticals. They also function as antioxidants and help in minimizing membrane-damage, and in controlling human diseases such as cancer, cataract and atherosclerosis.

Astaxanthin is a red orange carotenoid produced by aquatic organisms such as algae and is also found in yeasts like *Phaffia rhodozyma* and *Rhodotorula rubra*. It is used as a pigment in feed for salmon and shellfish and also enhances immune response of fish and shrimp. Among yeasts, *R. rubra* TP1 is a good source of red pigment and whole cells induce pigmentation in fish. It has been shown in earlier work that *R. rubra* has faster growth rate, shorter incubation-time and yields more biomass than *P. rhodozyma*. Further, previous feeding-trial experiments carried out using rainbow trout have been successful and therefore *R. rubra* TP1 has economic potential.

In the current work the mutants M1, M2 and M3 of *R. rubra* TP1 were characterized and their properties compared with those of the wild type yeasts. The optimal pigment production was determined by growing the mutants and wild type yeasts under different growth conditions, such as different substrates, temperatures, initial pH and light. The maximum pigment recovery was achieved by using different extraction methods which include French Press method, Freeze- dried cells, sonication and

enzymatic cell breakage. The spectrophotometer graph and Thin Layer Chromatography (TLC) techniques were used to estimate the total carotenoid concentration and to analyse the pigment in each sample.

The experimental results showed that light enhances pigment production. Yeast malt broth with peat extract as a nitrogenous source showed more biomass yield. Bacto czapex dox broth was found to be inhibitory to growth of the mutants of *R. rubra* TP1. The cells gave more pigment at 25 °C in the initial pH range of 5.0 to 7.0. The French press method was found to be more efficient to extract the optimum pigment for M1, M2 and M3 with values 250.6, 254.4 and 193.2 µg/g, respectively. Mutant 2 alone gave higher recovery of the pigment with Freeze-dried method. Sonication method gave less pigment recovery. The enzymatic method with a pH of 7.0 for all mutants gave recovery values of 184.4, 164.2 and 129.4 µg/g for M1, M2 and M3, respectively. The pigment analysis confirmed that all the mutants contain β-carotene, torulene and torularhodin carotenoids in their pigments.



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## ABBREVIATIONS

Yeast Malt Broth	YMB
Bacto Czapek Dox Broth	BCDB
Gram./Litre	g/L
Micro gram/ gram	$\mu\text{g/g}$
Thin Layer Chromatography	TLC
Nanometer	nm
Beta mercapto ethanol	BME
Dithiothreitol	DTT
Degree centigrade	$^{\circ}\text{C}$

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## CHAPTER-1

### INTRODUCTION

The quality of food, aside from the microbiological aspects, is generally based on its colour, flavour, texture, and nutritive value. Depending on the particular food, these factors may be weighted differently in assessing overall quality. However, one of the most important sensory quality attributes of food is colour, because no matter how nutritious, flavourful, or well textured a food is, it is unlikely to be accepted unless it has the appropriate colour. The acceptability of food is reinforced by economic worth since in many cases raw food materials are judged on the basis of their colour.

Pigments are chemical compounds which reflect only certain wavelengths of visible light, making them appear "colourful". Flowers, corals, and even animal skin contain pigments, which give them their colors. The ability of pigments to absorb light of certain wavelengths is more important than reflection by them.

The term "pigment" is used to refer to a material of known or unknown physical state or to an unanalyzed coloured material (Sangha, 1994). Colours of various carotenoids are related to the number of alternating carbon-carbon double-bond pairs in the long polyene chain of the molecule, known as the chromophore (Fig. 1). Specifically, light energy is absorbed by the carotenoid polyene system between 400 – 700 nm, and is converted into vibrational energy and heat. Each carotenoid has a unique resonance in this regard (Fox, 1976) through the isoprenoid pathway (Fig. 2) and they produce diverse compounds such as essential fatty acids, steroids, sterols, and vitamins A, D, E, and K.

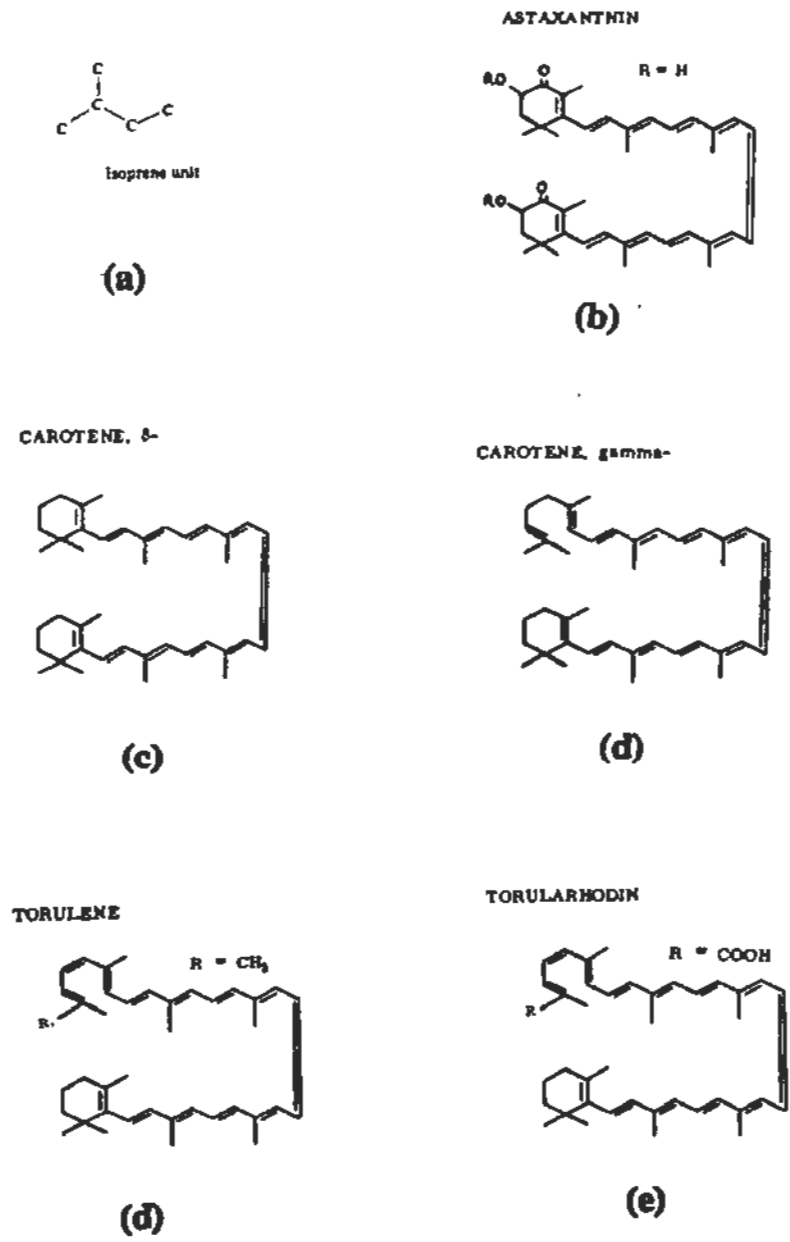


Fig. 1 Chemical structure of Carotenoids (Hari *et al.*, 1992)



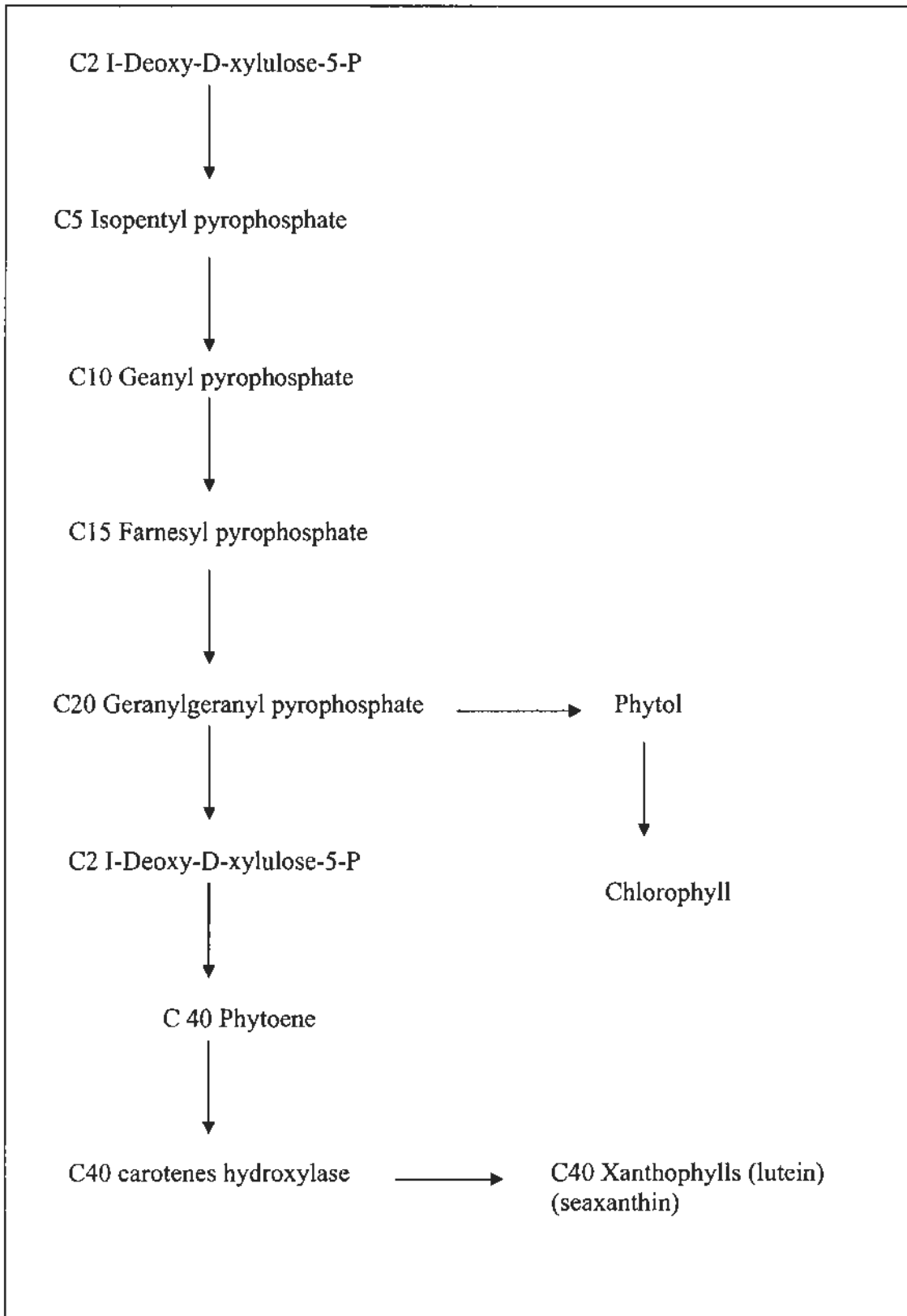


Fig. 2. Isoprenoid pathway (Tanaka.,1995)

Within the various classes of natural pigments, the carotenoids are the most wide spread and structurally diverse pigmenting agents. They are responsible, in combination with proteins, for many of the brilliant yellow to red colors in plants and the wide range of blue, green, purple, brown and reddish colors of fish and crustaceans. The general distribution and metabolic pathways of carotenoids have been extensively detailed (Goodwin 1984). Carotenoids are widespread throughout biological systems. They are found in the plants, algae, bacteria, animals and fungi (Goodwin, 1980). Several species of yeasts produce carotenoids and are grouped as the 'red yeasts'. These carotenogenic ascomycetes, basidiomycetes and deuteromycetes all tend to accumulate predominantly hydrocarbon carotenoids, such as beta-carotene and gamma-carotene (Goodwin, 1980).

### **1.1 Manifestation of colour:**

Colour is displayed by organisms in two ways, namely, (1) physically, by colourless particles or ultramicroscopic structures called "schemochromes", and (2) chemically, by naturally occurring chemical substances possessing a coloured molecule, called "biochromes" (Fox, 1979).

Schemochromes are exhibited by both colourless, randomly scattered, light-diffracting submicroscopic bodies. These give rise to the Tyndall blues of scattering and various striations or ultrathin successive films or layers which resolve incident light into its components producing interference colors (Fox, 1979).

Biochromes absorb wavelength, while reflecting and/or transmitting other wavelengths of visible light (Fox, 1979). The structural feature of a biochrome

responsible for the absorption of light is the chromophore. For example, in carotenoids the chromophore is the conjugated carbon-carbon double bond system.

Other functional groups or substituents in a biochrome, which possess the ability to modify the absorption maximum of the molecule are termed auxochromes. Vision in humans and animals is a complex chemical phenomenon. The human eye, for example is roughly spherical with an opening to admit light, which falls on a rear surface lined with millions of cells. The molecules responsible for vision are attached to the cells. Discrimination between colours is possible because cone cells occur in three groups: those receptive to blue light, those receptive to green light and those receptive to yellow-red light. Each type can absorb light in a range around its primary color. When an object absorbs these wavelengths (visible range 400 and 750 nm), certain molecules within the object become excited. A molecule is excited when one of its outer orbital electrons is raised to a higher orbital. These electron transitions are characteristic of most biological materials but are particularly pronounced in biochromes (Needham, 1974a).

## **1.2 Major Pigment Types:**

There are six major groups of pigments occurring in biological systems. These are carotenoids, tetrapyrroles, indolic biochromes, N-heterocyclic biochromes (other than tetrapyrroles), oxygenous heterocyclic biochromes (the flavonoids) and quinones.

Carotenoids are nature's most widespread pigments, with the earth's annual biomass production estimated at 100 million tons (Fennema, 1996). In nature over 560 carotenoid structures have been identified and compiled. They derive their names from

the fact that they constitute the major pigment in the carrot root, *Daucus carota*, one of the first foods observed to possess this class of pigments (Klaui *et al.*, 1981).

### **1.2.1 Functions:**

Most of the functions of carotenoids are a consequence of their ability to absorb visible light. It has been established that carotenoids play a role in photoreception (vision), photosynthesis, photoprotection, phototaxis and integumental colors (Burnett, 1965; Needham, 1974; Goodwin, 1980; Sangha, 1994; Britton *et al.*, 1995). The luminous carotenoid colours of tropical fish are not only keys for species identification and mating signals they have significant physiological roles as well. The seasonal astaxanthin levels in the carapace have shown that the eggs parallel with the exposure to sunlight, indicating that the carotenoids serve to protect external proteins and eggs from ultraviolet exposure. Beta-carotene is converted to vitamin A, which is required for the biochemical processes involved in vision (Goodwin, 1980). Furthermore, vitamin A plays an important role in the growth, development, and integrity of mucous surfaces. However, the majority of research concerning astaxanthin and other carotenoids has been aimed at its role in photoprotection and as an antioxidant in quenching of oxygen radicals.

Carotenoids owe their color to the absorption of light by the feature of their molecular structure known as the 'chromophore'. In most carotenoids the chromophore consists entirely of a conjugated system of carbon-carbon double bonds, referred to as the 'polyenechain' (Fig. 3). It is possible to have up to 15 conjugated double bonds in the chromophore of a C<sub>40</sub> carotenoid, although structures with 7 to 11 such bonds are

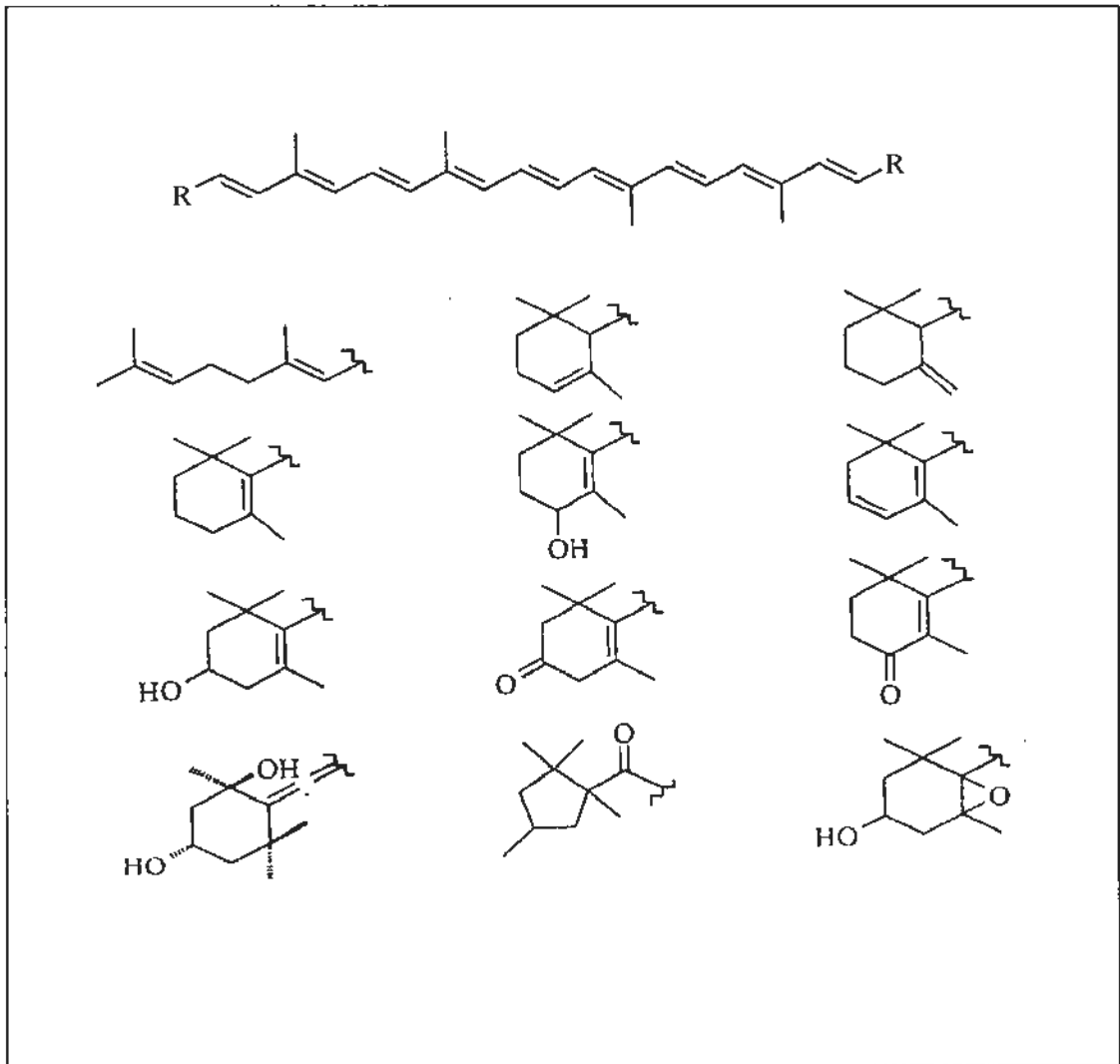


Fig. 3. Chemical structure of polyene chain with a variation in the end groups.  
(Weedon et al., 1995)

more common. Other features of carotenoid molecules that may constitute part of the chromophore are triple bonds, terminal allene groups, substituted phenyl end groups, and carbon-oxygen double bonds (Weedon *et al.*, 1995).

### **1.3 Applications of Carotenoids:**

Carotenoids have commercial application in various industries such as aquaculture, food industry, pharmaceutical, cosmetic, and medicine (Bauernfeind and Klaui, 1981; Munzel, 1981; Sangha, 1994). The use of carotenoids as pigments in aquaculture is well documented. It appears that their broader functions include a role as an antioxidant and provitamin A activity as well as enhancing immune response, reproduction, growth, maturation and photoprotection. An extensive body of data stresses the vital role of carotenoids in the physiology and overall health. It concludes that carotenoids are essential nutrients that should be included in all aquatic diets at a minimum level of 5 –10 ppm (Torrissen, 1989).

### **1.4 Taxonomy of *Rhodotorula rubra*:**

Yeast is defined as a unicellular fungus that reproduces by budding or fission (Kreger van-Rij, 1984). Yeasts are taxonomically diverse and classified in the division Eumycota, which includes the classes Ascomycotina, Basidiomycotina and Deuteromycotina (Kreger van-Rij, 1984). The ascomycetes are recognized as unpigmented yeasts possessing asci with ascospores, and reproduce by holoblastic budding (Kratochvilova, 1990).

The system of taxonomy used today is the result of the development and integration of various avenues of approach to the problem of yeast identification and classification (Lodder, 1970). Morphological and reproductive attributes are utilized to decide the main taxonomy- to designate higher taxa while physiological evidence is used to differentiate lower taxa, and in particular, species classification. Most of these characteristics are defined based on a particular test, such as fermentation and assimilation (Kreger-van-Rij, 1984). The isolation of the mutants of *Rhodotorula rubra* TP1 used in this work has been reported (Acheampong, 2000).

The carotenoid-producing yeasts include genera such as *Cryptococcus*, *Rhodotorula*, *Rhodospiridium*, *Sporidiobolus*, *Sporobolomyces*, *Phaffia* (Johnson and Lewis, 1979) and *Saitoella* (Komagata *et al.*, 1987). Yeasts belonging to the genera *Cryptococcus*, *Rhodotorula*, *Rhodospiridium*, *Sporidiobolus*, and *Sporobolomyces* typically contain  $\beta$ -carotene,  $\gamma$ -carotene, torulene and torularhodin as major carotenoids (Simpson *et al.*, 1971). The genera *Rhodospiridium* and *Rhodotorula* may also produce carotene, phytoene, and phytofluene, 2-hydroxyplectanixanthin have been found in a strain of *Rhodotorula aurantiaca* (Lui *et al.*, 1973). Some species of *Rhodotorula* also synthesize  $\beta$ -carotene,  $\beta$ -zeacarotene and plectanixanthin, which are also found in *Cryptococcus laurentii* (Lui *et al.*, 1973).

The yeast *Phaffia* produces astaxanthin as its most abundant carotenoid. Other characterized carotenoids are  $\beta$ -carotene,  $\gamma$ -carotene, neurosporene, lycopene, echinenone, 3-hydroxyechinenone, 3-hydroxy-3', 4'-didehydro-  $\beta$ -carotene – 4 – 1 and phoenicoxanthin (Andrewes *et al.*, 1976).

## **1.6 Mutagenesis:**

Several methods are available for genetic manipulations of biological cells. Newer techniques include protoplast fusion, pulsed field electrophoresis and recombinant DNA techniques. However, difficulty arises in applying these methods when genetic information of a species is lacking. More fundamental approaches for strain improvement involve genetic mutations (Crueger and Crueger, 1989).

To enhance the potential of a microorganism, the genotype can be manipulated by inducing mutations in the genome. Common mutagenic agents include ultraviolet and ionizing radiations and chemical agents. These affect non-replicating DNA and cause frame-shifts in DNA and base substitution by analogs (Crueger and Crueger, 1989).

Short wavelength ultraviolet rays between 200 – 300 nm, with an optimum wavelength at 265nm, are effective in causing mutations. The absorption maximum of DNA is 265 nm. The most important products of this type of radiation are pyrimidine dimers, formed between adjacent pyrimidine bases on complementary strands of DNA. Long wavelength ultraviolet rays between 300 – 400 nm are less lethal mutagens. However, if cells are exposed to this type of radiation in the presence of various dyes, increased mutation frequency is induced (Crueger and Crueger, 1989). Ionizing radiation includes x-rays,  $\gamma$ -rays,  $\beta$ -rays. These types of radiations are seldom used for mutagenesis as the rays cause a much greater percentage of single and double strand breaks in DNA than the other mutagens, which can result in major structural changes in the chromosome. A variety of chemicals are known mutagens and are used in genetic studies. These chemicals are classified according to their mode of action. Frame-shift mutagens intercalate into the DNA molecule, causing errors in the reading frame and



result in the formation of faulty proteins or no proteins at all. Examples of this type of mutagen are acridine dyes, such as acridine orange, proflavine and acriflavine. Although useful in research, frame-shift mutagens are not very suitable for isolation of mutants in strain development, because they have little or no mutagenic effect in bacteria and yeasts (Crueger and Crueger, 1989).

Base analogs, such as 5-bromouracil and 2-aminopurine, act as mutagens by being incorporated into replicating DNA in place of the corresponding bases thymine and adenine because of their structural similarity. These cause transitions to occur, resulting in the wrong base pair being incorporated into the replicated DNA. Conditions for the development of this type of mutants are costly and as such, base analog mutagens are rarely used in practical applications.

Many carotenogenic, or red yeasts have also been genetically altered using N-methyl-N-nitro-N-nitrosoguanidine (NTG). An *et al.*, (1989) evaluated the effectiveness of UV light, ethylmethanesulfonate (EMS) and NTG in generating greater pigment producing mutants of *Phaffia rhodozyma*. NTG was reported to be the best mutagen. However, most of the mutants were unstable. In another attempt to obtain hyper pigment producing mutants, Lewis *et al.* (1990) exposed *Phaffia rhodozyma* to NTG and then screened the astaxanthin-overproducers using beta-ionone. Acheampong (2000) successfully treated *Rhodotorula rubra* *TPI* with NTG in order to produce mutants with enhanced pigmentation and a better capacity to utilize cheaper substrates for growth.

### **1.7 Astaxanthin:**

Astaxanthin is the main carotenoid pigment found in some aquatic animals. This red-orange pigment is closely related to other well-known carotenoids such as beta-carotene or lutein, but has a stronger antioxidant activity (10 times higher than beta-carotene). Studies suggest that astaxanthin can be 1000 times more effective as antioxidant than vitamin E. In many of the aquatic animals where it can be found, astaxanthin has a number of essential biological functions, ranging from protection against oxidation of essential polyunsaturated fatty acids to enhance immunity and growth. In species such as salmon or shrimp, astaxanthin is even considered as essential for normal growth and survival, and has been attributed to have vitamin-like properties. Some of these unique properties have also been found to be effective in mammals and open very promising possibilities for nutritional and pharmaceutical applications of astaxanthin in humans. It can be found in many of seafoods such as salmon, trout, shrimp, lobster and fish eggs. It is also found in a number of bird species. Astaxanthin cannot be synthesized by animals and must be provided in the diet as is the case with other carotenoids. While fish such as salmon are unable to convert other dietary carotenoids into astaxanthin, some species such as shrimp have a limited capacity to convert closely related dietary carotenoids into astaxanthin, although they will benefit strongly from being fed astaxanthin directly. Mammals are also unable to synthesize astaxanthin. Some microorganisms can be quite rich in astaxanthin.

A ubiquitous micro-algae, *Haematococcus pluvialis* is believed to be the organism, which can accumulate the highest levels of astaxanthin in biological system. The function of astaxanthin appears to be to protect the algae from adverse environment

changes, such as increased UV-light photooxidation and evaporation of the water pools in which it lives. *Haematococcus* algae can accumulate as high as 10 to 30 g of astaxanthin per kg of dry biomass. This level is 100 to 3000 fold higher than in salmon fillets. Some strains have even been observed to accumulate as much as 70 to 80 g of astaxanthin per kg of dry biomass. Esterified astaxanthin from *Haematococcus pluvialis* algal meal is the preferred form in several oral prophylactic and therapeutic formulations for muscular dysfunction, such as exertional rhabdomyolysis (also known as exertional myopathy, tying-up syndrome, azoturia, or Monday morning sickness) in horses (Lignell, 1999), as well as for mastitis (mammary inflammation) in dairy cows (Lignell, 1999).

Astaxanthin is one of a group of natural pigments known as carotenoids. The astaxanthin molecule is similar to that of the familiar carotenoid, beta-carotene. The small differences in structure of these confer large differences in the chemical and biological properties of these two molecules. In particular, astaxanthin exhibits superior antioxidant properties to beta-carotene in a number of in vitro studies (Krinsky, 1992). Higher survival rate in red sea-bream was found to be that astaxanthin enhanced liver cell structure. Glycogen storage in red tilapia increases fertilization and survival rates of eggs. Higher growth rates during the early-feeding period of young salmonids have all been associated with dietary astaxanthin supplementation (Sommer *et al.* 1991; Torrissen and Christiansen 1995; Kawakami *et al.* 1998). When astaxanthin was included in poultry feeds, dietary astaxanthin was reported to improve egg production, the general health of hens and also increase in the hatching percentage, resistance to *Salmonella* infection, and shelf life of eggs. (Lignell *et al.* 1998).

## **1.8 Significance of Carotenoids:**

The food and pharmacological industries are potential users of large amounts of natural antioxidants. One of the advantages for the food industry is that these antioxidants may be used as preservatives against both enzymatic and spontaneous oxidation of foods, thereby extending their shelf life. Astaxanthin, which belongs to the carotenoid group, is a very valuable natural red dye used as a feed additive for deepening the pigmentation of salmon and organic chicken eggs. Initial results also show that astaxanthin is a promising cancer preventing agent and hence has potential for use as an additive for promoting good health (Tanaka, 1995).

In nature, like other pigments, astaxanthin is synthesized only by microalgae and then passed up the food chain. Salmon and other marine animals cannot make the compound themselves and must get it in their food. Traditionally astaxanthin has been added to commercial aquaculture diets to improve the pigmentation of the flesh of fish. This use remains by far the largest market in terms of volume and market value. However a number of studies (Klaui H. and Bauernfeind, J.C., 1981) have shown that astaxanthin was much more than a pigment and in fact had vitamin-like properties. As a result, astaxanthin is now also used to enhance the immune response of fish and shrimp to secure maximum survival and growth. Recent studies (Ito *et al.*, 1986) with young shrimp and other fish species have shown a superior uptake of natural astaxanthin from microalgae compared to the synthetic form. Another reason for aquaculturists to prefer natural astaxanthin is the growing demand from consumers for fish being fed natural pigments, identical to those fish that acquire natural astaxanthin from the environment.

### 1.9 Carotenogenic Yeasts as sources of Carotenoids:

The pink to red color of the flesh of salmonids is an important factor in consumer preference for coloured fish. Colour is not an intrinsic component of the fish, but results from the deposition of dietary carotenoids. Astaxanthin is an abundant carotenoid in the marine environment. Salmonids, like most animals, are unable to synthesize or biologically transform carotenoid precursors into the pigments found in their tissues. Wild salmon obtain their carotenoids from marine zooplankton, nekton, and their natural foods. Pen-raised salmonids, in turn, must derive this pigmentation from sources in their feed.

The dominant pigment source in aquaculture is synthetic astaxanthin and canthaxanthin, commercially produced by Hoffman La Roche (Basle, Switzerland), which are marketed under the trade names of 'Carophyll pink' and 'Carophyll red', respectively (Torrissen *et al.*, 1989). However, the use of synthetic feed colorants is quickly declining due to strict regulations and the increasing reluctance of consumers to accept chemicals as food additives.

In recent years, yeasts have been used as a pigment source for fish. The species *Phaffia rhodozyma* possesses high levels of carotenoids, of which astaxanthin is the most abundant. In feeding trials, the incorporation of this yeast's pigment into the diets has achieved high levels of pigment deposition in rainbow trout, lobsters and salmon (Johnson and Lewis, 1977). However, three major obstacles have prevented the commercial use of *Phaffia rhodozyma* as a natural source of carotenoids in fish feeds: a rigid cell wall, which limits the pigment extractability, a slow growth rate and poor digestibility of the whole *Phaffia* cells by the fish.

A strain of the *Rhodotorula* species, *Rhodotorula rubra* TP1, was also found to be a good source of pigments for rainbow trout. Unlike *Phaffia rhodozyma*, whole cells of *Rhodotorula rubra* TP1 were able to induce pigmentation. In addition, this has been found to have a faster growth rate and easier pigment extractability than *Phaffia rhodozyma* (Sangha, 1994).

#### **1.10 The Genus *Rhodotorula*:**

The genus *Rhodotorula* belongs to the class Deuteromycotina, family Cryptococcaceae (Kreger van-Rij, 1984) and sub-family Rhodotoruloideae (Lodder and Kreger-van Rij, 1954).

Yeasts are classified in the family Cryptococcaceae by the constant presence of budding cells- although a pseudomycelium, true mycelium and arthrospores may be formed. Culture cells are hyaline, red, orange or yellow due to carotenoid pigments, and are seldom brown or black. Dissimilation is strictly oxidative or oxidative and fermentative (Kreger van-Rij, 1984).

Members of the genus *Rhodotorula* have ovoidal, spheroidal or elongate cells. They reproduce vegetatively by multilateral budding and variants of some species form pseudohyphae or true hyphae. Neither ascospores nor ballistospores are formed. Red or yellow carotenoid pigments are synthesized in malt agar cultures (Kreger van-Rij, 1984). Regarding culture appearance, some strains appear mucoid due to capsule formation, while others seem pasty or dry and wrinkled (Kreger-van Rij, 1984).

### **1.11 Commercial Importance of *Rhodotorula* Species:**

The metabolic capabilities of some *Rhodotorula* species have indicated possible applications of this genus in the commercial industry. Two *Rhodotorula rubra* strains were found to degrade 4-hydroxy-benzoate and as such could be used in oil sludge treatment (Wright and Ratledge, 1991).

Ogrydziak (1993) reported the production of extra cellular proteases by a strain of *Rhodotorula rubra*. It was proposed that these proteases could be used to degrade the proteins responsible for protein hazes that form in wines and beers during storage.

### **1.12 The red yeast, *Rhodotorula rubra*:**

The species *Rhodotorula rubra* was first discovered in 1889 by Demme under the name *Saccharomyces ruber*. Like all *Rhodotorula* species, ascospores or ballistospores are not produced and reproduction is by multilateral budding. As described by Kreger van Rij (1984), *Rhodotorula rubra* assimilates glucose, sucrose, trehalose, raffinose, D-xylose, ribitol, melezitose and succinic acid. Galactose, maltose, cellobiose, L-arabinose, D-ribose, L-rhamnose, D-mannitol and citric acid are assimilated by some strains while lactose, soluble starch, erythritol, inositol, melibiose and nitrate are not assimilated (Kreger van Rij, 1984).

Cells grown in malt extract or on malt agar vary from short ovoidal to elongate, 2-5.5 µm in width, and occur singly, in pairs, short chains or in clusters. Colony color ranges from deep coral to pink or salmon-colored. Colony surface is glistening and usually smooth, but is sometimes reticulate, corrugated and the texture varies from soft to mucous Kreger van Rij, (1984).

The carbohydrate patterns of *Rhotorula rubra* whole cell hydrolysates show the presence of fucose and mannose as the dominant sugars in this yeast, while hexitol and pentitol also occur in high concentrations (Weijman and Miranda, 1988). The total lipid content of *Rhodotorula rubra* is about 6.0% of dry weight, with palmitic acid, oleic acid and linoleic acid as the major lipids (Perrier *et al.*, 1995). This carotenogenic yeast also contains about 100 mg carotenoids/g dry weight, which includes beta-carotene, beta-zeacarotene, torulene and torularhodin as the major components (Perrier *et al.*, 1995). The G+C content is 60 – 63.5 mol% (Nakase and Komagata, 1971).

### **1.13 Description of *Rhodotorula rubra* TP1 :**

*Rhodotorula rubra* strains have been isolated from leaves, flowers, soil, atmosphere and marine sources (Cook, 1958; Ingram, 1955; Kreger van Rij, 1984). Recently a new strain has been isolated from yogurt (Hari *et al.*, 1992). A new strain of red yeast contaminating a home-fermented yogurt was isolated and, using the Analytical Profile Index (API) clinical yeast system, was identified as *Rhodotorula rubra* (Hari *et al.*, 1992). The results confirmed by Microcheck Inc. Northfield, VT using a technique involving cell wall fatty acid analysis. The isolate was named *Rhodotorula rubra* TP1 (Hari *et al.*, 1992). *Rhodotorula rubra* mutants were isolated by Achempong (2000). He used three different mutagens including UV irradiation, ethyl methane sulfonate (EMS) and nitrosoguanadine NTG. He found NTG to be a better mutagen and he was able to isolate 8 mutants of *R.rubra* TP1.

Like other *Rhodotorual rubra* strains, *Rhodotorula rubra* TP1 does not form ascospores or ballistospores, and reproduces by multilateral budding (Sangha, 1994).



However, in one study, Sangha, (1994) observed the presence of ascospores in this strain of yeast. As such, this experiment needs to be repeated. As described by Hari *et al.*, (1992). *Rhodotorula rubra* TP1 assimilates melezitose, melebiose, maltose, mannitol, trehalose, D-ribitol, raffinose, citric acid, sucrose, arabinose, D-xylose, succinic acid, soluble starch, galactose, and nitrate. It is unable to assimilate glucose, erythritol, inositol, rhamnase, cellobiose, and lactose.

Cells grown in yeast extract/malt extract (YM) broth are circular or ellipsoidal and average 2 to 4  $\mu$ m in diameter. Colony color is best described as salmon-colored and the colony surface is glistening and smooth. The absorption spectrum of the pigment from *Rhodotorula rubra* TP1 shows that the pigment belongs to the family of carotenoids.  $R_f$  values of the pigment on a thin-layer chromatography plates were similar to those obtained for standard astaxanthin, while a mass spectrometry analysis showed a molecular mass similar to that of astaxanthin (Hari *et al.* 1992).

#### **1.14 Potential Commercial Applications of *Rhodotorula rubra* TP1:**

Sangha (1994) found *Rhodotorula rubra* TP1 to be an efficient source of pigments and nutrients for aquacultured rainbow trout. The yeast was found to be more economically favorably over *Phaffia rhodozyma*, which has also been successful in pigmenting pen-raised salmonids. However, *Rhodotorula rubra* TP1 has a faster growth rate with greater levels of pigment production compared to *Phaffia rhodozyma* under similar conditions of growth. Moreover, whole cells of *R. rubra* TP1 were able to pigment rainbow trouts but cells of *Ph. rhodozyma* showed no pigmentation (Hari *et al.* 1993). They also found that *Rhodotorula rubra* TP1 could be successfully grown on

various industrial and agricultural by-products for biomass production. This has important implications, as the cost of growing sufficient amounts of yeast cultures for commercial use has always been a concern. These raw material by-products are readily available, relatively low in cost while pure sugars like glucose and sucrose, which are often used for microbial growth in laboratory situations, are too expensive for use on an industrial scale.

#### **1.15 Research Objectives:**

An increased production of carotenoids by microorganisms such as red yeasts will make its industrial applications cost effective and competitive. With this in mind, mutant strains of *R. rubra* TP1 were examined with the following objectives:

- (i) To determine optimal growth conditions for pigment production under the influence of pH, light, temperatures, and different sources of carbon and nitrogen.
- (ii) To investigate efficient methods for optimal extraction of pigments from mutants and parent cells using Freeze-drying, French Press, Sonication and enzymatic cell breakage methods in order to determine their efficiency on pigment recovery.

## CHAPTER 2

### METHODS AND MATERIALS

#### 2.0 Materials

#### 2.1 Chemicals:

Acetone, dimethyl sulphoxide (DMSO), sodium chloride, sulphuric acid, sodium hydroxide, petroleum ether, hexane, iodine crystals, citrate phosphate, ethyl methane disulphate (EDTA), trizma base (Tris HCl), dithiothreitol and beta mercapto ethanol which were purchased from Fisher Scientific Company Ltd., Fair Lawn, N.J., U.S.A. All the chemicals were of Analar Grade and were used without further purification.

#### 2.2 Sources of Microorganisms:

The test strain used in the experiments was *Rhodotorula rubra* TPI from earlier collection from Dr. T. R. Patel's Laboratory, Department of Biology, Memorial University of Newfoundland (MUN), NL, Canada. Mutants strains, Mutant 1 (M1), Mutant 2 (M2) and Mutant 3 (M3) were isolated earlier by Achcampong (2000) working in the same laboratory. These mutants, maintained on Rose Bengal Agar plates (purchased from Difco Laboratories, Detroit, MI, U.S.A.) and stored at 40° C. These were transferred once a month onto new plates. *Rhodospiridium toruloids* (10657) and *Rhodotorula minuta* (10658) were from American Type of Culture Collection (ATCC).

#### 2.3 Peat Extract and Cane Molasses:

Peat extract was a gift from Dr. A.M. Martin's Laboratory, Department of Biochemistry, MUN. Cane molasses was procured from Lalle Nand Inc., Montreal, PQ.

## **2.4 Lysing Enzymes:**

Lysing enzymes from *Trichoderma harzianum*, *Aspergillus species*, *Cytophaga species* and *Rhizoctonia solani* were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.

## **2.5 Media:**

Yeast Malt Broth (YMB), Potato Dextrose Agar (PDA), Bacto Czapek Dox Broth (BCDB) and Rose Bengal Agar base (RBA) were purchased from Difco Laboratories.

## **2.6 Methods:**

### **2.6.1 Preparation of Media and Inoculum:**

Yeast malt (YM) broth was prepared according to the instructions given by the manufacturer. Loop-fulls of yeast from RB agar plates were aseptically added to 10 ml of saline water and vortexed. This suspension was used for inoculating growth media.

### **2.6.2 Growth of Cultures and Harvesting of Yeast Cells:**

Yeast cells were grown in liquid media of different types. Erlenmeyer flasks (2 L) containing 500 ml liquid YM broth were inoculated with yeast suspension and were incubated at 28° C for 5 days in a Psychrotherm Temperature Control Shaker (New Brunswick Scientific Co. Inc., Edison, New Jersey, U.S.A). The cultures were agitated at 150 rpm. Liquid cultures were centrifuged at 10,000 rpm for 10 minutes to pellet the cells. The pelleted cells were used for pigment extraction after washing three times in a saline solution.

### **2.6.3 Optimization of Growth Conditions:**

Different growth conditions such as substrate concentration, initial pH of the culture medium, temperature, light, fermentation time, initial optical density of the inoculum and agitation speed were tested to determine the optimum growth parameters for the mutants of *R.rubra* TP1.

### **2.6.4 Growth on Molasses and Peat Substrates:**

(i) Crude molasses diluted at a ratio of 1: 10 was used to determine the effect of carbon source on pigment production. YM broth medium (500 ml) contained 50 ml cane molasses, as a supplement of carbon source was incubated in 2 L flasks at 28° C on a shaker at a speed of 150 rpm for 4 days. Aliquots (1 ml) were removed in 3 hour intervals and optical density was measured on a Pharmacia LKB Novaspec II spectrophotometer at a wavelength of 600 nm.

(ii) Peat extract diluted at a ratio of 1: 10 was used to determine the effect of nitrogen source on pigment production. YM broth (500 ml) contained 50 ml peat extract as a supplement of nitrogeneous source. Broth cultures were incubated at 28° C on a shaker at a speed of 150 rpm. Aliquots (1 ml) were removed in 3 hour intervals and the optical density was measured using a spectrophotometer.

### **2.6.5 Growth Measurement and Generation Times:**

Growth and biomass of the wild type *R.rubra* TPI and the mutants were measured using methods such as (i) optical density measurement, (ii) dry weight and wet weight determinations. Growth in liquid media were examined as follows: YM broth (500 ml) in

2 L flasks were incubated at 28° C on a shaker (at 150 rpm) after inoculation with 2 ml suspension of the yeast. Aliquots (1 ml) were removed at 3 hour intervals and optical density readings were used to establish growth curves. Generation times were calculated using the logarithmic growth phase of each culture. Readings were taken in triplicates for each yeast sample. After 5 days of incubation the yeast cells were collected by centrifugation in a pre-weighed centrifuge bottles, washed twice with saline, weighed and dried in a hot air oven at 80° C (Oven, Blue M electric company, Blue Island, Illinois, U.S.A.). The dry weight was recorded after three constant readings were observed. Growth table gives the generation time values given by  $T = (t_2 - t_1) / \log (y/x)$ , where  $x = \text{cells/ml at time } t_1$  and  $y = \text{cells/ml at time } t_2$ .

#### **2.6.6 Effect of Temperature on Pigment Production of the *R. rubra* TP 1 mutants:**

To study the effect of different temperatures on growth and pigment production, the culture flasks (70 ml liquid medium in 250 ml flasks) were incubated at 15, 20, 25, 28, 30 and 35° C on a shaker for 5 days. Growth was determined by wet weight and dry weight of the cells.

#### **2.6.7 Effect of Initial pH of the Growth Media on Pigment Production of Mutants:**

To study the effect of different initial pH on growth and pigment production, the pH of the growth medium was adjusted between pH 3 to 10. This was achieved by adding NaOH (1 M) or by adding HCl (1 M) to the broth. Yeast cell suspension (2 ml) was added to 100 ml of YM broth contained in 250 ml flasks. These flasks were

incubated at 28° C on a shaker for 5 days. Growth was determined by the optical density method as well as by the wet weight and dry weight methods.

#### **2.6.8 Effect of Light on Growth and Pigment Production of *R.rubra* TPI Mutants:**

To study the effect of light on growth and pigment production, the culture flasks were incubated in dark or in the presence of light on a shaker at 28° C for 5 days. Biomass-yield was obtained by using wet weight and dry weight methods.

#### **2.6.9 Pigment Extraction:**

##### **2.6.9.1 Extraction using French Press:**

Wet cells (4 g) were placed in the French Press Cell (SLM Instruments, Chicago, Illinois, U.S.A.) and chilled by placing the cylinder in a freezer (- 70° C) for 15 minutes. Partially frozen cells were ruptured at 20000 psi. The broken cell mass was collected in a 125 ml flask and 20 ml of acetone was added to it. After shaking the cells suspension thoroughly the mixture was centrifuged at 5000 rpm in Sorvall RC-5B Plus centrifuge (Dupont-Sorvall Instruments, Newark, DE, U.S.A). The supernatant was decanted into a clear flask and 20 ml fresh acetone was added to the pellet. It was then mixed and centrifuged as before. The extraction protocol is shown in Fig. 4. The acetone extracts were pooled (60 ml, approx.) and filtered through Whatman No 1 filter paper. Carotenoid containing acetone solution was added to 50 ml of n-hexane and mixed in a separatory funnel. Sodium chloride was (0.5%, 100 ml) added to maximize the extraction of the carotenoids. Carotenoid containing hexane solution was concentrated using an evaporator (Roto vapour-R, Brinkmann, Buchi Laborotoriums, Ontario) to 3 ml.

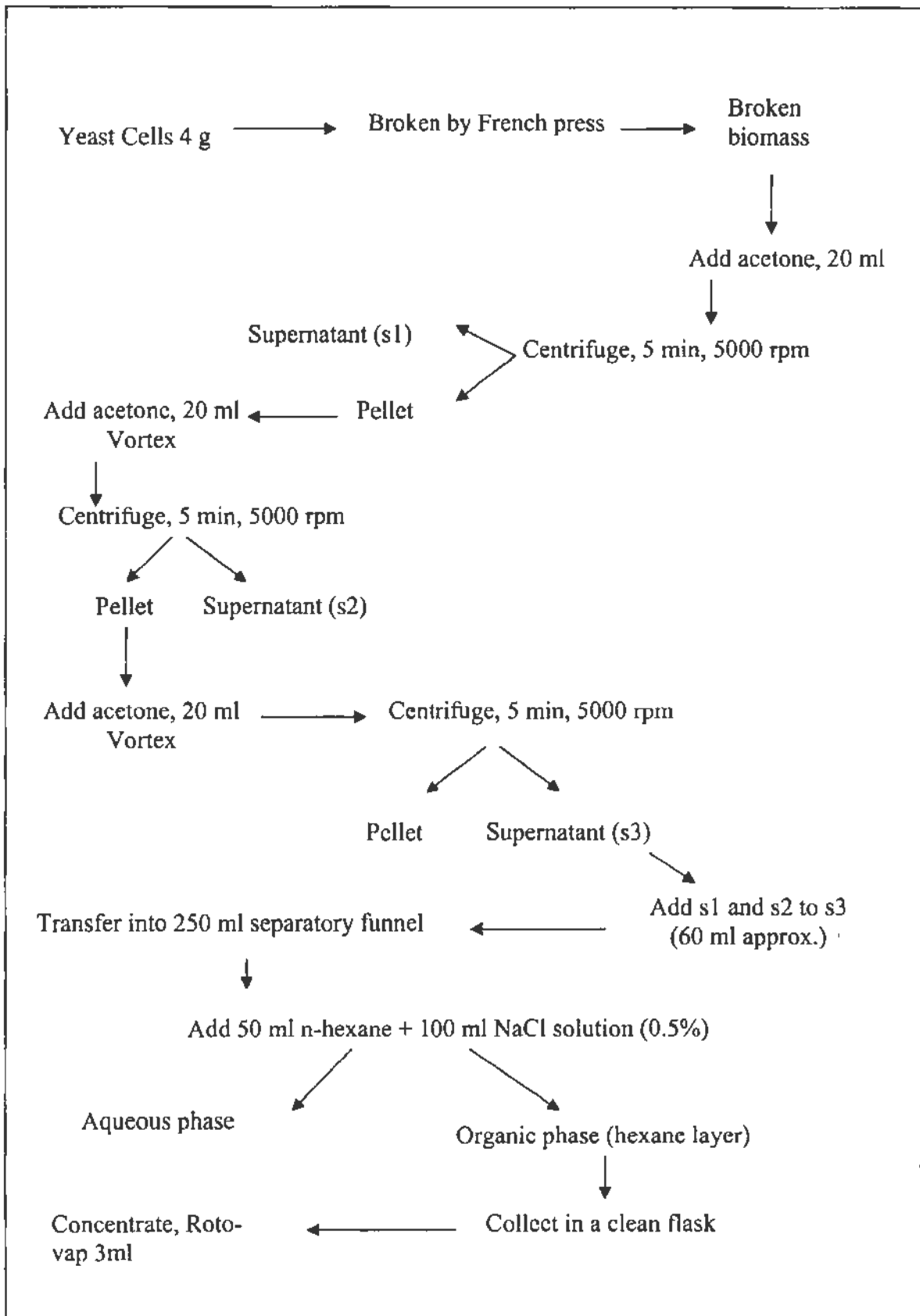


Fig 4. French press procedure for pigment extraction.



The absorption spectrum was recorded in the region 400 to 600 nm using a spectrophotometer (Shimadzu photo spectrometer UV-260, Kyoto, Japan).

#### **2.6.9.2 Extraction by Freeze Drying:**

In freeze-drying methods, 10 g frozen cells were dried using a lyophilizer LABCONCO, Freeze Dry System, Indiana, U.S.A.). The dried powder (1 g) was treated with 6 ml of warmed dimethyl sulphoxide in a 40 ml test tube. The tube was kept in the dark at room temperature for 20 minutes by covering it with aluminum foil. The mixture was centrifuged at 5000 rpm for 5 min and the supernatant was collected. The pellet was extracted with 5 ml of additional acetone and centrifuged as before. The supernatant was collected and the pellet was treated once again with 5 ml acetone and centrifuged. The supernatants obtained were pooled together (15 ml) were filtered through a No.1 Whatman filter paper. Petroleum ether (30 ml) and 15 ml water were added to this filtered supernatant in a separatory funnel. After thorough mixing the organic phase was allowed to separate. The bottom aqueous phase was removed and discarded. The organic phase (30 ml) containing carotenoids was dried with anhydrous sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) and then concentrated using an evaporator to 3 ml as showed in Fig. 5.

#### **2.6.9.3 Extraction by the Method of Sonication:**

In sonication method fresh cells (1 gm) were suspended in 2 ml acetone and sonicated for a period of 3 minutes at intervals of 30 seconds using Braun-Sonic, B Braun, Model 2000 sonicator. The suspension was centrifuged for 5 minutes (5000 rpm). Separate the supernatant (sl) from the pellet and add 2 ml of acetone to it and vortexed.

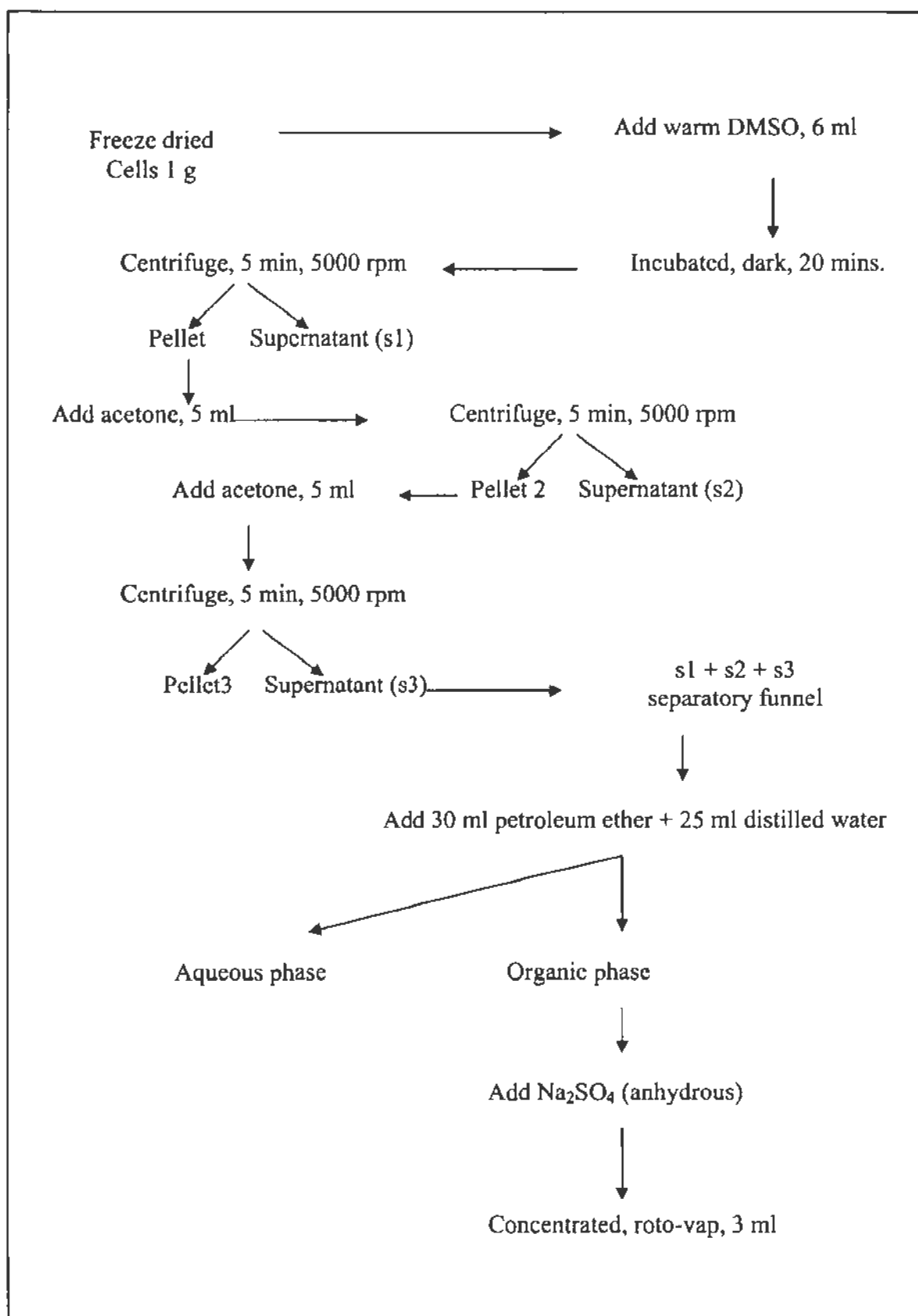


Fig 5. Freeze drying procedure for pigment extraction.

This suspension was again sonicated and centrifuged as before and added the supernatant (s2) to s1. From this acetone extraction mixture 1 ml was taken to run the spectrum for the analysis of the pigment.

#### **2.6.9.4 Extraction using Enzymes:**

In enzymatic cell breakage method 1 g of wet cell mass was suspended in 2 ml of Tris HCl (pH 7) buffer or Citrate Phosphate buffer (pH 7) in a centrifuge tube (15 ml). Lysing enzyme (3.5 mg) was added to the tube and was incubated for 24 hours in a water bath (Precision scientific Company, U.S.A.) at 25° C. Reaction mixtures were centrifuged for 10 minutes, at a speed of 5000 rpm. The supernatant was then decanted off and 2 ml acetone was added to the pelleted cells. It was then vortexed and sonicated for 3 minutes and centrifuged again as before. The acetone layer (supernatant 1, s1) was collected in a fresh bottle, and the pellet was resuspended in 2 ml acetone. After thorough mixing, it was once again centrifuged and the supernatant s2 was obtained, then mixed with s1 in a round bottom flask and concentrated to 3 ml using an evaporator (Fig. 6).

##### **2.6.9.4.1 Freeze and Thaw method:**

In this method yeast cells were frozen at -70° C for 3 hours and were then thawed. Thawed cells (1 g) were separately suspended in 2 ml of Tris HCl buffers with pH ranging between 7 and 9 or citrate phosphate buffer (pH, 5 to 7). These suspensions were treated with a 3.5 mg lytic enzyme (*Rhizoctonia solani*) and were incubated for 24 hours at 25° C in a water bath. The incubated cell suspension was sonicated for 3 minutes and 2 ml of acetone was added to it. This suspension was centrifuged at 5000 rpm, for 10

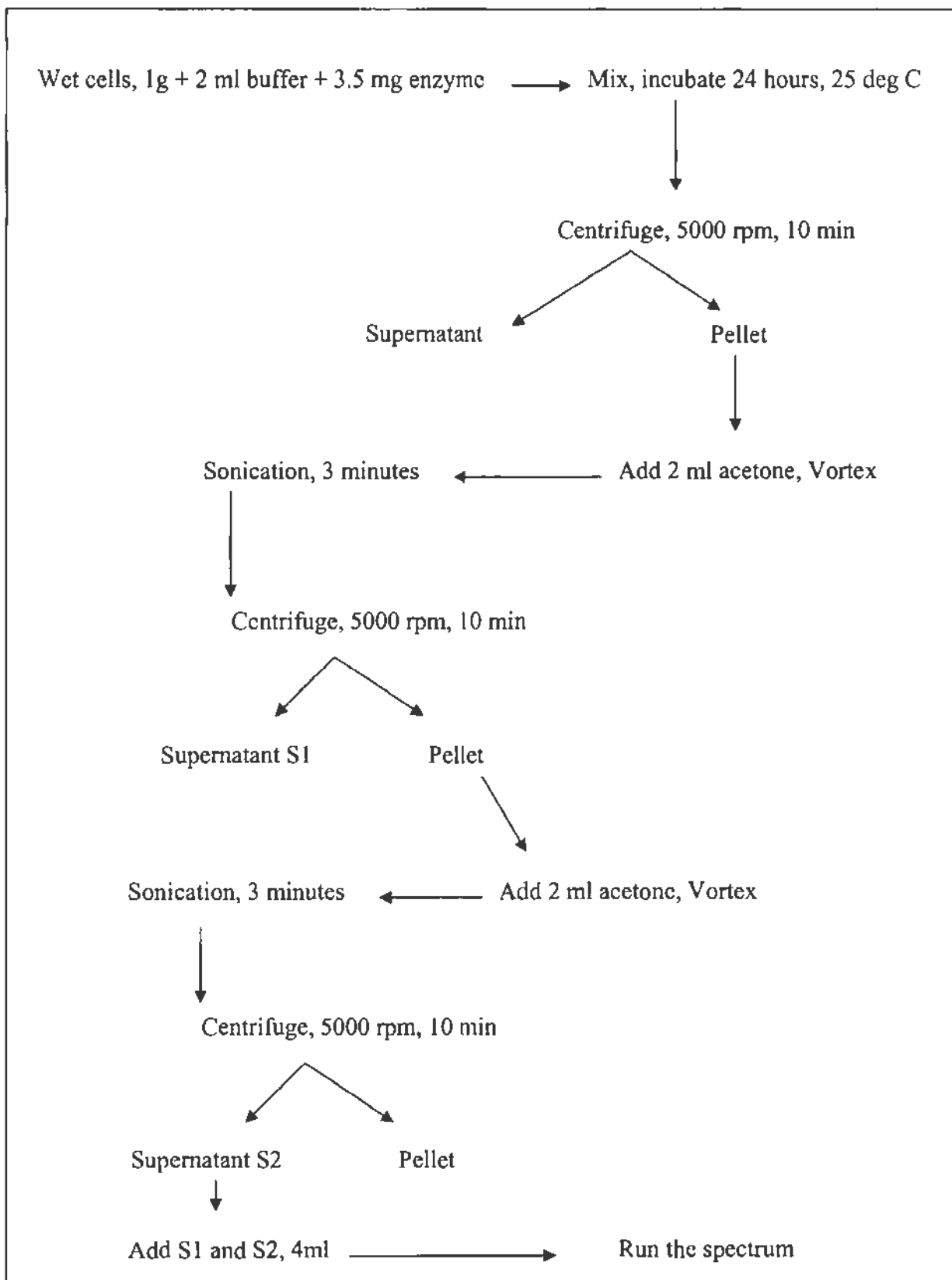


Fig 6. Enzymatic cell-breakage procedure for pigment extraction.

minutes. The supernatant was decanted off and the pellet was suspended in 2 ml acetone. This was vortexed and was sonicated for 3 minutes. The sonicated cell suspension was centrifuged at 5000 rpm for 10 minutes. The supernatant (s1) was separated from the pellet and 2 ml of acetone was again added to the pellet. This cell suspension was sonicated for 3 minutes and was centrifuged as mentioned above. The supernatant (s2) was mixed with s1 (4 ml). Spectrum of this mixture was obtained between 200 – 600 nm. All the experiments were done in triplicates.

#### **2.6.9.4.2 Effect of Thiol Group on Pigment Recovery:**

Reducing agents such as dithiothreitol (DTT) and beta mercapto ethanol (BME) were used at different concentrations to evaluate their effects on pigment extraction. Wet cells (1 g) were separately added to 2 ml citrate phosphate buffer (pH, 7.0) in four different tubes. The concentrations of BME in the buffer were 50 mM, 150 mM and 200 mM. One g of cells were separately added to 2 ml citrate buffer (pH, 7.0) in four different tubes. The concentrations of DTT in the buffer were 15, 20, 25 and 30 mM respectively. To this cell suspension 3.5 mg lysing enzyme was added and incubated for 24 hours at 25° C. The cell suspension was sonicated for 3 minutes and then 2 ml acetone was added to it. This was then centrifuged at a speed of 5000 rpm, for 10 minutes and decanted. Two ml of acetone was then added to the pelleted cells. The cells were vortexed and sonicated for 3 minutes and centrifuged at 5000 rpm for 10 minutes. The supernatant was separated from the pellet and 2 ml of acetone was added to the pellet. It was then vortexed, sonicated for 3 minutes and centrifuged again. The two supernatants were

mixed and the spectrum for the sample-mixture was recorded for the analysis of the pigments.

#### **2.6.10 Measurement of Pigment:**

The total carotenoid concentration in yeast cells was calculated using the formula,

$$\text{Carotenoid}(\mu\text{g})/\text{dry yeast}(\text{g}) = \frac{100AV}{EW}$$

where A is the absorbance maxima at 474 nm, V is the total volume of the sample (ml), E is the extinction coefficient and W is the dry weight of the cells. Since the crude extracts usually contained a variety of carotenoids an average coefficient of 2100 was used in the calculations and the concentrations of the individual pigments were calculated using the method according to An *et al.* 1989. The absorbance values of the pigment extracts in acetone were measured by spectrophotometer. The maximum absorbance determined by scanning from 600 to 300 nm in a Shimadzu Ultra Violet 260 Recording spectrophotometer. Identification of the individual pigments was done by comparison of their absorption maxima with those of standard carotenoids reported by other researchers (Davies, 1976; Bauerfeind and Klaui, 1981).

#### **2.6.11 Thin Layer Chromatography:**

The pigments were separated by means of Thin Layer Chromatograph. Pre-coated silica gel (Whatman International Ltd., Maidstone, England) plates were used to chromatograph the samples. The solvent used was a 10% toluene mixed in 90% petroleum ether (v/v). The spotted TLC plate was developed in this solvent until the solvent front was about 1 cm below the top of the plate. The spots were visualized under

ultra-violet rays and also by iodine vapours.  $R_f$  (Retardation factor) values were calculated by using the ratio of the distance traveled by the substance to the distance traveled by the solvent.

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1 Growth and pigment production by *R.rubra* mutants:

The growth rate was determined in YM broth. *R. rubra* mutants (M1, M2 and M3), showed less growth than the parent TP1. The generation times for mutants M1 and M2 were 12.0 and 11.52 hours, respectively. Figure 7 illustrates the generation times for the wild type and mutant yeasts. The time required for the population to double in the case of TP1 was less, indicating faster growth rate compared to the mutants. *R. rubra* TP1 had a shorter generation time and greater biomass yield than that of *P. rhodozyma*, in an earlier investigation (Sangha, 1994).

The growth curves for the mutants and the wild type organisms at 15<sup>o</sup> C are shown in Fig.8. All the isolates showed a lag period of about fifteen hours as shown in the figure. Figure 9 examines the growth curves at 25<sup>o</sup> C. The cell yields were greater for cultures grown at 25<sup>o</sup> C (Table 1). The time to reach stationary phase for the mutants M1, M2 and M3 were 42.8, 41.2 and 43.2 hours, respectively (Fig. 10). M3 showed more time as parent TP1 to reach stationary phase than the other two mutants, M1 and M2. This figure shows the differences in times to reach stationary phase by the mutants and the wild type yeasts.

##### 3.1.1 Growth on Cane Molasses and Peat Extracts:

Greater biomass yield was obtained upon addition of peat extract to the YM broth. Figure 11 shows the effect of 1 % peat extract, on M1, M2, M3 and TP1. The yields



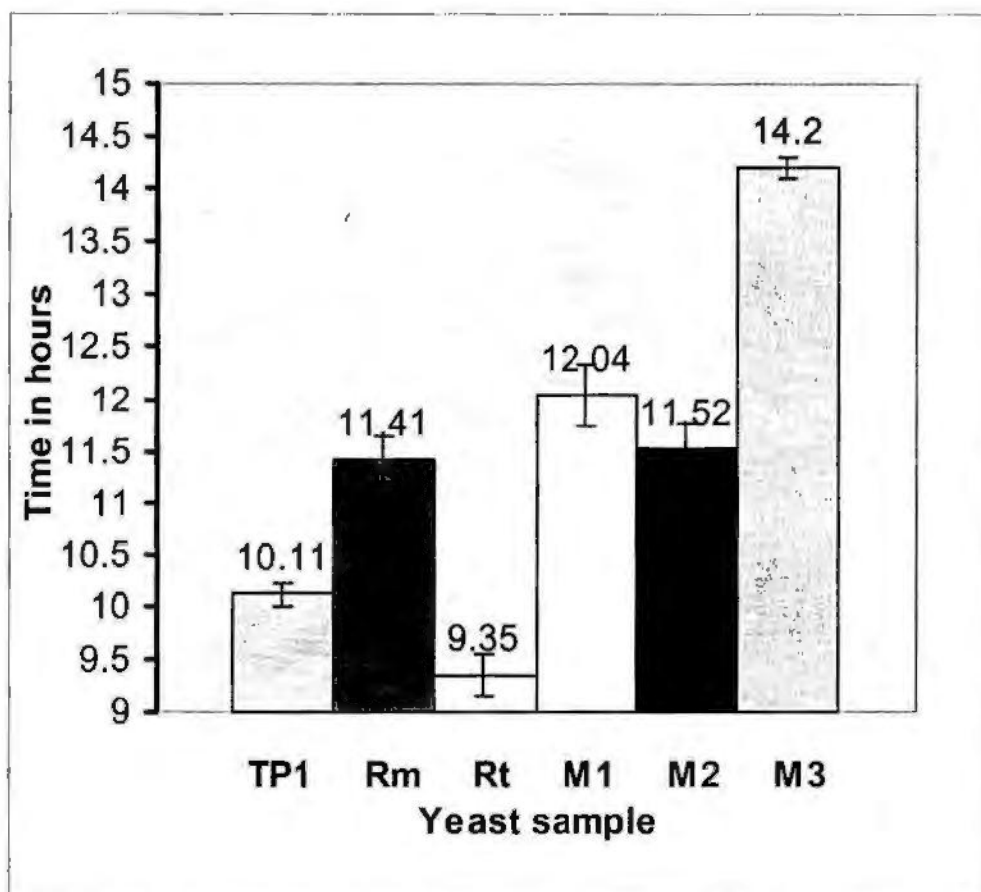


Fig. 7. Generation times of different yeasts.

Yeast cells were growing in liquid media as described under Materials and Methods. Each of the data points represents the mean value of three determinations. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 0.12$ ,  $\pm 0.23$ ,  $\pm 0.20$ ,  $\pm 0.31$ ,  $\pm 0.25$  and  $\pm 0.11$  hour, respectively.

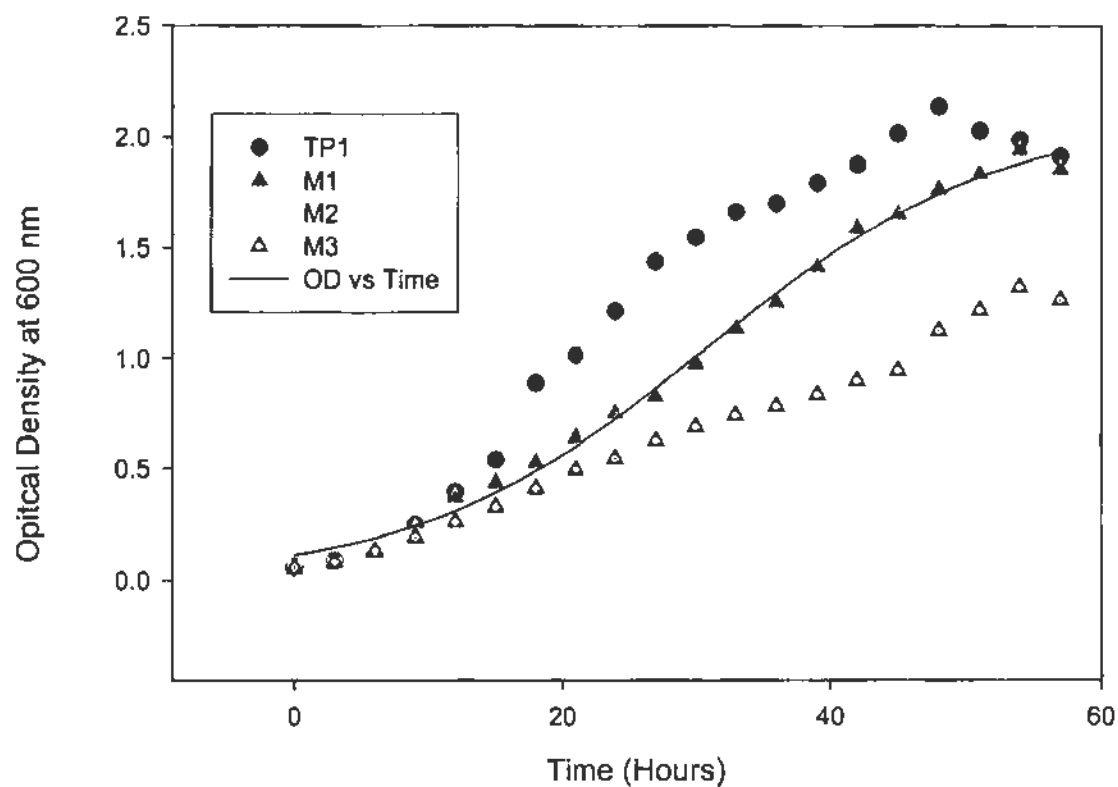


Fig.8 Growth of yeasts on YM-broth at 15° C.

Erlenmayer flasks (500 ml) containing 300 ml of the liquid medium were inoculated with freshly grown yeasts on RB agar and incubated on a Psychrotherm, agitated at 150 rpm. The solid curve is shown for M1 only. The data points are averages of three determinations (standard deviation,  $\pm 0.5$  O.D. units).

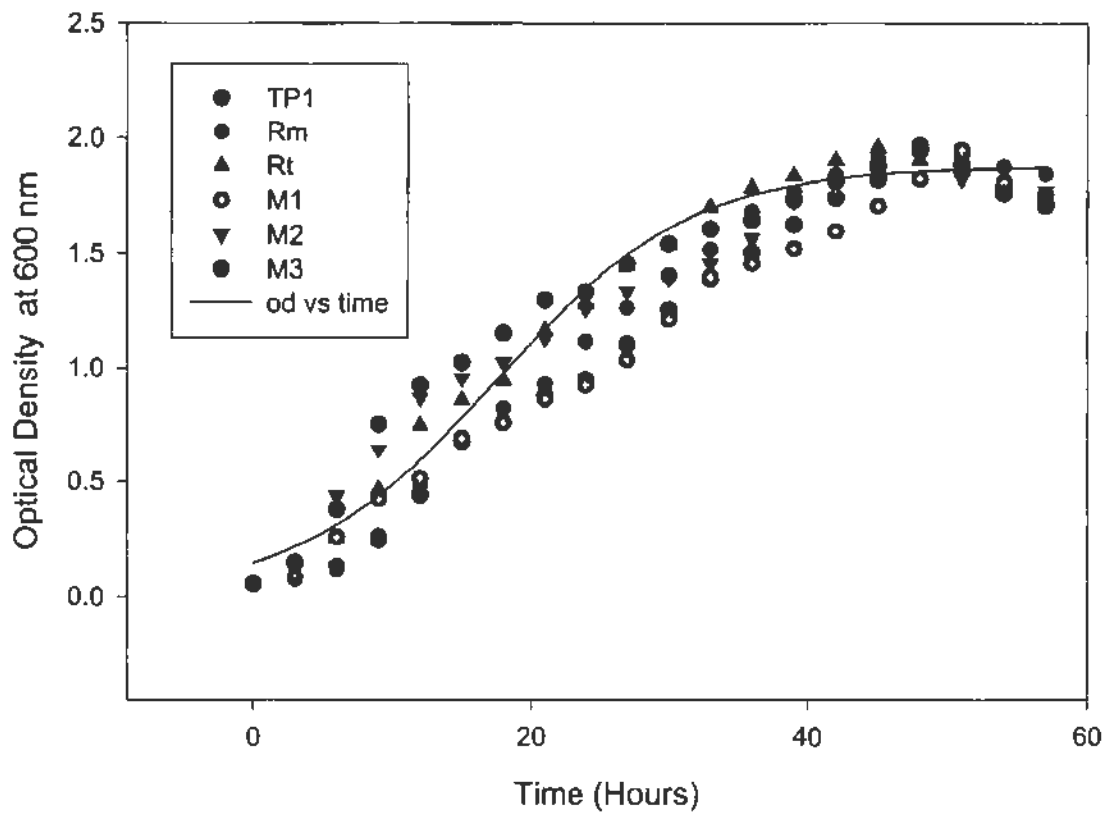


Fig. 9 Growth of different yeasts on YM-broth at 25° C.

Growth conditions were similar to those given in the figure caption for Fig. 8 except temperature. The figure represents growth measured at different time intervals. The solid curve is shown for TP1, yeast only. The data points are averages of 3 determinations (standard deviation,  $\pm 0.5$  O.D. units).

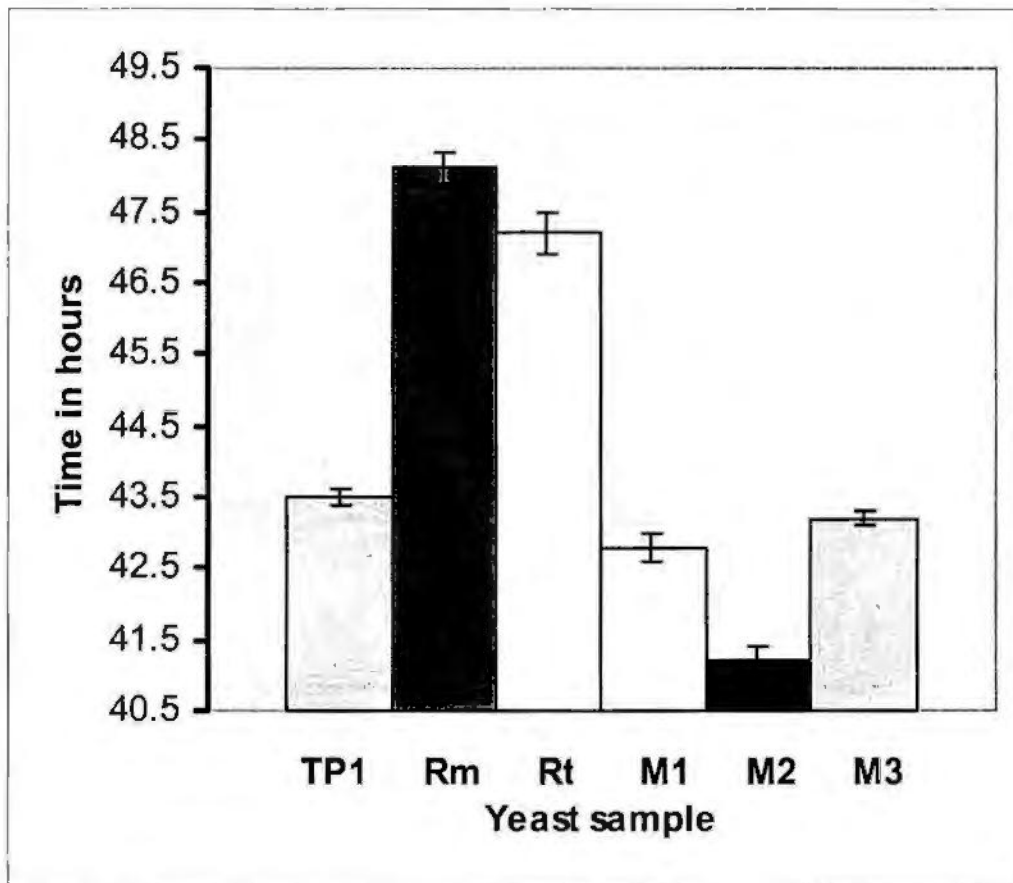


Fig. 10 Time needed for achieving stationary phase by different yeasts in liquid cultures.

Each of the data points represents the mean value of three determinations. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 0.2$ ,  $\pm 0.3$ ,  $\pm 0.3$ ,  $\pm 0.5$ ,  $\pm 0.1$  and  $\pm 0.1$ , respectively.

Table 1. Biomass yield (g/L) in wild type and mutant yeasts at various temperatures.

Yeast	TP1	Rm	Rt	M1	M2	M3
Temp.	Biomass yield					
15	4.0 ± 0.2	4.1 ± 0.3	3.5 ± 0.2	4.6 ± 0.2	3.7 ± 0.1	3.2 ± 0.3
20	8.3 ± 0.2	6.9 ± 0.1	8.0 ± 0.1	7.6 ± 0.1	8.4 ± 0.2	7.3 ± 0.2
25	10.2 ± 0.1	11.2 ± 0.6	12.5 ± 0.4	9.3 ± 0.3	8.6 ± 0.2	8.4 ± 0.2
28	12.5 ± 0.2	12.0 ± 0.4	13.1 ± 0.3	11.0 ± 0.3	10.2 ± 0.2	9.5 ± 0.1
30	9.4 ± 0.2	10.9 ± 0.1	12.7 ± 0.2	7.1 ± 0.1	7.1 ± 0.2	7.6 ± 0.2
35	7.0 ± 0.1	10.3 ± 0.3	11.2 ± 0.3	6.3 ± 0.3	5.9 ± 0.2	6.1 ± 0.2

Yeasts were grown in a liquid media in flasks (500 ml) containing 300 ml YM-broth. Inoculated flasks were incubated at various temperatures (15 to 35° C) separately, in a Psychrotherm shaker at 150 rpm. Cells were collected by centrifugation after 5 days. Wet weights and dry weights were determined in pre-weighed glass centrifuge tubes. Each experimental point represents average of three determinations. Errors given are standard deviations.

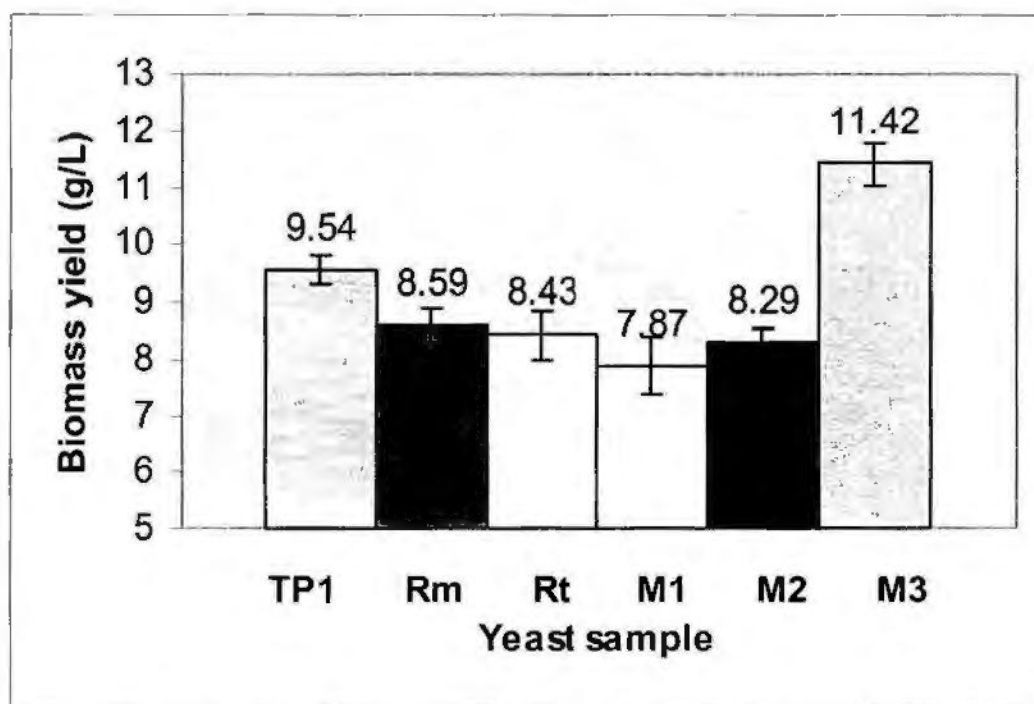


Fig. 11 Biomass yield of different yeasts grown in YM-broth with 1 % peat extract.

Yeast cells were grown in liquid cultures in Erlenmeyer flasks (500 ml) containing 300 ml liquid media inoculated with different yeasts. Culture flasks were incubated at 25° C and shaken in a Psychrotherm at 150 rpm. Yeast cells were harvested by centrifugation as described under Materials and Methods.

Each experimental point represents an average of three readings. The standard error in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 0.25$ ,  $0.32$ ,  $0.42$ ,  $0.51$ ,  $0.23$  and  $0.39$  g/L, dry weight, respectively.

were 7.87, 8.29, 11.42 and 9.54 g/L (dry weight), respectively. The effect of 2 % peat extract in YM broth was observed to be better for larger biomass yields. At this concentration M2 and M3 resulted in biomass yield of 9.71 and 9.84 g/L, respectively.

In contrast under similar conditions the parent TP1 gave 10.12 g/L, as shown in Fig. 12. Further increases in peat extract concentrations for the growth medium did not give corresponding increases in the biomass yields except in the case of M1 (Fig. 13).

The yeasts were able to utilize a wide variety of inorganic nitrogen sources with an optimum growth in the presence of ammonium sulphate and ammonium hydroxide (Sangha, 1994). However, an organic nitrogen source like peptone was assimilated much better than an inorganic-nitrogen source (Sangha, 1994). Abour-Zeid and Yousef (1972) also reported similar behavior with *Streptomyces caespitosus*. The yeast preferred molasses may be because of the presence of lower amount of reducing sugars in the peat hydrolysate. Anderson (1979) grew *Candida utilis* on a commercial scale using sulfite waste liquor. Nitrogen supplementation of sulphite waste liquor in the form of urea or ammonium sulfate and phosphorus as phosphoric acid was found to enhance the biomass yield and substrate consumption (Simard and Cameron, 1974).

Figure 14 shows the effect of different concentrations of cane molasses in YM broth on biomass yield. With 1 and 2 % cane molasses the mutants and the parent organism showed much greater cell growth than with the higher concentration (3 %). The biomass yield by mutants showed considerable variation as shown in figure 14.

Earlier workers found that cane molasses were better than beet molasses in supporting the growth of the yeast (Peppler, 1979). It is postulated that the higher

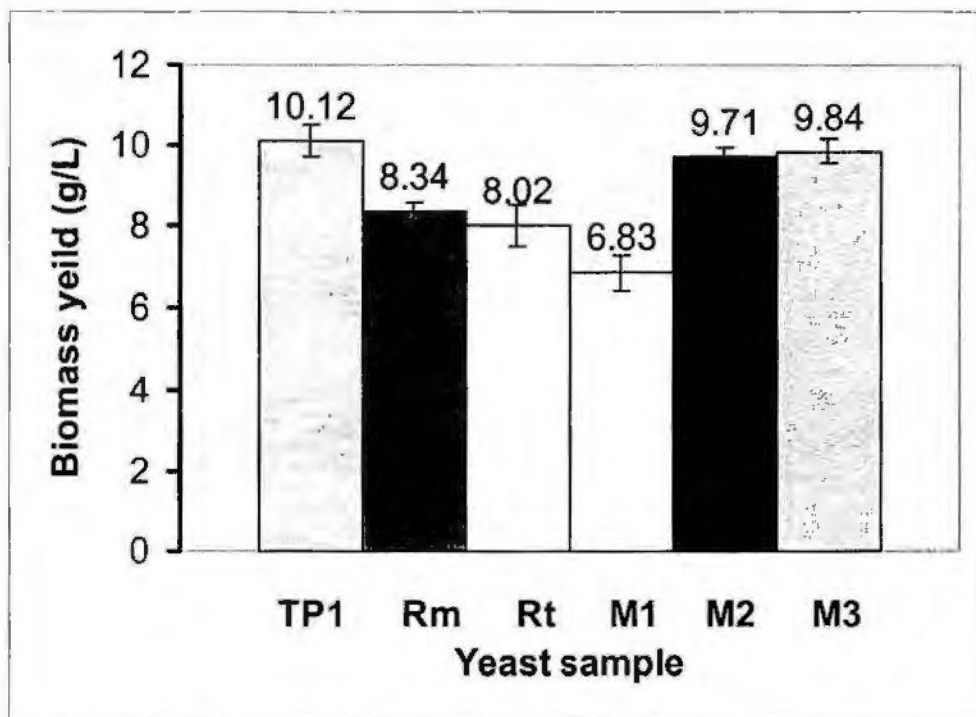


Fig. 12 Biomass yield of yeast cells grown in YM-broth with 2 % peat extract.

Growth conditions were similar to those described for caption for Fig. 11 except the concentration of peat extract in the growth medium.

Each experimental data represents an average of three readings. The standard error of mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 0.39$ ,  $0.23$ ,  $0.51$ ,  $0.42$ ,  $0.23$  and  $0.32$  g/L, dry weight, respectively.



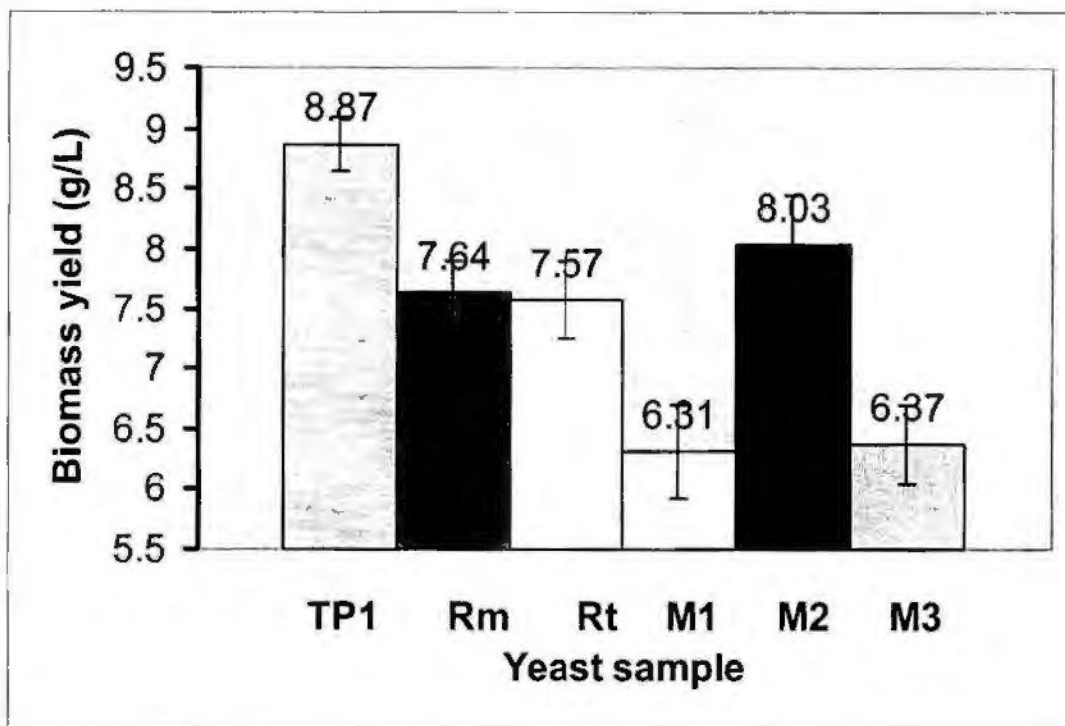


Fig. 13 Effect of 3 % peat extract on the biomass yield of yeast cells.

Growth conditions were similar to those described for caption for Fig. 11 except the concentration of peat extract in the growth medium.

Each experimental data represents an average of three readings. The standard error of mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 0.23$ ,  $0.25$ ,  $0.32$ ,  $0.39$ ,  $0.42$  and  $0.32$  g/L, dry weight, respectively.

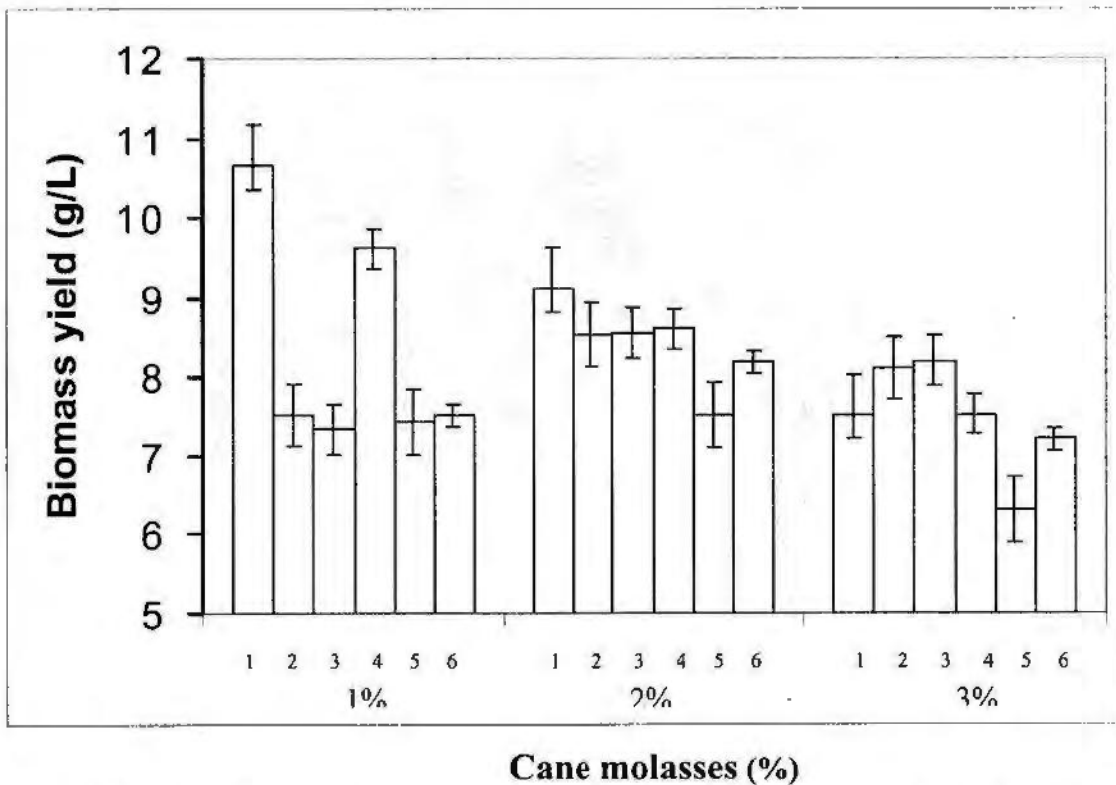


Fig. 14 Effects of cane molasses concentrations on the biomass yield in yeasts.

(1) TP1 (2) Rm (3) Rt (4) M1 (5) M2 (6) M3

Yeasts were grown in YM-broth plus different concentrations of cane molasses as indicated. Liquid cultures were grown as described under Materials and Methods. Each experimental point represents an average of three readings. The standard errors of mean for TP1, Rm, Rt, M1, M2 and M3 are 0.51, 0.42, 0.32, 0.25, 0.42 and 0.14 g/L dry weight, respectively.

content of sugars (55 – 62 % compared to 48 % for beet molasses) in cane molasses may have a role in growth of yeast. Cane molasses are also richer in biotin, pantothenic acid, thiamin, magnesium and calcium (Pepler, 1979). These may also have a stimulatory effect on growth parameters of a yeast. Cane molasses have been used as a substrate for the production of bio mass. Rolz (1984) reported a fed batch system with molasses to optimize cell yields and substrate utilization using *S. cereviceae*. Estevez and Almazan (1973) used a continuous culture system with high test-molasses and crude sugarcane juice as the substrate and reported excellent biomass yields. Moreira *et al.* (1976) supplemented molasses with urea and inorganic phosphorus to grow *R. gracilis* and *Candida utilis*.

In the present study also the highest biomass yield was obtained with the YM broth medium (Difco) than with YM broth supplemented with cane molasses. All three mutants responded similarly. The presence of growth-limiting impurities in the molasses plus the deficiency of some nutrients may account for the differences in growth response of the yeast. Molasses and wort also have been used to grow *P. rhodozyma* (Okagbue and Lewis., 1985) while wort has been used to boost astaxanthin production by the same yeast (Johnson and Lewis, 1979).

### **3.1.2 Effects of the Media on Biomass yield:**

Bacto Czapek Dox Broth (BCDB) was used to grow the yeast cultures. It was found that there was very less growth and practically no pigment. Rose Bengal agar was found to be better medium and promoted greater growth when compared to BCDB.

*R. rubra* TP1 was found to grow readily on the common laboratory media while *P. rhodozyma* showed reduced growth on many of the media tested, including PDA, SDA and malt agar (Sangha, 1994). The mutants, M1, M2 and M3 did not grow either in Bacto Czapek broth or on Sabroux dox agar plates. Under similar and the same conditions and the same substrates, the parent TP1 showed reduced growth with no pigment production.

### 3.1.3 Effects of Temperature:

The optimum temperature was found to be 28° C for the parent organisms and the mutants. At this temperature, both, cell biomass as well as pigment production was enhanced with all test organisms (Table 1). The extracted pigment from these cells was analysed by using thin layer chromatography (Table 2). From the table it is evident that mutants produced four of the carotenoids present in the wild type, TP1 but lacked phytoene. The R<sub>f</sub> values determined for the individual carotenoids also coincided with those reported in literature (Acheampong, 2000). The differences in R<sub>f</sub> values observed for pigments in mutants and wild –type yeasts were very minute.

In a study using mutants of *R. mucilaginosa*, Villoutreix (1960) reported that torulene, torularhodin,  $\gamma$ -carotene and  $\beta$ -carotene were the principal pigments of the parental strain, whereas phytoene and phytofluene were absent. Nakayama *et al.*, (1954) also examined the pigments from several *Cryptococcus* and *Rhodotorula* species and concluded that quantities of the red and yellow pigments varied depending on cultural condition specially, by temperature. According to these authors the concentrations of the red pigments decreased at 5° C but increased as the temperature was increased.

Table 2. R<sub>f</sub> values of carotenoids from various yeasts .

Yeast		TP1	M1	M2	M3
Carotenoids	R <sub>f</sub> values Literature *	Experimental R <sub>f</sub> values			
Phytoene	0.84	0.86 ± 0.1 #	-	-	-
Phytofluene	0.62	0.62 ± 0.05	0.64 ± 0.02	0.61 ± 0.05	0.62 ± 0.03
β-carotene	0.50	0.49 ± 0.06	0.48 ± 0.07	0.51 ± 0.06	0.50 ± 0.07
Toluene	0.30	0.32 ± 0.04	0.30 ± 0.05	0.28 ± 0.02	0.29 ± 0.04
Torularhodin	0.19	0.19 ± 0.03	0.16 ± 0.04	0.17 ± 0.03	0.18 ± 0.06

Pre-coated silica gel plates were used to spot the yeast samples. The solvent is the mixture of 10 % toluene in petroleum ether. Pigments were visualized by Iodine vapour and the R<sub>f</sub> values were calculated.

# Each of the data point is an average of 3 determinations.

\* Reported values are from Acheampong, 2000.

Inspection of the carotenoid composition of *R. rubra* TP1 as well as the mutagenized cells allowed the identification of most of the carotenoid previously described in other *Rhodotorula* species (Bonner *et al.*, 1946; Hayman *et al.*, 1974., Simpson *et al.*, 1964). In the present study, torulene, torularhodin and  $\beta$ -caroten were determined to be the major pigments produced by the new yeast isolate *R. rubra* TP1, as reported earlier (Hari, 1994). In *Rhodotorula* and *Rhodospiridium* species, carotenoids, torulene and torularhodin are produced in high amounts even though several others carotenoids including  $\beta$ ,  $\gamma$  and  $\delta$ -carotene, phytoene, phytofuluene and  $\beta$ -zeacarotene may also be present (Ciegler, 1965; Hayman *et al.* 1974).

In a study to re-examine the pigments produced by *R. glutinis* strain 48-23T which had been studied earlier by Nakayama *et al.*, (1954). Simpson *et al.*, (1964) reported that the total carotenoid concentration, on a dry weight basis, was nearly equal at both room temperature and 5° C, however, the level of torulene and torularhodin coupled with a decrease in the levels of  $\beta$ -carotene when the yeast was cultured at a higher temperature, 25° C. The gain in the levels of torulene and torularhodin were nearly equal to the decrease in the level of  $\beta$ -carotene. According to Simpson and group (1964), these results suggest that  $\gamma$ -carotene lies at the branch point in the carotenoid biosynthesis sequence, and that intermediates can be channelled through it either to  $\beta$ -carotene or to the red pigments, torulene and torularhodin, depending on the growth temperature. Similarly, in *R. pallida* 62-506, it was shown that there was an increase in the level of torulene and torularhodin as the level of  $\gamma$ -carotene decreased.

### 3.1.4 Effects of Initial pH:

Optimum biomass production was achieved when the initial pH range was between 5 and 7 (Table 3). The differences in biomass yields of all the mutants and the parent were statistically significant. In an earlier study (Sangha, 1994) it was found that the amount of growth decreased for *R. rubra* TP1 as the pH increased from 7.2 to 10. This was also the case in the present study. Sangha (1994) also reported that yeasts had difficulty in growth at pH between 3.0 and 4.9.

### 3.1.5 Effects of Light:

It is known that light influences pigment production in biological systems. The effect of light enhanced biomass yields and pigment production in the parent and the mutant yeasts. Mutants M1, M2 and TP1 gave high carotenoid concentration than mutant M3. Figure 15 shows the effect of light on total carotenoid concentrations in yeasts grown at 28<sup>0</sup> C in YM broth. The light source was a tungsten lamp in a Psychrotherm. Effect of darkness on pigment production was observed by growing the yeast in dark brown flasks and incubated in a psychrotherm with lights turned off. The pigment concentrations in yeast grown were very low in darkness compared to that of samples grown under light. Figure 16 shows the total carotenoid concentration (TCC) of all the yeast samples tested. Parent TP1 and M2 showed almost the same TCC, 148.4 and 152.5 µg/g (dry weight), respectively.

Girard *et al.* (1994) observed that yellow mutants of *P. rhodozyma* accumulated high concentrations of β-carotene while the white mutants produced no carotenoids. In *Rhodotorula* species, γ-carotene produced in then biosynthetic pathway is usually

Table 3. Effect of initial pH on biomass yeilds- in yeasts.

Yeast	TP1	M1	M2	M3
Initial pH	Biomass yeild (g/L)			
3	5.2 ± 0.1	4.8 ± 0.1	4.4 ± 0.3	3.8 ± 0.6
5	5.9 ± 0.1	5.2 ± 0.1	5.6 ± 0.2	4.8 ± 0.3
7	8.8 ± 0.1	7.9 ± 0.1	7.4 ± 0.2	6.7 ± 0.3
9	5.4 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	3.8 ± 0.2

Yeasts were grown in pre-adjusted pH liquid media flasks (500 ml) containing 300 ml YM-broth. Inoculated flasks were incubated at 25° C in a Psychrotherm shaker (150 rpm). Cells were collected by centrifugation after 5 days. Wet weights and dry weights were determined in pre weighed glass centrifuge tubes. Each experimental point represents an average of three readings. Errors given are standard deviations.



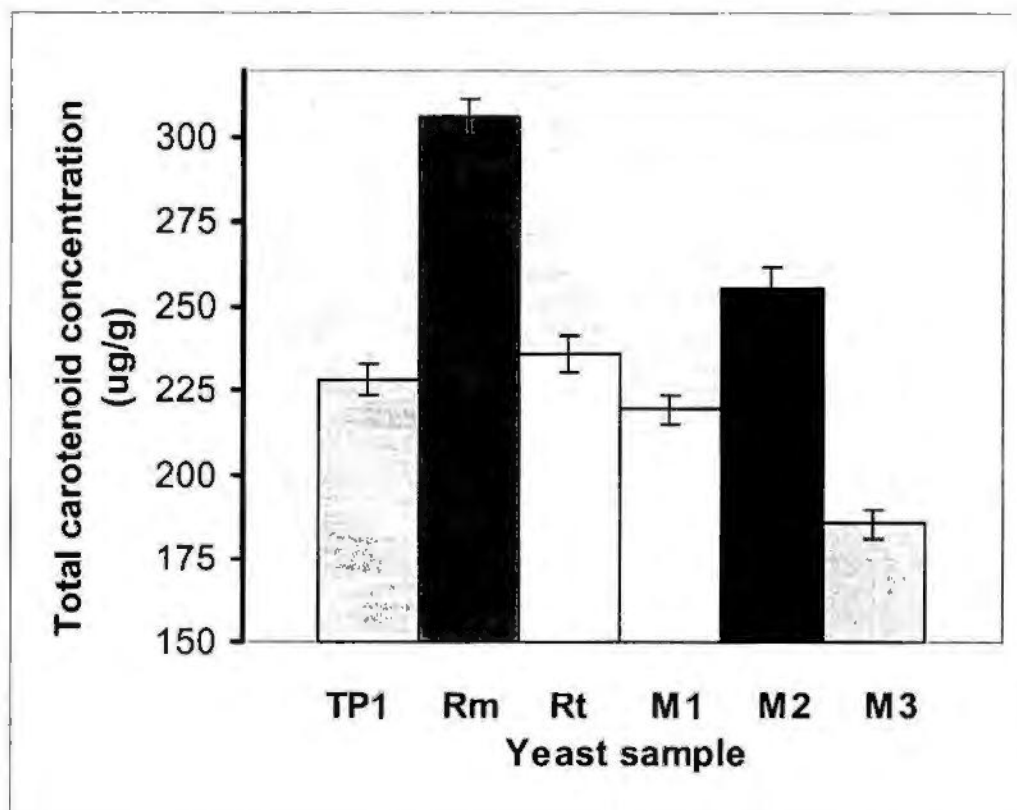


Fig. 15 Effect of light on total carotenoid concentration in different yeasts.

Yeasts were grown in liquid media in flasks (500 ml) containing 300 ml YM-broth as described under Materials and Methods. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 4.6$ ,  $5.3$ ,  $5.6$ ,  $4.5$ ,  $6.2$  and  $4.3$ ,  $\mu\text{g/g}$ , dry weight, respectively.

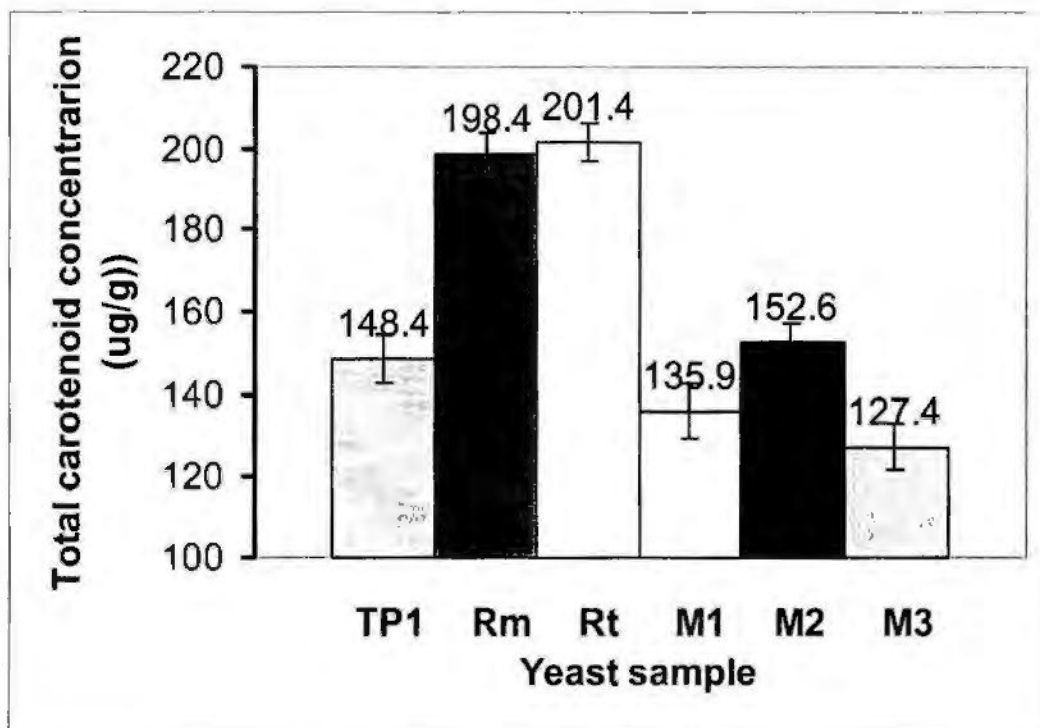


Fig. 16 Effect of darkness on total carotenoid production in different yeasts.

Yeasts were grown in liquid media in flasks (500 ml) containing 300 ml YM-broth as described under Materials and Methods. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 5.2$ ,  $5.7$ ,  $4.7$ ,  $5.4$ ,  $4.8$  and  $5.4$ ,  $\mu\text{g/g}$ , dry weight, respectively.

transformed to yield either  $\beta$ -carotene or torulene (Goodwin, 1965). The torulene can then be oxidized to form torularhodin (Simpson *et al.* 1964). In a study (Kayser and Volloutreix, 1961), it was found that the isolated  $\beta$ -carotene over producing mutants were similar to  $\beta$ -carotene accumulating mutants of the yeast *R. glutinis*.

Although the mutants appeared to produce pigments similar to those encountered in the parent strain the total quantity was reduced in them. Girard *et al.*, (1994) postulated that low pigment production in mutant is due to inhibition of the early steps of carotenogenesis and the enzyme, phytoene synthetase, may be affected. In the present study mutants M2 and M3 in which  $\beta$ -carotene in the total carotenoid content was detected may represent similar condition.

Spectral analysis (Table 4) and the wild-type reported that all the mutants contain torulene, torularhodin and  $\beta$ -carotene. Simpson *et al.* (1964), in their investigation of pigment production in *P. rhodozyma* reported that  $\gamma$ -carotene is converted to torulene which is in turn converted to torularhodin. Nakayama *et al.* (1954) determined the content of individual carotenoids present in several species of *Rhodotorula* and reported that torulene, torularhodin,  $\beta$ -carotene and  $\gamma$ -carotene to be the principal pigments in these yeasts. Although all investigators agree on the presence of these three components, torulene, torularhodin, and  $\beta$ -carotene, the data with respect to concentrations reported was variable. Perhaps, all these researchers used different strains of yeast and cultural conditions in their studies.

Since it has been reported that pigment composition depends on the strain of yeast and particular cultural conditions (Nakayama *et al.*, 1954; Kavanikov *et al.*, 1978; Bonner *et al.*, 1946), the variations in the concentration of the different pigments should

Table 4. Absorption maxima ( $\lambda$  max) of carotenoids from different yeasts.

Carotenoid	Literature value ( $\lambda$ max)	Absorption peaks ( $\lambda$ max)
Torularhodin	465, 501, 537 a*	467 (M1), 502 (M3), 535 (M2)
Toluene	454-460, 480-484, 513-518 b*	454 (M1), 483 (M3), 516 (M2)
$\beta$ -carotene	425, 448-453, 475-482 a*	426 (M1), 451 (M3), 476 (M2)

a\* Goodwin, 1955; b\* Ligen-Jensen, 1965.

Absorption spectra were obtained on a Shimadzu spectrophotometer using visible light between 400 - 600 nm.

M1: Mutant 1., M2: Mutant 2., and M3: Mutant 3.

not be surprising. In the current work the major pigments produced by these mutants have been identified to be torulene, torularhodin and  $\beta$ -carotene. The findings of present investigations are supported by the earlier work (Simpson, 1964; Nakayama *et al.*, 1954; Kavanikov *et al.*, 1978 and Bonner, 1946).

### **3.2 Effect of Extraction Methods on Pigment Recovery:**

#### **3.2.1 French Press:**

Mechanical disruption of yeasts has traditionally been accomplished by using either a French press or a Bead beater. In the present study, the recovery of carotenoids from wild-type yeasts, TP1, Rm, Rt and mutants M1, M2 and M3, were 326.7, 277.5, 291.6, 245.4, 242.5 and 193.2  $\mu\text{g/g}$ , dry weight yeast, respectively, using French press method (Fig. 17). Mutants M1 and M2 showed comparable pigment recovery. The least yield of pigment recovery was obtained with M3 i.e. 59.1 % compared to that from TP1. The wild type yeasts gave superior yields compared to the mutant yeasts.

#### **3.2.2 Freeze-drying:**

Figure 18 shows the recovery of carotenoids from freeze-dried cells of TP1, Rm, Rt, M1, M2 and M3. The yields were 282.2, 196.3, 243.5, 158.6, 237.4 and 214.4  $\mu\text{g/g}$ , dry weight yeast, respectively. In the present study it was observed that M2 gave higher recovery than other two mutants M1 and M3. Compared to yield from TP1, the recovery of carotenoids from M3 was 84 % while those from M1 and M3 were 56.2 and 75.8 %, respectively. The yeasts, Rt and M2 gave comparable yields of carotenoids with values of 86.1 % and 84.1 %, respectively, compared to that from TP1. M3 gave slightly better

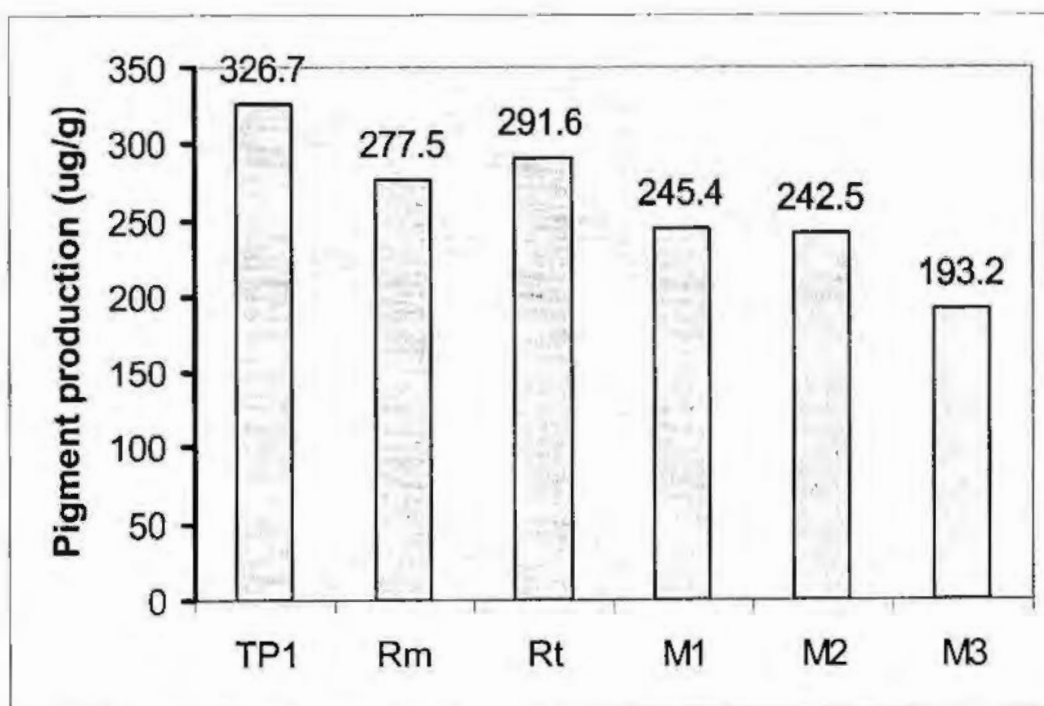


Fig. 17 Effects of French press method on pigment recovery in different yeasts.

Known amounts of yeast cells were broken in a French press and the broken cell mass extracted with acetone as described under Materials and Methods.

The values represent averages of three determinations. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 8.1$ ,  $4.7$ ,  $9.2$ ,  $5.4$ ,  $6.2$  and  $4.2 \mu\text{g/g}$ , dry weight, respectively.

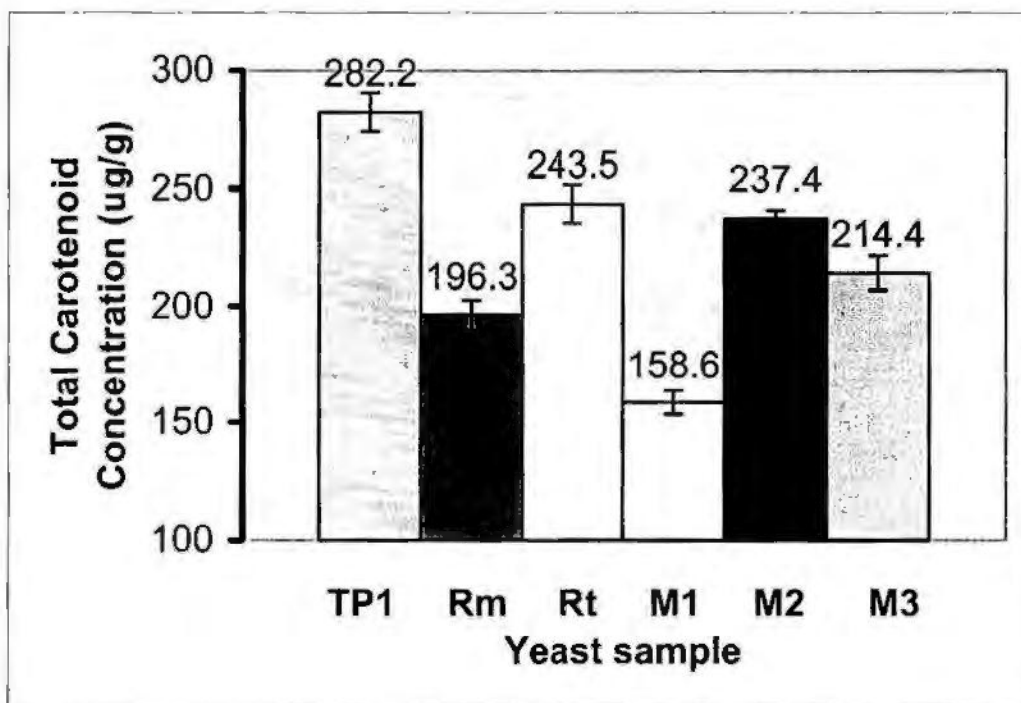


Fig. 18 Pigment extractability using Freeze drying method in yeasts.

Known amounts of frozen yeast cells were lyophilized for 24 hours and the dried powder was treated with DMSO and petroleum ether. The pigment was collected by centrifugation and the total carotenoid concentration was achieved by using spectrophotometer results as described under Materials and Methods. The values represent averages of three determinations. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm 8.2$ ,  $6.3$ ,  $8.1$ ,  $5.2$ ,  $3.4$  and  $7.3 \mu\text{g/g}$ , dry weight, respectively.

recovery of pigments than wild type yeast, Rm. Compared to other yeasts M1 gave least amounts of pigment recovery, with a value of only 56.2 %.

### **3.2.3 Sonication:**

Figure 19 shows pigment recoveries from various yeasts broken by sonication. Pigment recoveries from Rt, Rm and M1 were 98.7, 86.5 and 92.3 %, respectively, when compared to the parent organism, TP1. Wild type yeast, Rt showed 1.3 % reduction and M1 showed 7.7 % reduction in their pigment recovery with values compared to that from TP1. Pigment recovery from Rm and M2 were similar with values of 86.5 and 84.0 %, respectively, when compared to the recovery from TP1. Least quantity of carotenoids were released from M3 with a value of 77.6 % compared to that from TP1.

Figure 20 shows comparative recovery of carotenoids using different extraction methods. It is evident from the figure that French press method is superior compared to the other two methods. The recovery of carotenoids from TP1, using French press method was greatest (326  $\mu\text{g/g}$ , dry weight). In contrast other methods decreased the amounts of carotenoids released. Example, yields using sonication method and freeze-drying method were 37 % and 86.4 %, respectively, compared to that from French press method. Compared to the other extraction methods, pigment recoveries from M1 and M3 were greater with French press method. Yields with sonication method for M1, was only 46 % while that with freeze-drying method was 64.6 % of the value obtained with the French press method (Fig. 21). Similarly percentile pigment recovery from M2, revealed decreased values with sonication and freeze-drying methods. Values observed were 57.5 % and 47 %, respectively (Fig. 22). In the case of M3, surprisingly, the highest yield of



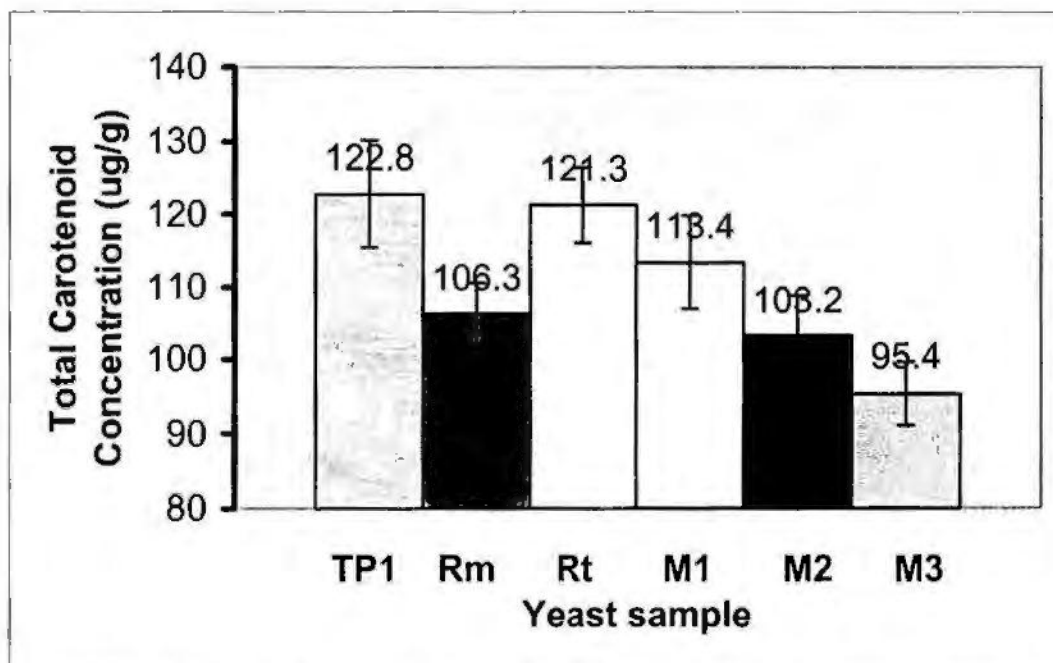


Fig. 19 Pigment extractability using sonication method in different yeasts.

Known amounts of yeast cells were treated with enzyme and incubated for 24 hours. This cell mass was sonicated for 3 minutes with 30 second intervals for each. This cell debris was treated with acetone and the pigment was collected by centrifugation as described under Materials and Methods. The values represent averages of three determinations. The standard errors in the mean for TP1, Rm, Rt, M1, M2 and M3 are  $\pm$  7.6, 4.3, 5.2, 6.4, 5.7 and 7.2  $\mu\text{g/g}$ , dry yeast, respectively.

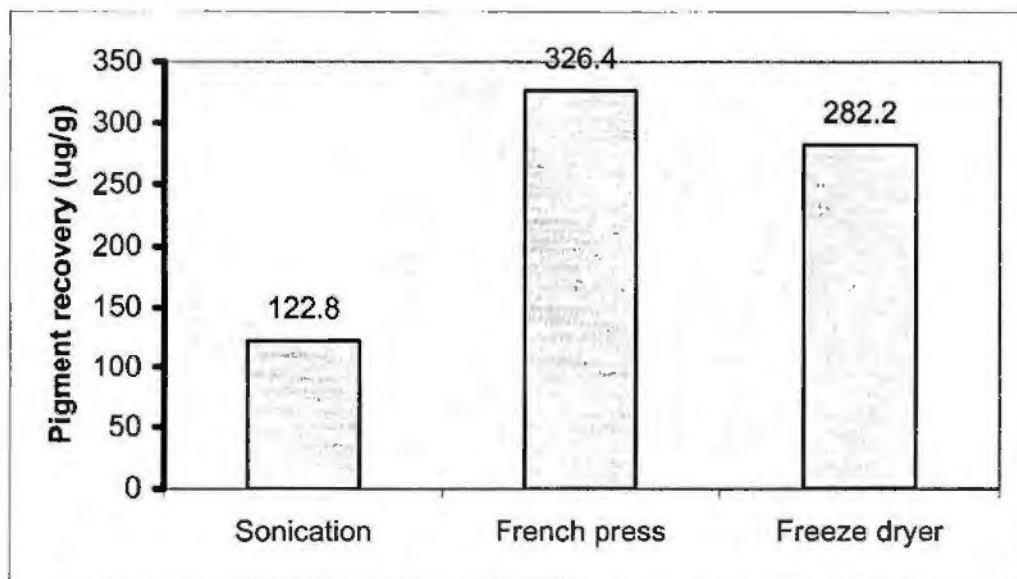


Fig. 20 Effects of extraction methods on pigment recovery from TP1, yeast.

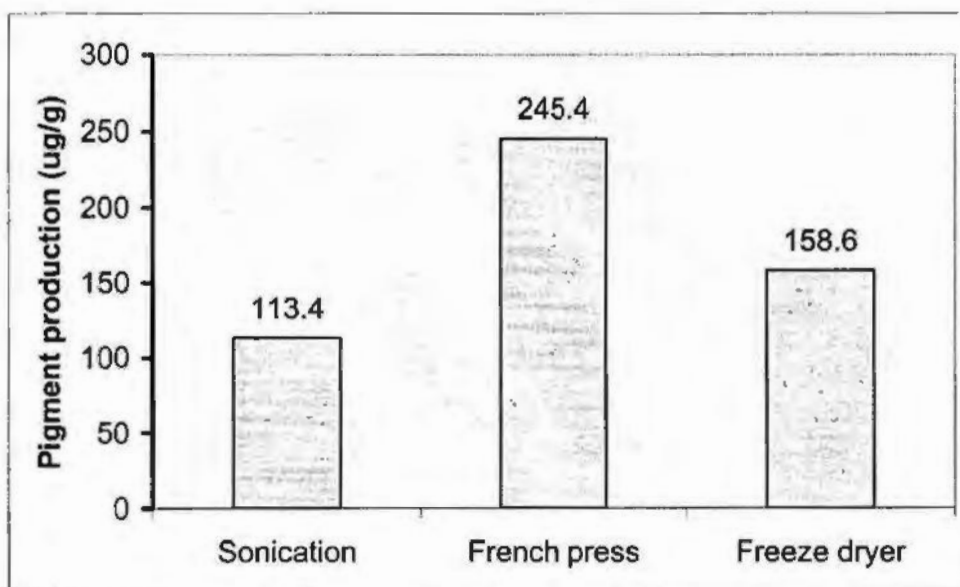


Fig. 21 Effects of extraction methods on pigment recovery from M1, yeast.

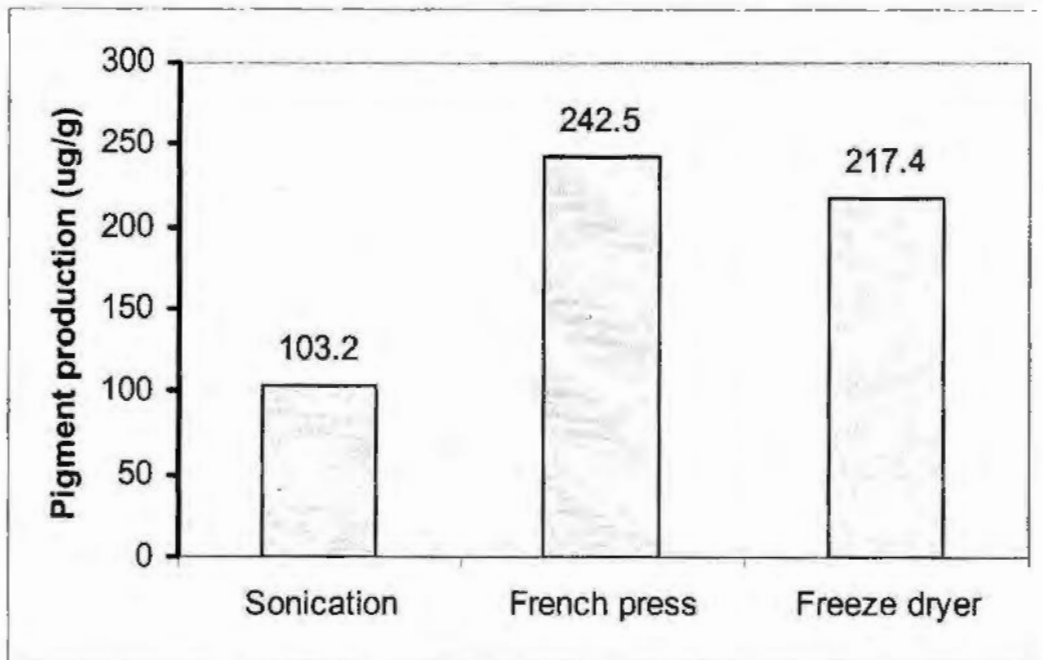


Fig. 22 Effects of extraction methods on pigment recovery from M2, yeast

carotenoids was obtained with freeze-drying method (Fig. 23). The percentile recoveries obtained with the French press and sonication method were 44.4 and 90 %, respectively, compared to that with the freeze-drying methods. Results of extraction of carotenoids from Rm using the three methods as described above are illustrated in Fig. 24. From this figure it is evident that French press method is superior compared to the other two methods. Pigments recovered using sonication and freeze drying methods represent 38.3 % and 70.7 %, respectively, when compared to quantity of pigment released using the French press method. Figure 25 compares quantities of pigment released from Rt with the three methods. Once again French press method caused highest amounts of pigments to be released from this yeast. The amounts of pigments released with sonication and freeze drying methods represented 41.6 % and 83.5 % compared to the French press method. Hari (1994) compared pigment release from *P. rhodozyma* and *R. rubra* using French pressure methods and found that the pigment yield from *R. Rubra* was more (2.07 mg/L of medium) than from *P. rhodozyma*(1.21 mg/L medium).

Several methods have been attempted to enhance pigment extraction from *P.rhodozyma* including mechanical breakage and chemical (acid or alkaline) hydrolysis (Simpson *et al.* 1971). These investigators found both methods to be very laborious. It was also observed that acid or alkaline hydrolysis resulted in denaturation of carotenoids (Davies, 1976). Phaff (1977) proposed that the digestion of yeast cell walls for the extraction of pigment protein employing microbial lytic enzymes.

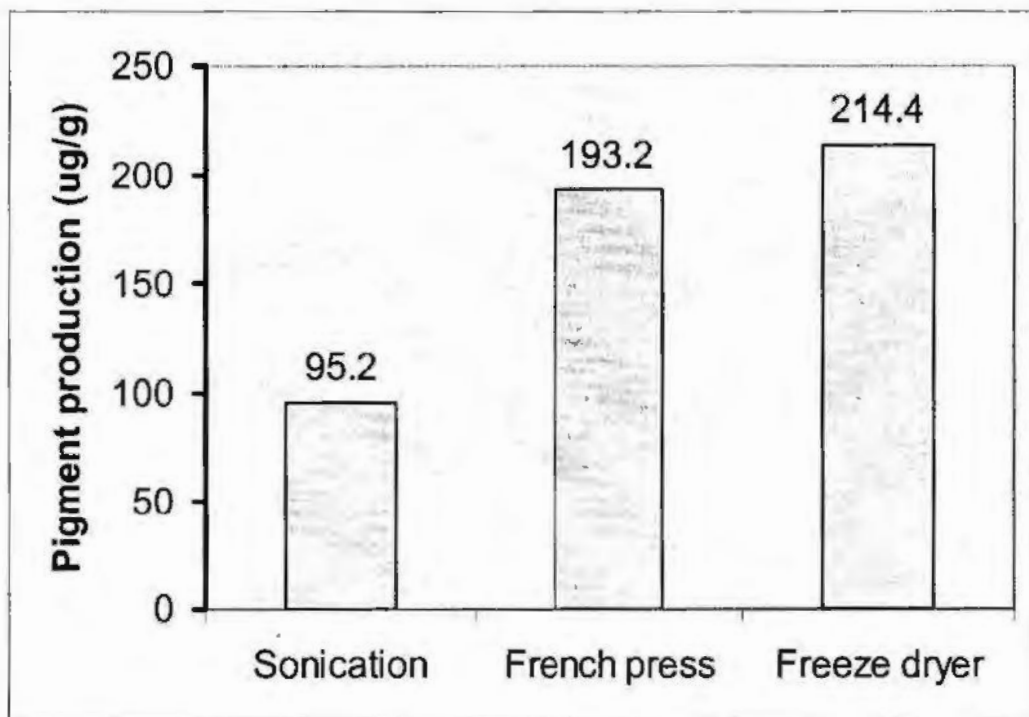


Fig. 23 Effects of extraction methods on pigment recovery from M3, yeast

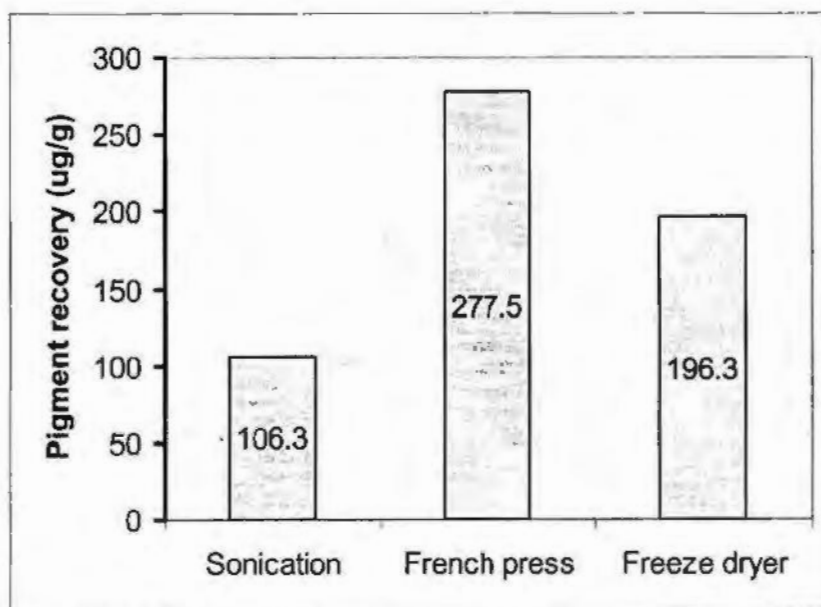


Fig. 24 Effects of extraction methods on pigment recovery from Rm, yeast

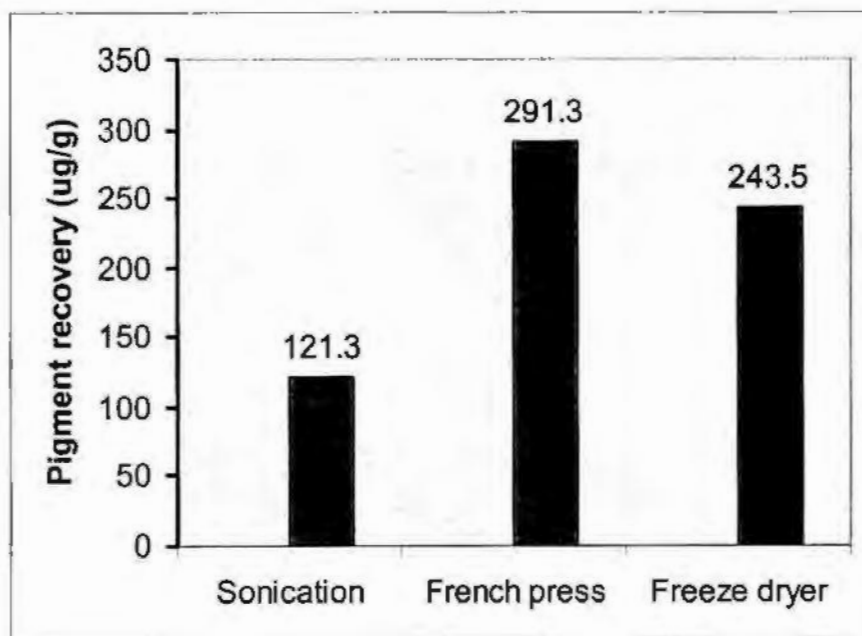


Fig. 25 Effects of extraction methods on pigment recovery from Rt, yeast



### **3.2.4 Extraction using Enzymes:**

#### **(i) Effect of different lytic enzymes on pigment release:**

Three different lytic enzymes used were from *Rhizoctonia solani*, *Aspergillus species*, and *Trichoderma hazarium*. Table 5 shows the effect of these enzymes on the recovery of carotenoids from different yeasts. From this table it is evident that the enzyme from *Rhizoctonia solani* is more efficient in lysing the cell walls of the mutants and the parent yeast, TP1 compared to lytic enzymes from other yeasts.

#### **(ii) Effect of Buffers:**

Breakage of various yeasts at different pHs using Tris-HCl (pH: 7.0, 7.5, 8.0, 8.5 and 9.0) and citrate phosphate (pH: 5.0, 5.4, 6.0, 6.6 and 7.0) buffers was examined. Table 6 and 7 show the total carotenoid recovery from different yeasts. It is apparent from these two tables that optimum cell breakage occurs at pH 7.0 with maximum pigment release. With Tris-HCl buffer (pH 7.0) M1, M2 and M3 gave 76 %, 68 % and 53.5 %, respectively, of pigment recovery compared to that from TP1. It is also observed that the recoveries decreased with higher pH values of the buffers (Table 6). In the case of citrate phosphate buffer (pH 7.0) the pigment yields from M1, M2 and M3 were 197.7, 162.1, 141.7 and 101.1  $\mu\text{g/g}$ , dry weight, respectively. When pigment recoveries from M1, M2 and M3 at this pH are compared to that from TP1, there is a reduction by 18.1, 28.4 and 48.9 %, respectively. In the case of M1 the pigment recovery at pH 5.4 and 6.0 were similar (105 and 106  $\mu\text{g/g}$ ).

Table 5 Effects of lysing enzyme from different sources on pigment recovery of yeasts

Enzyme Source		Total Carotenoid concentration ( $\mu\text{g/g}$ )		
Yeast Sample	TPI	M1	M2	M3
<i>Aspergillus species</i>	182.2 $\pm$ 6.4 *	154.4 $\pm$ 5.2	88.5 $\pm$ 7.8	104.3 $\pm$ 5.0
<i>Trichoderma Hazarium</i>	176.3 $\pm$ 7.5	104.5 $\pm$ 8.7	142.2 $\pm$ 6.8	96.5 $\pm$ 7.1
<i>Rhizoctonia Solani</i>	194.5 $\pm$ 6.2	114.3 $\pm$ 5.8	104.1 $\pm$ 6.3	102.3 $\pm$ 7.0
No enzyme	82.7 $\pm$ 5.5	85.5 $\pm$ 6.0	62.7 $\pm$ 6.5	45.9 $\pm$ 5.7

The yeast cells (1 g) were suspended in 2 ml of tris HCl and treated with 3.5 mg of enzyme from different sources separately. This cell suspension was sonicated after 24 hours of incubation. The pigment was then extracted as described in Materials and Methods.

\* Standard errors are for averages of three determinations.

Table 6. Recovery of carotenoids by using lytic enzyme (*R. solani*) in Tris-HCl buffer

	Carotenoids ( $\mu\text{g/g}$ )				
	pH				
	7.0	7.5	8.0	8.5	9.0
Isolate					
TP1	241.5 $\pm$ 3.4*	212.7 $\pm$ 4.3	192.3 $\pm$ 6.4	182.2 $\pm$ 5.2	147.3 $\pm$ 6.7
M1	184.4 $\pm$ 4.1	158.5 $\pm$ 6.4	132.4 $\pm$ 5.4	112.6 $\pm$ 4.8	98.7 $\pm$ 3.2
M2	164.2 $\pm$ 5.2	127.6 $\pm$ 3.7	113.9 $\pm$ 5.0	102.3 $\pm$ 4.3	85.3 $\pm$ 2.7
M3	129.4 $\pm$ 4.9	106.5 $\pm$ 5.4	96.5 $\pm$ 4.1	87.4 $\pm$ 2.8	78.2 $\pm$ 3.5

The yeast cells (1 g) were suspended in 2 ml of tris HCl and treated with 3.5 mg of enzyme. This cell suspension was sonicated after 24 hours of incubation. The pigment was then extracted as described in material and methods. The procedure is same as mentioned in the caption of Table 3 except the concentrations for each sample were varied between 7.0 and 9.0.

\*Standard errors are for averages of three determinations.

Table 7 Recovery of carotenoids by lytic enzyme (*R. solani*) in citrate phosphate buffer

Carotenoids ( $\mu\text{g/g}$ , dry weight)					
pH					
	5.0	5.4	6.0	6.6	7.0
Isolate					
TP1	114.3 $\pm$ 4.1	129.6 $\pm$ 3.6	144.2 $\pm$ 3.4	170.5 $\pm$ 4.2	197.7 $\pm$ 4.9
M1	75.7 $\pm$ 4.1	106.5 $\pm$ 4.7	105.4 $\pm$ 3.4	134.5 $\pm$ 3.6	162.1 $\pm$ 3.1
M2	64.2 $\pm$ 5.2	81.9 $\pm$ 5.8	94.2 $\pm$ 4.8	104.5 $\pm$ 6.5	141.7 $\pm$ 6.0
M3	56.2 $\pm$ 4.7	63.5 $\pm$ 4.5	77.4 $\pm$ 4.2	89.6 $\pm$ 3.6	101.1 $\pm$ 5.3

The yeast cells (1 g) were suspended in 2 ml of citrate phosphate buffer and treated with 3.5 mg of enzyme. This cell suspension was sonicated after 24 hours of incubation. The pigment was then extracted as described in material and methods. The procedure is same as mentioned in the caption of Table 3 except the concentrations for each sample were varied between 5.0 and 7.0.

\*Standard errors are for averages of three determinations.

### **(iii) Effect of reducing reagents:**

Different Molarities of reducing reagents were obtained using dithiothreitol (DTT, 5.0, 10, 15.0, 20.0, 25.0 and 35.0 mM) and beta-mercapto ethanol (BME, 50, 100, 150, and 200 mM). Table 8 shows the effect of DTT on pigment recovery in TP1, M1, M2 and M3 yeasts. The table shows that the best concentration of DTT for higher enzyme activity is 25 mM and the yields observed for M1, M2 and M3 were 90.5, 85.9 and 79 %, respectively, compared to that from TP1. It is observed that the pigment recovery increased with the increasing concentrations of the reducing agent (5 to 25 mM).

Table 9 shows effect of BME on pigment recovery in the isolates. The pigment recovery decreased in M1, M2 and M3 by 8.9, 14.6 and 26.2 %, respectively, when compared to that from TPI. From the table it is evident that the best recovery was observed with BME concentration of 100 mM. When 150 to 200 mM BME was used with the lytic enzymes the pigment release was reduced. Of the two reducing agents, DTT was found to be superior in stimulating enzyme activity. The total carotenoid recovered with DTT using TPI, M1, M2 and M3 were 96.5, 87.4, 82.9 and 76.3  $\mu\text{g/g}$ , respectively.

### **(iv) Effect of freezing and thawing:**

Table 10 shows that freeze and thaw method enhances pigment release from cells treated with lytic enzymes and then sonicated. TPI showed more recovery than the mutants. Pigment recovery in frozen and thawed cells doubled or tripled when compared to unfrozen yeast cells (Table 10). The recoveries in M1, M2 and M3 were about 85 %, 78 % and 73 %, respectively, compared to that from TPI.

Table 8 Recovery of carotenoids by using lytic enzyme (*R. solani*) in Dithio threitol (DTT) buffer

DTT Concentration (in mM)	Carotenoids ( $\mu\text{g/g}$ , dry weight)			
	TP1	M1	M2	M3
5	63.2 $\pm$ 3.2 *	54.1 $\pm$ 4.3	48.3 $\pm$ 5.2	42.7 $\pm$ 4.9
10	73.6 $\pm$ 4.1	69.1 $\pm$ 5.1	55.4 $\pm$ 4.2	57.4 $\pm$ 5.4
15	81.3 $\pm$ 5.2	74.5 $\pm$ 4.7	55.4 $\pm$ 4.2	64.2 $\pm$ 4.6
20	87.6 $\pm$ 3.5	81.9 $\pm$ 5.3	76.5 $\pm$ 5.4	71.4 $\pm$ 5.1
25	96.5 $\pm$ 4.1	87.4 $\pm$ 3.7	82.9 $\pm$ 3.4	76.3 $\pm$ 2.9
30	82.4 $\pm$ 2.0	73.9 $\pm$ 2.3	67.5 $\pm$ 2.7	58.9 $\pm$ 2.6
35	63.4 $\pm$ 3.2	54.2 $\pm$ 2.7	50.6 $\pm$ 3.9	43.2 $\pm$ 3.4

The yeast cells (1 g) were suspended in 2 ml tris HCl contained DTT with various concentrations (5 to 35 mM) and treated with 3.5 mg of enzyme. This cell suspension was sonicated after 24 hours of incubation. The pigment was then extracted as described in material and methods.

\* Standard errors are for averages of three determinations.

Table 9. Recovery of carotenoids by using lytic enzyme (*R. solani*) in Beta Mercapto Ethanol (BME) buffer

BME Concentration (in mM)	Carotenoids ( $\mu\text{g/g}$ , dry weight)			
	TP1	M1	M2	M3
50	81.8 $\pm$ 3.2 *	74.6 $\pm$ 2.3	63.8 $\pm$ 2.6	54.3 $\pm$ 3.5
100	92.7 $\pm$ 2.7	84.5 $\pm$ 2.1	79.2 $\pm$ 3.1	68.5 $\pm$ 2.7
150	79.3 $\pm$ 2.4	73.2 $\pm$ 2.6	64.7 $\pm$ 2.7	56.2 $\pm$ 3.3
200	64.2 $\pm$ 2.8	61.4 $\pm$ 3.1	53.2 $\pm$ 2.4	48.9 $\pm$ 2.1

The yeast cells (1 g) were suspended in 2 ml tris HCl contained BME with various concentrations (50 to 200 mM) and treated with 3.5 mg of enzyme. This cell suspension was sonicated after 24 hours of incubation. The pigment was then extracted as described in material and methods.

\* Standard errors are averages of three determinations.

Table 10. Recovery of carotenoids by using lytic enzyme (*R. solani*) by freeze and thaw method

Isolate	Carotenoids ( $\mu\text{g/g}$ , dry weight)	
	Fresh cells Method	Freeze and thaw Method
TP1	82.5 $\pm$ 2.6 *	194.3 $\pm$ 1.7
M1	85.9 $\pm$ 2.4	165.5 $\pm$ 2.3
M2	62.8 $\pm$ 2.1	81.9 $\pm$ 5.8
M3	45.7 $\pm$ 3.1	142.5 $\pm$ 2.2

Known amounts of yeast cells were allowed to thaw after being frozen and treated with enzyme. The cell suspension was then incubated for 24 hours and the pigment was then extracted by centrifugation after sonication as described in material and methods.

\* Standard errors are averages of three determinations.



Figure 26 illustrates effects of increasing substrate (one gram of yeast cells) on the activity of lytic enzymes. From the figure it is observed that with a constant amount of lytic enzyme, the pigment recovery increased as substrate amounts were raised from 1 g to 4 g of yeast cells. Optimum pigment recovery resulted with 4 g of yeasts and 3.5 mg of lytic enzyme.

Substrate concentration greater than 4 g per assay did not increase pigment recovery. Figure 27 illustrates effects of increasing enzyme concentration on the recovery of pigments from frozen and thawed yeast cells. Optimum pigment release occurred with enzyme concentrations of 2.5 - 3.0 mg per assay. In the case of TPI maximum pigment release was observed with 2.5 mg enzyme, while in the case of other yeasts, this concentration was found to be 3.0 mg per assay. Higher enzyme concentration did not show corresponding increases in pigment release. French press method showed better pigment recovery with all the isolates. Pigment recovery in cells broken by French press doubled when compared to enzymatic breakage (Table 11). Sangha (1994) also found in a study that *R. rubra* cell wall was resistant to the enzyme treatment and practically no pigment was extractable from the enzyme treated. Hence, *P. rodhomya* cells were much more amenable to rupture by the funcelase than to mechanical rupture in a French press. It was also noted that, the enzyme had a pH optimum of 4 to 5 and a temperature optimum of 30° C. In the present study enzyme activity was tested at 25° C using a water bath. Sangha, (1994) also found that the enzyme treatment requires a more scrupulous control of experimental conditions in order to be effective than the French press rupture method. Also, the economic feasibility of enzyme treatment is questionable as reported by

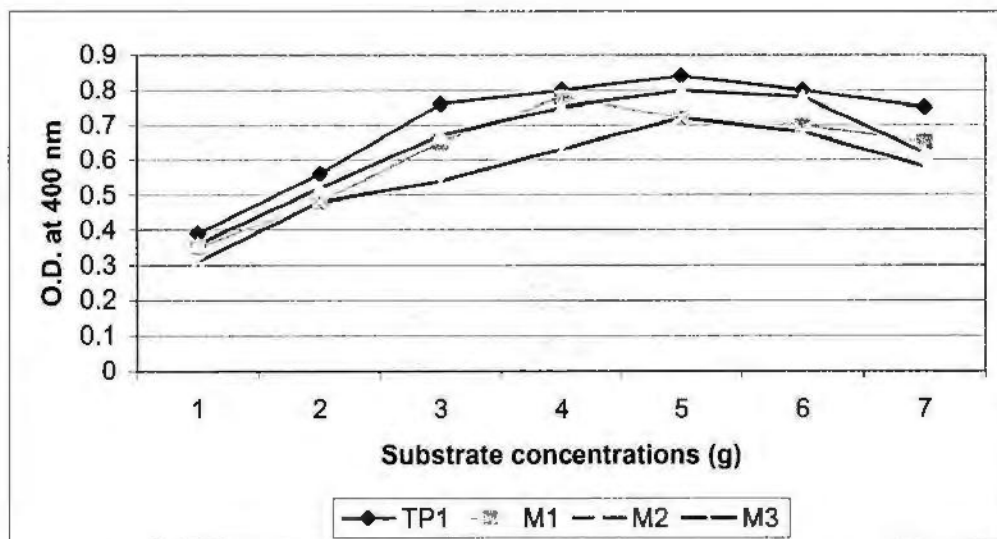


Fig. 26. Effects of cell biomass on the activity of the lytic enzyme.

Different amounts of yeast cells (wet weight) were treated with the same amount of (3.5 mg) enzyme and were incubated for 24 hours and the pigment was extracted as described in Material and Methods. Amounts of carotenoids released were qualitatively measured by determining absorption by the extract preparations at 400 nm.

Each data point represents an average of 3 readings. The standard errors of the mean for TP1, M1, M2 and M3 are  $\pm 0.1$ ,  $0.2$ ,  $0.1$ , and  $0.1$ , respectively.

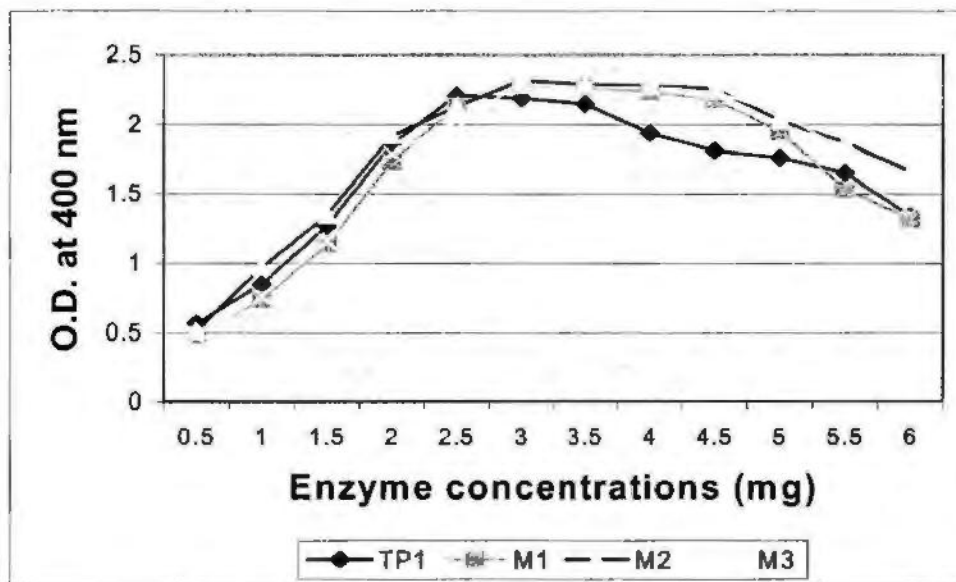


Fig. 27 Effects of enzyme concentration on release of carotenoids from yeast cells.

Fixed amount of cell mass was treated with the different volumes of enzyme and incubated for 24 hours. The pigment was extracted as described in the Materials and Methods.

Each data point represents an average of 3 readings. The standard errors of the mean for TP1, M1, M2 and M3 are  $\pm 0.2$ ,  $0.2$ ,  $0.1$  and  $0.3$ , respectively.

Gentles and Haard (1991). These factors tell that the enzymatic technique is less commercially practical than the French press. French press method showed better pigment recovery with all the isolates. Pigment recovery in cells broken by French press doubled when compared to enzymatic breakage (Table 11). Sangha (1994) also found in a study that *R. rubra* cell wall was resistant to the enzyme treatment and practically no pigment was extractable from the enzyme treated cells. Gentles and Haard (1991) treated *P. rhodozyma* with the enzyme funcelase and reported that the yeast capsule, not the cell wall, was removed by enzyme treatment. This would explain the susceptibility of the *P. rhodozyma* cell wall, by the enzyme treatment perhaps, that the apparent differences in the structure of capsule in these two red yeasts (Gentles and Haard, (1991). In the present study, cells were incubated for 24 hours before extracting the pigment. French press ruptured method showed more extractability than with the enzymatic cell breakage.

Table 11. Recovery of carotenoids by enzymatic breakage and French press

Isolate	Carotenoids ( $\mu\text{g/g}$ , dry weight)	
	French press method	Lytic enzyme method
TP1	326.7 $\pm$ 1.2 *	194.3 $\pm$ 1.7
M1	245.4 $\pm$ 1.6	165.5 $\pm$ 2.3
M2	242.5 $\pm$ 1.4	81.9 $\pm$ 5.8
M3	193.2 $\pm$ 2.1	142.5 $\pm$ 2.2

\*Standard errors are for averages of three determinations.

## CONCLUSIONS

The yeast cells were grown under different growth conditions and observed that the optimum temperature for all the mutants was 28°C. Parent TPI yielded 12.5 g/L biomass and other the mutants M1, M2 and M3 13.1, 11.0 and 9.5 g/L, respectively. Yeast malt broth with 2 % peat extract as a nitrogenous source was observed to be a good substrate concentration. The cane molasses concentration 1 to 2 % as a carbon source also gave good biomass yield, whereas bacto czapex dox broth was found to be inhibitor of the growth and pigment production in all the yeast samples. The initial pH of the media was found to be 5.0 to 7.0 for the optimum growth. In all the growth conditions light enhanced the pigment production.

The French press method was found to be an efficient extraction method for the mutants as well as the wildtype yeasts. The total carotenoid concentrations for M1, M2 and M3 are 250.6, 245.4 and 193.2 µg/g (dry yeast), respectively. Mutant 2 gave high amount of pigment when it was extracted with Freeze dryer method. Sonication method alone did not give much extraction of the pigment in all the mutants. The cells that were treated with lysing enzyme from *Aspergillus* species gave higher yields of pigments. Lysing enzymes tested with Tris- HCl and Citrate-phosphate buffers at pH 7.0 resulted in relatively greater pigment release. The freeze and thaw method also enhanced the total carotenoid concentration compared to fresh, unfrozen/thawed yeast cells. Yeast cells when treated with different concentrations of thiol reagents like beta mercapto ethanol (100 mM) and Dithio thritol (25 mM) showed less pigment recovery.

In the presence of 1 to 2 % cane molasses or 2 % peat extract supplements in YM broth, better growth yields combined with higher pigment production were noted. All yeast cultures grew well between pH 5.0 and 7.0, at 28° C.

From the above it is concluded that French press method is superior extraction technique as compared to other methods. Enzymatic methods did not improve the extractability of pigments when compared to other methods. For better understanding of the biology of the mutant strains the following directions may be adopted:

- (i) Proximal analysis.
- (ii) Regulation of the biosynthetic pathway for carotenoids.
- (iii) Characterization of the key enzymes in the biosynthetic pathway for carotenoids.

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