STUDIES ON A NEW STRAIN OF RHODOTORULA RUBRA ISOLATED FROM YOGURT

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STUDIES ON A NEW STRAIN OF *Rhodotorula rubra* ISOLATED FROM YOGURT

BY

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ABSTRACT

A new strain of red yeast was isolated from yogurt and identified as Rhodotorula rubra TP 1. Studies were conducted on its pigment production, morphology, sexuality, growth kinetics and possible use in aquaculture.

Studies on the sexuality indicated a basidiomycetous affinity of the isolate and hence the first report of a sexual stage in Rhodotorula rubra.

The isolate showed good growth on different substrates. It grew readily on a molasses and wort medium. Peat hydrolysate also supported a satisfactory growth of the yeast. The pH profiles were also studied and growth was found within a broad pH zone of 3 to 10.

Studies on the genetics of the isolate included mutagenesis with nitrosoguanidine. One of the two mutants obtained utilized additional substrates for growth and pigmentation. This mutant also showed a higher value for productivity, yield coefficient and economic coefficient.

The kinetics of growth and pigment formation of the isolate showed pigmentation in the exponential phase, like Phaffia rhodozyma. The isolate was grown in a 1500 L batch fermenter using molasses and wort as the growth medium and the influence of various kinetic parameters on the yield coefficient and specific growth rate was observed. The

fermenter culture showed a higher value for the specific growth rate and productivity than that of the shake flask cultures.

Studies on aquaculture involved a feeding trial for 9 weeks using rainbow trout. The new yeast was found to be a good source of pigments as well as nutrients for the fish when fed as intact cells. Though the commercial astaxanthin containing diet induced better pigmentation than that containing the test yeast, the total color difference between the fish fed with the two diets declined to almost one-third at the end of the feeding trial. The test yeast fed fish had higher growth rates than those fed with the commercial pigment. The proximate analysis of the fish was done wherein the test yeast-fed fish showed an increase in the protein amount at the end of the feeding trial while the synthetic astaxanthin-fed fish showed a decrease. The lipid levels of both groups decreased at the end of the experiment.

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LIST OF ABBREVIATIONS

4, 6-Diamidino-2-phenyl-indole	DAPI
Diazonium Blue B	DBB
Yeast extract/malt extract medium	YM
Sabouraud's dextrose agar	SDA
Potato dextrose agar	PDA
Tryptic soy agar	TSA
Total ion current	TIC
Sulfite waste liquor	SWL
N-methyl-N'-nitro-N-nitrosoguanidine	NTG
Maintenance coefficient	m
High performance liquid chromatography-mass spectrometry	HPLC-MS
Scanning electron microscopy	SEM
Transmission electron microscopy	TEM

LIST OF MANUSCRIPTS

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CHAPTER 1

REVIEW OF LITERATURE

1.1 General introduction

Since times immemorial, colors in the living world have always fascinated and amazed mankind leaving it wonderstruck; the present study has also been inspired by their provocative and conspicuous nature.

The visible perception of color is caused by the pigments or biochromes, the chemical compounds absorbing specific wavelengths of visible light.

1.1.1 Chemistry of pigment

The term 'pigment' is applied to a material of known or unknown physical state or to an unanalysed and unknown coloured material. However, a more appropriate and scientific term is 'biochrome' which is defined as a specific chemical substance with a colored molecule, synthesized by living organisms (Needham, 1974a).

The structural feature of a biochrome which is responsible for the absorption of light is called the chromophore, for example, in the carotenoids, the chromophore is the conjugated double-bond system. Other functional groups or substituents in the molecule which are able to modify the absorption maximum are called auxochromes (Britton, 1983).

The absorption of visible light by a molecule results in the excitation of an electron in the outer orbital to a higher

orbital. These transitions are characteristic of most biological materials but are particularly pronounced in the biochromes. This is because the energy increment is minimized by a number of factors like: large molecular size, conjugated double bonding, polar structure and high dipole moment (Needham, 1974b).

1.1.2 Types of pigments

The six major groups of pigments found in the biological systems include carotenoids, tetrapyrroles, indolic biochromes, N-heterocyclic biochromes (other than tetrapyrroles), oxygenous heterocyclic biochromes (the flavonoids) and quinones. The most important group, carotenoids, will be discussed later in the chapter.

1.1.2.1 Tetrapyrroles

The tetrapyrroles consist of the N-heterocyclic pyrrole, a very stable heteroaromatic system. These are of two types, cyclic tetrapyrroles having the basic structure of porphin with four pyrrole residues linked together (Fig. 1.1) and linear tetrapyrroles, also called bilins (Fig. 1.2). Tetrapyrroles play important roles in plants and animals. Cyclic tetrapyrroles like chlorophyll have function in photosynthesis in green plants while haem and haem proteins such as haemoglobin, myoglobin and leghaemoglobin are oxygen carriers. Cytochromes are essential in the electron transport chain and the two haemoprotein enzymes, catalase and

peroxidase, contribute to the redox reactions (Britton, 1983).

1.1.2.2 Indolic biochromes

The indolic biochromes contain the indole nucleus as in tryptophan. A common example is melanin, a polymer of indole-5, 6 quinone (Fig. 1.3).

The role of melanins is that of providing photoprotection, capturing stray light and protection in general. Eumelanins afford defense to cuttle fish, allow the capture of stray light of all wavelengths in the back of eye and afford photoprotection in animals. All melanins common to plants form the protective coating of many ripe seeds and serve as directional guides for pollinating insects (Britton, 1983).

1.1.2.3 N-Heterocyclic biochromes (other than tetrapyrroles)

This group of biochromes is represented by compounds with very complex structures found in purines, pterins, flavins, phenazines, phenoxazines and betalains (Fig. 1.4). The purines adenine and guanine are found in nucleic acids and nucleotides. The two pterin derivatives, biopterin and folic acid, are important in the redox reactions and as an essential vitamin, respectively. Riboflavin (belonging to the flavin group of biochromes) occurs universally in living organisms as a component of two coenzymes FMN and FAD. Iodinin and pyccyanin are examples of phenazines, synthesized by Pseudomonas spp. and having bacteriostatic properties.

Actinomycin, an example of phenoxazine and produced by Streptomyces, has antibiotic properties. Two groups of betalains, betacyanins (e.g. betanidin) and betaxanthins (indicaxanthin), help in seed dispersal and pollination (Britton, 1983).

1.1.2.4 Oxygenous heterocyclic biochromes - the flavonoids:

They have the basic structure of flavone and flavan (Fig. 1.5). These have been found in all plant tissues-leaves, wood, roots, fruits, seeds and all parts of the flower, especially petals. They occur as anthocyanins, chalkones, aurones and flavan derivatives and are important in coloration, pollination, protection and disease resistance (Britton, 1983).

Applications of flavonoids in food industry:

In the USA, only two anthocyanin preparations have been legalised as food colorants; one is enocyanin from skins of wine grapes and the other is from lees in the bottom of tanks of grape juice (Fracis, 1989).

1.1.2.5 Quinones

The basic quinone structure is that of an unsaturated cyclic diketone derived from a monocyclic or polycyclic aromatic hydrocarbon. Examples include benzoquinones, naphthaquinones and anthraquinones (Fig. 1.6). Colorations by quinones are observed in the animal kingdom, contributors

being spinochromes and echinochromes found in echinoderms (Thomson, 1971; Thomson, 1976). Quinones have found applications in industry as dyes, food colorants and medicinals.

1.1.2.6 Carotenoids

Carotenoid pigments belong to the class of polyenes and are probably the most widely distributed. Almost all carotenoids either are, or are derived from, tetraterpenes, C-40 compounds with a carbon skeleton built up from eight C-5 isoprene units (Fig. 1.7), e.g. carotenes, xanthophylls, retro-carotenoids, seco- and apo-carotenoids, nor-carotenoids and higher or homo-carotenoids (Britton, 1983).

Functions of carotenoids:

The carotenoids play a role in photoreception (vision), photosynthesis, photoprotection, phototaxis, sporangiophore formation and integumental colors (Moss and Weedon, 1976; Britton, 1979).

Applications of carotenoids:

1. Aquaculture: The color of fish is an important factor in consumer acceptance of aquacultured fish. Hence the use of carotenoid pigment in the diets of farmed salmonids has increased dramatically in the past few years.
2. Food industry: Xanthophylls of the algae Spirulina have

been used for chicken egg yolk pigmentation (Anderson et al. 1991). Pigments from Phaffia rhodozyma have similarly been used in poultry feeds to color egg yolks. Table 1.1 summarizes the sources of pigments used as food additives.

3. Pharmaceutical and cosmetic products: Carotenoid formulations for cosmetics and pharmaceuticals have been produced by Hoffman La Roche, eg, tablet coatings, suppositories, gelatin capsules, fat-based ointments and creams, vitamin emulsions, lipsticks and toothpastes (Taylor, personal communication).

4. Medical applications:

a. Carotenoids are recommended for erythropoietic protoporphyria and congenital porphyria (Taylor, personal communication).

b. Retinoids have been demonstrated to be chemopreventive agents for experimental carcinogenesis of mammary gland, urinary bladder, lungs, skin, liver, pancreas, colon and esophagus (Moon, 1989).

c. ß-Carotene has been shown to be protective against the development of lung cancer (Ziegler, 1989) as well as enhance immune function (Bendich, 1989a).

d. Certain carotenoids with antioxidant activities have been shown to act as anti-mutagens and anti-carcinogens, protect against radiation damage and block the damaging

effects of photosensitizers (Bendich, 1989b).

Business implications and value of the market :

i. The carotenoid market, which generated world-wide sales of $100 million in 1989, is expected to expand more because of the proposed link between carotenoids and cancer prevention (Table 1.2).

ii. Natural ß-carotene now constituting 10% of the total ß-carotene market, is expected to capture 25% of the market because of increasing demand (Taylor, personal communication).

Distribution of carotenoids:

Carotenoids are well spread amongst biological systems including the following:

A. Higher plants and algae: The pigments of higher plants and green algae include β-carotene, lutein and violaxanthin (Britton, 1983) while other classes of algae produce acetylenic carotenoids, e.g, fucoxanthin (Stewart, 1974).

B. Bacteria: The non-photosynthetic bacteria produce glycosides of C-30, C-40 and C-50 carotenoids while the photosynthetic bacteria synthesize acyclic, aromatic and glycosidic carotenoids (Britton, 1983).

C. Animals: Most animals are characterized by oxycarotenoids

as well as carotenes (Britton, 1983).

D. Fungi: Most carotenogenic fungi accumulate carotenes like β-carotene and γ-carotene. The yeasts which accumulate carotenoids belong to families Deuteromycetes and Basidiomycetes (Britton, 1983).

The present piece of work is devoted to the pattern of carotenogenesis in the yeast Rhodotorula rubra as well as its growth kinetics, morphology and sexuality, among other characteristics.

1.2. Yeasts:

A yeast is defined as a unicellular fungus which reproduces by budding or fission (Kreger-van Rij, 1984).

1.2.1 Taxonomy

Yeasts are classified in the division Eumycota and include Ascomycetes, Basidiomycetes and Deuteromycetes (Table 1.3) (Kreger-van Rij, 1984).

1.2.2 Cultivation of yeast

Substrates for yeast biomass production:

Different substrates have been used to grow yeast for biomass production (Table 1.4). The substrate chosen should have a few desirable characteristics, e.g., it should give maximum yield of product and minimum yield of undesirable products; it should be inexpensive, of consistent quality and readily available throughout the year; it should cause minimum

problems in aspects of production process like aeration, agitation and waste treatment (Stanbury and Whitaker, 1984).

1.2.3 Yeast genetics

Winge (1935) demonstrated the haploid and diploid phases in the life cycle of Saccharomyces ellipsoideus Hansen while Mendelian segregation was first observed in Saccharomyces ludwigii (Mortimer and Hawthorne, 1969).

Yeasts have been found to present many advantages for genetic studies, e.g., rapid growth, ease of cloning, handling and storage, adaptability to replica plating, micromanipulation and an array of biochemical procedures (Mortimer and Hawthorne, 1969).

Methods for genetic manipulation:

1. Mutagenesis:

A. Mutagenesis through radiation:

a) *Ultra-violet radiation*: Short wavelength ultra-violet rays between 200-300 nm have been found to be effective for mutagenesis, with an optimum at 254 nm, absorption maximum of DNA. The products of UV action are dimers between adjacent pyrimidines or pyrimidines of complementary strands.

Long wavelength UV radiation between 300 and 400 nm is less lethal; however, in the presence of various dyes which interact with DNA, it induces increased mutation frequency.

b) *Ionizing radiation*: Ionizing radiations which include X-rays, ß-rays and γ-rays, are seldom used for mutagenesis in

industrial strain development (Crueger and Crueger, 1989).

B. Mutagenesis with chemical agents:

a) *Mutagens affecting non-replicating DNA:* These include nitrous acid, hydroxylamine and alkylating agents. Nitrous acid deaminates adenine to hypoxanthine and cytosine to uracil while hydroxylamine reacts with pyrimidines. The alkylating agents have been found to be one of the most potent mutagenic systems. These include ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), diethylsulfate (DES), diepoxybutane (DEB), N-methyl-N′-nitro-N-nitrosoguanidine (NTG), N-methyl-N-nitroso-urea and mustard gas.

b) *Base analogs*: Base analogs such as 5-bromouracil (BU) and 2-aminopurine (AP) are incorporated into replicating DNA because of structural similarity. However, these are not important for practical application.

c) *Frameshift mutagens*: These intercalate into the DNA molecule causing errors and resulting in an alteration of the reading frame and thus lead to the formation of faulty protein or no protein at all. Some examples of such mutagens are acridine orange, proflavine and acriflavine. Though useful in research, these are not suitable for routine purposes in strain development (Crueger and Crueger, 1989).

2. Protoplast fusion: This involves cell fusion followed by nuclear fusion occurring between protoplasts of strains which normally do not fuse. Thus, protoplast fusion is used to

overcome recombination barriers. Protoplast fusion has been shown in Streptomyces spp. (Hopwood et al. 1977), filamentous fungi (Ferenczy and Zsolt, 1974) and yeasts (Sipiczki and Ferenczy, 1977). Intergeneric fusion of R. rubra with S. cereviceae has been successful (Evans and Conrad, 1987).

3. Recombinant DNA techniques: In these techniques, a plasmid from a yeast carrying the desired gene to be introduced into the recipient strain is used to transform Escherichia coli and the plasmid DNA is amplified. DNA from the bacterial strains is amplified, reisolated and used to transform the recipient yeast. The transformants are selected and then tested for the presence of the desired gene (Spencer et al. 1988).

4. Pulsed field electrophoresis: This technique permits separation of intact chromosomes and information about their size. Here, the yeast cells, embedded in agarose gel blocks and lysed enzymatically, are subjected to electrophoresis wherein, pulses of current of unequal duration are applied and then reversed at intervals of several seconds producing homogeneous electric fields and giving sharper separations of the bands. This technique has also been used for karyotyping of a number of yeast species (Spencer et al. 1988).

1.3 Red yeasts

The carotenogenic yeasts fall into seven genera, Sporobolomyces, Sporidiobolus, Rhodosporidium, Rhodotorula, Phaffia, Cryptococcus (Phaff et al. 1978) and Saitoella (Komagata et al. 1987).

These can be differentiated biochemically and morphologically (Table 1.5).

1.3.1 Carotenoids of red yeasts:

Phaffia is the only yeast studied to date that contains astaxanthin as its major pigment (Fig. 1.7). Other carotenoids synthesized are ß-carotene, neurosporene, γ-carotene, lycopene, echinenone, 3 hydroxy echinenone, 3-hydroxy-3-4 - didehydro-ß- caroten-4-one and phoenicoxanthin (Andrewes et al. 1976).

The yeasts of genera belonging to Cryptococcus, Rhodotorula, Rhodosporidium, Sporidiobolus and Sporobolomyces mainly synthesize ß-carotene, γ-carotene, torulene and torularhodin (Fig. 1.7) (Goodwin, 1972; Simpson et al. 1971). The genera Rhodotorula and Rhodosporidium may also produce ζ-carotene, phytoene, phytofluene and ß-zeacarotene (Hayman et al. 1974). Plectaniaxanthin has also been found in a few Rhodotorula spp (Ratledge and Evans, 1987) as well as Cryptococcus laurentii (Bae et al. 1971). A species of R. aurantiaca has been found to produce 2-hydroxy

plectaniaxanthin (Liu et al. 1973). The carotenoids of saitoella are yet unknown.

1.3.2 Analysis and identification of carotenoids:

A. *Extraction of carotenoids*:

1) Mechanical methods: Haard (1988) ground the freeze-dried P. rhodozyma cells with fine sand and 60% methanol in water; Johnson and Lewis (1979) mixed Phaffia cells with glass beads followed by vibration in a Braun homogenizer; The French pressure cell has been used as a method for cell rupture by Hari et al. (1992).

2) Chemical methods: Okagbue and Lewis (1984a) treated the yeast cell pellet with 2N HCl followed by mild heat treatment in a boiling water bath for 1-2 minutes and then rapid cooling and extraction; Okagbue and Lewis (1984b) used autolysis, of P. rhodozyma, in distilled water and 0.02M citrate buffer with or without dithiothreitol as a method to extract astaxanthin from the yeast cells; Bonner et al. (1946) shook the cell pellet with benzene and 20% KOH in methanol.

3) Enzymatic methods: Johnson et al. (1978) induced the lytic enzymes of Bacillus circulans WL-12 by the growth of the organism on heat-killed P. rhodozyma cells. Okagbue and Lewis (1985) used a mixed culture of B. circulans WL-12 and P. rhodozyma to render the yeast pigment extractable by the bacterial enzyme complex. Gentles and Haard (1991) treated the P. rhodozyma cells with a commercial enzyme from a mutant of

Trichoderma viride (funcelase enzyme) for the extraction of the pigment.

B. *Separation of carotenoids*:

1) Column chromatography: The adsorption of pigments onto packed columns of powdered solids was first applied to carotenoids by Tswett (1906). The adsorbents most commonly used for carotenoids are calcium carbonate, magnesium oxide, calcium hydroxide and aluminum oxide.

2) Thin-layer chromatography (TLC): For the TLC, magnesium oxide (Sadowski and Wojcik, 1983), silica (Singh et al. 1973) and kieselgel (Liaaen-Jensen and Andrewes, 1985) are the commonly used materials and different developing systems are used depending on the polarity of the pigment.

C. *Identification and characterization of carotenoids*:

1) Ultra-violet visible spectroscopy: The characteristic maximal absorption peaks in visible spectrum gives valuable information about the kind of carotenoid. The characteristic absorption spectrum is defined by the number of double bonds, various additional structural features and the type of solvent used (Vetter et al. 1971).

2) Infra-red spectroscopy (IR): This technique is not used extensively in the carotenoid identification. It is used for the assignment of different functional groups and different carbonyl functions, and allenes are readily revealed by this technique (Bellamy, 1975).

3) Mass spectroscopy (MS): The MS spectrum reveals three kinds of information, namely, molecular weight and elementary composition of a compound is obtained, structural features are deducible from the fragmentation pattern and the proof of the identity of different samples is possible (Vetter et al. 1971).

4) High-performance liquid chromatography (HPLC): In the carotenoid field, the use of this technique began in 1971 when Stewart and Wheaton reported the separation of complex mixtures on precipitated zinc carbonate and magnesium oxideusing steel columns. Some of the advantages of HPLC are rapid analysis time, high sensitivity, high resolving power, high recovery, non-destructive conditions (Taylor et al. 1990), selectivity and efficiency (Stewart and Wheaton, 1971). Silica and bonded nitrile columns are commonly used for normal-phase HPLC. In recent years reverse-phase HPLC is usually the method of choice; alumina columns are commonly used, typical columns being 15-30 cm in length with an internal diameter of 3-8 mm. Guard columns which are 20% of the length of analytical column are also employed to protect the life of the column (Taylor et al. 1990).

5) HPLC-MS: In this technique, HPLC separation and mass spectral amplification is achieved in one procedure. However, it entails high cost of instrumentation and maintenance (Taylor et al. 1990).

6) HPLC-MS-MS: This technique is usually employed when high amounts of contaminants are present in the carotenoid mixtures. Here, mass spectrometer provides two sequential stages of mass separation and hence chemical analysis, separation and identification are obtained in one technique. The HPLC-MS-MS has been used for the identification of α- and ß-carotene from the alga Dunaliella salina (Taylor et al. 1990).

7) Resonance Raman (RR) spectroscopy: This technique is used for investigating carotenoid-protein interactions (Salares et al. 1977). RR spectroscopy reveals the specific vibrational modes of the chromophore even when it is present in a complex biological medium at a low concentration (Merlin, 1985). RR spectroscopy has many advantages over IR spectroscopy, namely, spectra are obtained in aqueous solutions as water exhibits very weak Raman lines; time resolution reduces the analysis time; analysis of very small amounts of material included in a heterogeneous medium is possible; spectra can be obtained from single living cells (Merlin, 1985).

8) Circular dichroism (CD): The CD spectra are usually employed for carotenoid-protein complexes (Zagalsky et al. 1983) and reveal the geometrical configuration of a carotenoid (Goodwin and Britton, 1988).

9) Nuclear magnetic resonance (NMR): This is a powerful technique for carotenoid structure elucidation. The carotenoid

end group assignment is possible with the ^{1}H NMR (Goodwin and Britton, 1988). However, location of cis-bonds is possible by ^{13}C NMR (Liaaen-Jensen and Andrewes, 1985). A number of one and two dimensional NMR techniques have been described by Englert (1991).

1.3.3 Yeast as a source of carotenoids and its nutritive value:

The pink to red color of the flesh of salmonids is of economic importance due to consumer preference for colored fish. Salmonid aquaculture has increased over the years and so has the use of carotenoid pigments in the fish feed. The dominant pigment source used in aquaculture is synthetic astaxanthin or canthaxanthin which are commercially produced by Hoffman La Roche (Basle, Switzerland) and marketed under the trade names of 'Carophyll pink' and 'Carophyll red' respectively (Torrissen et al. 1989). Crustacean by-products have also been used as an alternative pigment source although because of their low astaxanthin content and higher mineral content, they have a limited potential (Johnson et al. 1980). The green algae high in astaxanthin have been used to color salmon. However, their high concentration of astaxanthin esters necessitates the development of a suitable hydrolysis process to increase the amount of free astaxanthin (Torrissen et al. 1989). In recent years yeasts have been tried as a

pigment source for fish and poultry. Laine and Gyllenberg (1969) used Rhodotorula sanneii to feed rainbow trout and found that the yeast yielded poor weight development in the fish but the color of the fish was enhanced. Savolainen and Gyllenberg (1970) fed Rhodotorula sanneii preparations to rainbow trout. However, they did not report any increase in the fish color. Johnson et al. (1977 & 1980) used lyophilized and freeze-dried P. rhodozyma to feed rainbow trout and the astaxanthin level was found to increase from 5mg/Kg to 10mg/Kg body weight. Johnson et al. (1980) also used the above red yeast to pigment egg yolks of laying hens and Japanese quail and reported that astaxanthin from broken yeast or prepared yeast oil but not from intact yeast cells was deposited in the egg yolks.

1.4 The genus *Rhodotorula*:

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

1.4.1 Description of Cryptococcaceae:

Budding yeast cells are always present; however, pseudomycelium, true mycelium and arthrospores may be formed. Cells are hyaline, or red, orange or yellow due to carotenoid pigments, very seldom brown or black. The break-down is

strictly oxidative or oxidative and fermentative (Kreger van-Rij, 1984).

1.4.2 Description of genus Rhodotorula:

As described by Harrison (1928), members of the genus show no fermentation, nitrate is sometimes assimilated, inositol is not assimilated, starch-like compounds are not produced and urease is produced.

Kreger Van Rij (1984) described the members of the genus as consisting of spheroidal, ovoidal or elongate cells. Reproduction is by multilateral budding and strains of some species form pseudo- or true mycelium. Ascospores or ballistospores are not formed. Red or yellow carotenoid pigments are synthesized in malt agar cultures. Many strains have a mucous appearance due to capsule formation but others are pasty, dry and wrinkled.

1.4.3 Sexuality in Rhodotorula:

The members of the genus do not form ascospores or ballistospores and reproduce vegetatively. Rhodotorula has been reported to have a haploid life cycle (Fowell, 1969). Kreger Van-Rij (1969) reported that the above genus may represent life-cycle stages of basidiomycetes. Fell (1970) provided biochemical evidence that certain species of Candida, Rhodotorula and Sporobolomyces are related to the heterobasidiomycetes rather than to the ascomycetes. The basidiomycetous origin has also been shown by Hamamoto et al.

(1987). The perfect forms of a number of strains of R. glutinis have been found and trasferred to Rhodosporidium toruloides (Banno, 1967). These strains had opposite mating types, dikaryotic mycelium with clamp connections and chlamydospores.

1.4.4 Commercial importance of *Rhodotorula*:

1. Some species of the genus have been found to metabolize aromatic compounds while others produce glycolipids containing polyhydroxy alcohols (Spencer et al. 1988).
2. A Rhodotorula spp. isolated from soil had the capacity to degrade aliphatic and aromatic hydrocarbons and hence could be used to treat oil sludge (Shailubhai et al. 1984).
3. Milk clotting enzymes have been investigated from strains of Cryptococcus and Rhodotorula. The milk clotting activity together with proteolytic ability could be used in cheese manufacture (Federici, 1982).
4. Some strains of R. minuta were found to produce isobutene, the starting material in petrochemical industry (Fujii et al. 1987).
5. Lipases have been reported in Rhodotorula spp. by Zvyagintseva and Pitryuk (1975) as well as Zvyagintseva (1972).

1.5 The red yeast *Rhodotorula rubra*

1.5.1 Description of Rhodotorula rubra:

As described by Lodder (1934), R. rubra assimilates sucrose, trehalose, raffinose, D-xylose, ribitol and succinic acid while galactose, maltose, cellobiose, L-arabinose, D-ribose, L-rhamnose, D-mannitol and citric acid are assimilated by some strains of the species (Kreger van Rij, 1984). The G+C content is 59.0-61.2 mole % (Hamamoto et al. 1986; Nakase and Komagata, 1971). The ubiquinone system is CoQ_{10} and the requirements for biotin as well as *p* aminobenzoic acid has been shown to be negative (Yamada and Kondo, 1973). The carbohydrate patterns of whole cell hydrolysates are found to contain fucose and mannose as the dominant sugars while other components include mannitol and arabinitol (Weijman and Rodrigues De Miranda, 1988).

1.5.2 Sources:

Rhodotorula strains have been isolated from leaves, flowers, atmosphere, soil and marine sources (Kreger Van Rij, 1969).

1.5.3 Growth and pigmentation in R. rubra:

Effect of cultural conditions on pigmentation:

1) *Carbon sources*: Different carbon sources have been shown to be effective in promoting pigmentation. In R. rubra, glycerol is effective in promoting carotenogenesis (Fromegeot

and Tchang, 1938). However, for Rhodotorula sp. no. 100, glycerol was less effective than glucose while phenol, resorcinol and kojic acid stimulated ß-carotene production in the above strain (Simpson et al. 1971). For another strain of R. rubra, the best yields of torularhodin were obtained on glycerol with asparagine as the nitrogen source while sucrose gave the highest yields of carotenoids torulene, ß-carotene and υ-carotene (Wittmann, 1957). A strain of Rhodotorula gracilis was found to produce the highest pigment on carrot juice (Vecher et al. 1965). Species of Rhodotorula have also been grown on petroleum hydrocarbons (Nikolaev et al. 1966; Vaskivnyuk and Kvasnikov, 1968). Strains of R. glutinis, R. aurantiaca and R. mucilaginosa (now R. rubra) have also been isolated from oil-containing soils.

2) *Nitrogen sources*: One strain of R. gracilis gave the highest pigment yield on leucine and glutamic acid, when used as the sole nitrogen source (Vecher et al. 1967) while an another strain of the same yeast showed maximum pigmentation on ammonium nitrate (Simpson et al. 1971). For a Rh-100 strain of Rhodotorula, the best yields of carotenoids were obtained with valine, leucine and asparagine. For an isolate of R. rubra, among a series of inorganic and organic nitrogen sources, the highest yield of total carotenoids per unit dry weight of cells was obtained on ammonium nitrate (Wittmann, 1957). However, the cell yield was only moderate. The highest

concentration of torularhodin was obtained with histidine while maximum yields of torulene and ß-carotene were obtained on ammonium nitrate while γ-carotene production was the highest on asparagine.

3) *Light*: Light has been shown to enhance carotenogenesis in R. rubra and R. gracilis (Simpson et al. 1971). Maxwell et al. (1966) showed that carotenoids in R. glutinis strain 48-23T afforded protection from photodynamic death by monochromatic light at 632 nm but not between 300 and 400 nm.

4) *Temperature*: With a change in the cultivation temperature, most yeasts show a decrease in the carotenoid level though the ratio of different pigments remains the same (Fromageot and Tchang, 1938; Nakayama et al. 1954, Bobkova, 1965a). However, two strains of R. glutinis (48-23T and 48-23W) were shown to be pink at 25°C and yellow at 5°C (Phaff et al. 1952). A Rhodotorula rubra strain also showed color changes at similar temperatures (Simpson et al. 1971). A change in the cultivation temperature of R. glutinis (str. 48-23T), from 5 to 25°C resulted in a decrease in the level of α- and ß-carotene while the amounts of torularhodin and torulene increased (Nakayama et al. 1954).

5) *Time of growth*: While carotenogenesis in Phaffia rhodozyma occurs in the exponential growth phase (Johnson and Lewis, 1979), pigment production in R. rubra (Goodwin, 1959, 1972) as well as other carotenogenic organisms like R.

glutinis (Vecher and Kulikova, 1968), Sporobolomyces roseus (Bobkova, 1965b), Phycomyces blakesleeanus (Leenheer and Nelis, 1991) and Dunaliella salina (Goodwin, 1959, 1972) has been shown to occur in the stationary phase.

6) *Other growth requirements*: Agar in a concentration of 2.5% along with yeast autolysate (10%, v/v) and glucose (5%) has been shown to enhance pigmentation in R. rubra, R. glutinis and many strains of Cryptococcus. The liquid medium of the same composition gave good growth of the yeast but lower yields of the carotenoids were obtained (Nakayama et al. 1954).

7) *Effect of inhibitors*: ß-Ionone in a concentration of 250mg/L inhibited the formation of carotenoids in R. rubra by destroying ß-carotene and torularhodin when added to mature cultures (Simpson et al. 1971). Other compounds found to inhibit carotenogenesis in R. mucilaginosa (R. rubra) were the diphenyl derivatives (Villoutreix, 1960).

1.5.4 Enzymology of carotenogenesis:

The pathways for carotenoid synthesis in Rhodotorula spp. have been worked out as shown (Fig. 1.8a, b, c and d). The effect of light on carotenogenic enzymes has been particularly studied in this yeast. In R. minuta the stimulation of carotenoid biosynthesis by light was found to occur in two phases: the first phase involves a photochemical reaction independent of temperature (light reaction) and the second

involves biochemical reactions independent of light (dark reaction) as shown by Tada & Shiroishi (1982a). The workers also reported the following aspects of photoregulative carotenogenesis in the above organism (Tada & Shiroishi, 1982b):

a. The quantity of carotenoid produced as well as the rate of carotenogenesis were regulated by light dose.

b. The photochemical product, postulated to serve as an inducer of carotenoid synthesis, was stable, not metabolized *in vivo* and decreased as the carotenoid synthesis progressed.

These researchers also showed that the photoregulation of carotenogenesis in R. minuta resulted from the photoregulation of HMG-CoA reductase synthesis while the enzymes required for the conversion of phytoene to carotenoid pigments were not induced by light (Tada & Shiroishi. 1982c).

1.5.5 Commercial significance of R. rubra:

1. Two R. rubra strains were found to degrade 4-hydroxy benzoate and therefore could be exploited for oil sludge treatment (Wright & Ratledge, 1991).

2. A strain of the red yeast has been reported to produce extracellular mannan which was found to have a moderate inhibitory action on the infectiousness of tobacco mosaic virus and hence could be used to control the plant

infections caused by this virus (Elinov et al. 1980).

3. A strain of R. rubra MGU 691 was found to produce extracellular capsular material that could be used in food, pharmaceutical, textile, paper, oil refining, paint and varnish industries (Golubev et al. 1980).

4. Production of extracellular proteases was reported from a strain of R. rubra. It was speculated that these proteases could be used in degradation of proteins remaining in beer and wine that form hazes during storage (Ogrydziak, 1993).

Table 1.1. Carotenoids used as food additives

Food products	Pigment	Amount used
A. Bakery products:	**ß-carotene**	**g/100 lb**
Cinnamon rolls		12.5
Frozen yeast dough		32.51
Yeast buns		11.9
Kaiser rolls		12.2
Shortbread cookies		12.6
Wafers		21.26
Doughnut		0.08
Soft cookies		1.0
B. Beverages:	**ß-carotene**	3.6%
Orange flavored drinks, fruit juice blends		
C. Fat and oil products:	**ß-carotene**	30%
Margarine, process cheese, winter butter, popping oil		
D. Dairy products:	**ß-carotene**	0.1-1.0 ppm
Imitation milk, whipped toppings, fluid & dry coffee whiteners, sour cream, frozen desserts, eg, custard, water ice, fruit sherbert & ice milk		
E. Frozen & dried egg yolk products:	**ß-carotene**	110 ppm
Frozen yolk, frozen whole egg & dried yolk		
F. Popcorn	**ß-carotene**	22%
G. Tomato based products:	**Canthaxanthin**	40 ppm
Tomato soup, vegetable cocktail spaghetti, barbecue & pizza sauce		
H. Dry spice & breading mixes	**Apo-carotenal**	4%
I. Confectionary products:		
Candy & fruit jelly	**ß-carotene**	0.0005%
	Apo-carotenal	0.001%
	Canthaxanthin	0.004%

Gordon (1982)

Table 1.2. Worldwide carotenoid markets

Carotenoid rate	$ million		Annual growth (%)
	1989	1995*	
ß-Carotene	60	100	8-10
Other carotenoids	45	95	10-15
Total	105	195	10-12

* Projected in 1989 dollars

Taylor (Personal communication)

Table 1.3. Taxonomy of yeasts.

Sub-division	Class	Order	Family
Ascomycotina	Hemiascomycetes	Endomycetales	Spermophthoraceae Saccharomycetaceae
Basidiomycotina		Ustilaginales	Filobasidiaceae Teliospore forming yeasts
		Tremellales	Sirobasidiaceae Tremellaceae
Deuteromycotina (Form sub-division)	Blastomycetes		Cryptococcaceae Sporobolomycetaceae

(Kreger-van Rij, 1984)

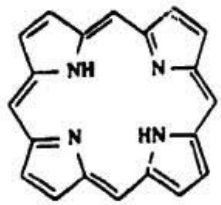
Table 1.4. Substrates for yeast biomass production

Substrate	Organism	Reference
Lactose whey	*Kluyveromyces fragilis*	Marzolf 1977
Molasses	*Candida utilis* *Saccharomyces cerevisiae*	Forage & Righelato 1979
Sulfite waste liquor	*Candida utilis*	Prescott & Dunn, 1959
Starch (Soybean meal, maize, wheat, barley)	*Endomycopsis fibuliger*, *C. utilis* *Schwanniomyces castellii*	Jarl 1969 Boze *et al*. 1987
Inulin (Dahlia, chicory, Jerusalem artichoke)	*Kluyveromyces marxianus*	Guiraud *et al*. 1979 Guiraud 1981 Guiraud & Galzy 1990
Ethanol	*C. ethanothermophilum*	Litchfied 1977
Methanol	*Pichia pastoris*, *Hansenula polymorpha*	Faust & Prave 1983
n-Alkane	*C. tropicalis*, *C. lipolytica*	Litchfied 1977
n-Hexadecane	*C. tropicalis*	Blanch & Einsele 1973
Lipids	*C. rugosa*	Ratledge & Tan 1990
Lignocellulosic material	*Candida* spp. *Rhodotorula* spp.	Kuhad & Singh 1993
Wood sugar	*C. utilis*	Saeman *et al*. 1945
Wood sugar stillage	*C. utilis*	Kurth 1946; Kurth & Cheldelin 1946

Table 1.5. Differentiation of red yeasts (Davenport, 1981; Komagata et al. 1987).

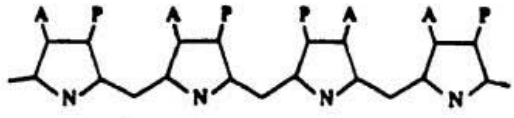
Organism	I	F	N	M	SS	T	B	Principal habitat	Subdivision
Phaffia	-	+	-	-	-	-	-	Tree exudates and cactus plants (R)	Deuteromycotina
Cryptococcus	+	-	±	-	-	-	-	Various (C)	Deuteromycotina
Rhodotorula	-	-	±	+	-	-	-	Ubiquitous	Deuteromycotina
Rhodosporidium	-	-	±	+	+	+	-	Antarctic sea water or plant surfaces (R)	Basidiomycotina
Sporobolomyces	±	-	±	-	-	-	+	Plant surfaces/ air (C)	Deuteromycotina
Sporidiobolus	±	-	+	+	+	+	+	Plant surfaces (R)	Basidiomycotina
Saitoella	-	-	+	-	-	-	-	Soil	Deuteromycotina

I= assimilation of inositol; F= fermentation of glucose; N= assimilation of nitrate; M= mycelium and/or psedomycelium formed; SS= sexual states; T= teliospores; B= ballistospores; R= rare (usually need special cultural conditions; C= common; ± = + & - strains; - = all strains negative; + = all strains positive



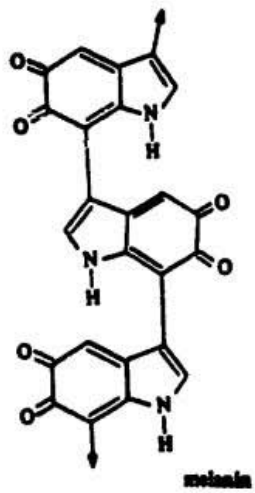
Porphin

Fig. 1.1. Cyclic tetrapyrrole



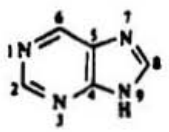
Basic bilin skeleton

Fig. 1.2. Linear tetrapyrrole



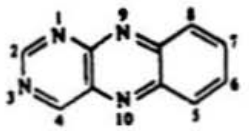
melanin

Fig. 1.3. Indolic biochrome

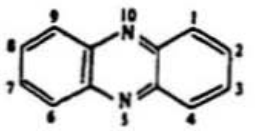


Purine

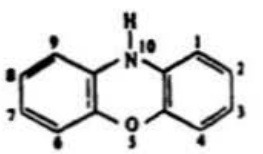
Pterin = 2-amino-4-hydroxypteridine



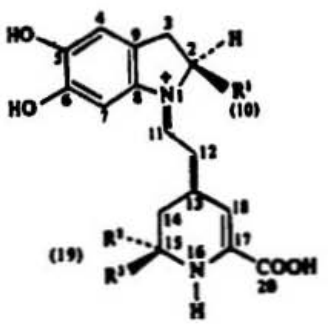
Isoalloxazine



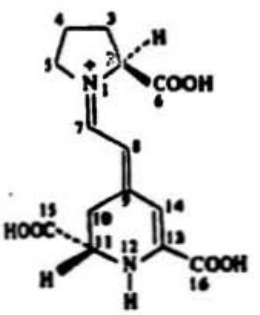
Basic dibenzopyrazine skeleton of phenazines



Phenoxazine ring system

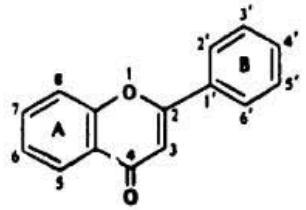


Betanidin ($R^1 = R^2 = COOH$; $R^3 = H$)

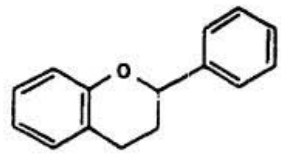


Indicaxanthin

Fig. 1.4. N-Heterocyclic biochromes



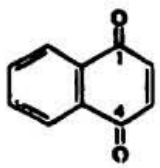
Flavone



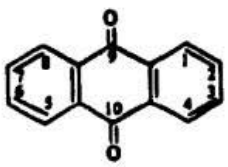
Flavan

Fig. 1.5. Flavonoids

1, 4-Benzoquinone

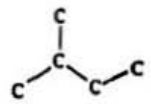


1, 4-Naphthaquinone

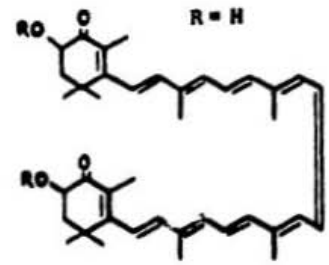


9, 10-Anthraquinone

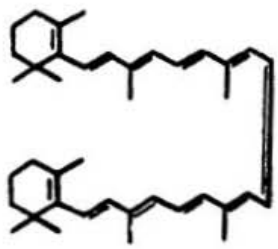
Fig. 1.6. Quinones



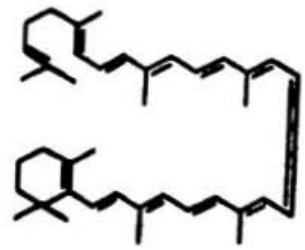
Isoprene unit



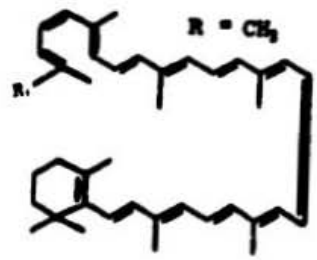
ASTAXANTHIN



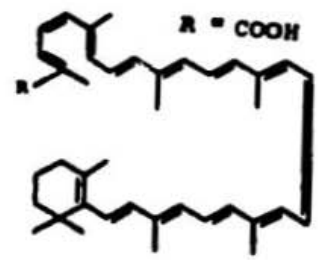
CAROTENE, β-



CAROTENE, gamma-



TORULENE



TORULARHODIN

Fig. 1.7. Carotenoids

Fig. 1.8a. *Conversion of acetyl and acetoacetyl CoA to geranylgeranyl pyrophosphate (Simpson et al. 1971; Britton, 1983).*

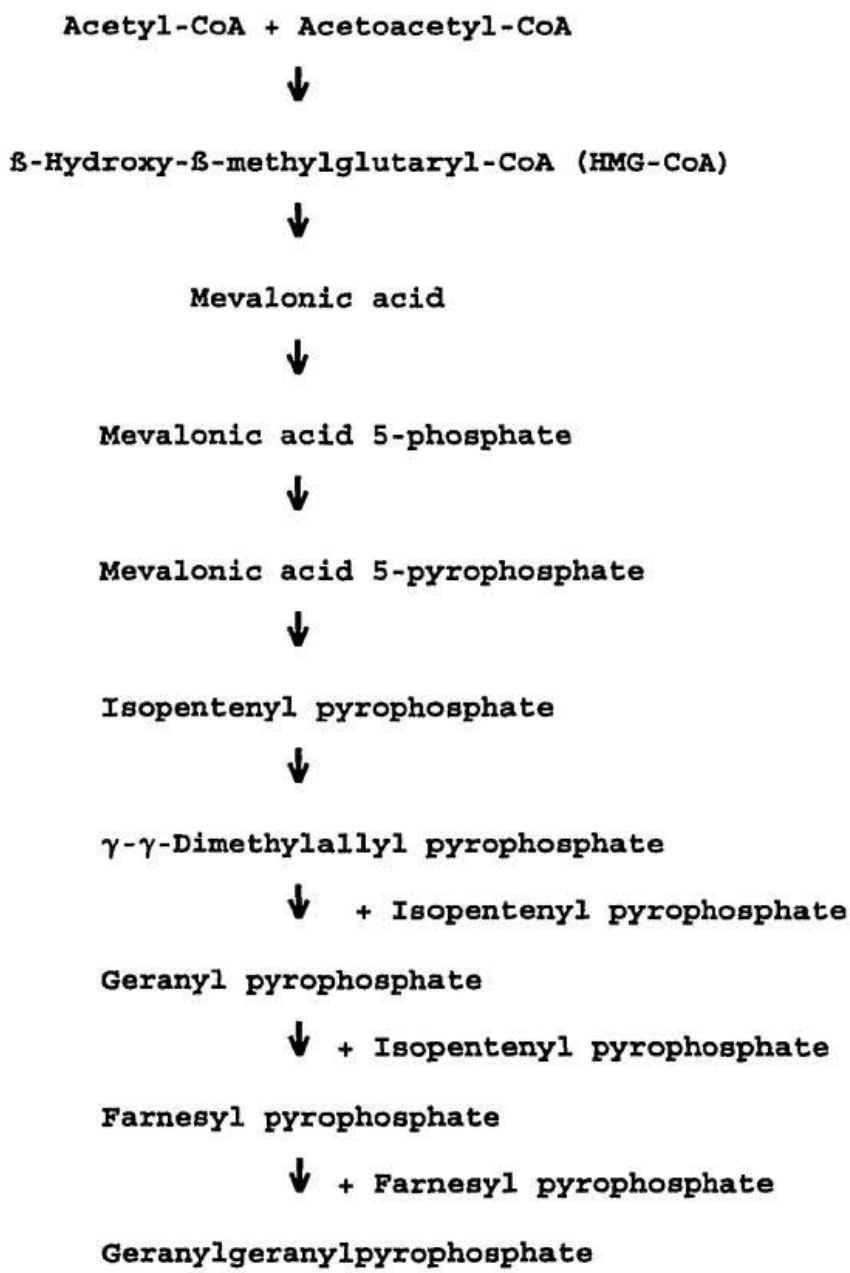


Fig. 1.8b. *Conversion of geranylgeranyl pyrophosphate to cis-phytoene*

2 X Geranylgeranyl pyrophosphate

↓ (Altman et al. 1972; Qureshi et al. 1972)

Prephytoene pyrophosphate

↓ (Altman et al. 1972; Qureshi et al. 1972)

Cyclo propylcarbinyl cation

↓ (Camara et al. 1980, 1982)

Cis-Phytoene

Fig. 1.8c. *Conversion of cis-phytoene to torulene and torularhodin (Simpson et al. 1971)*

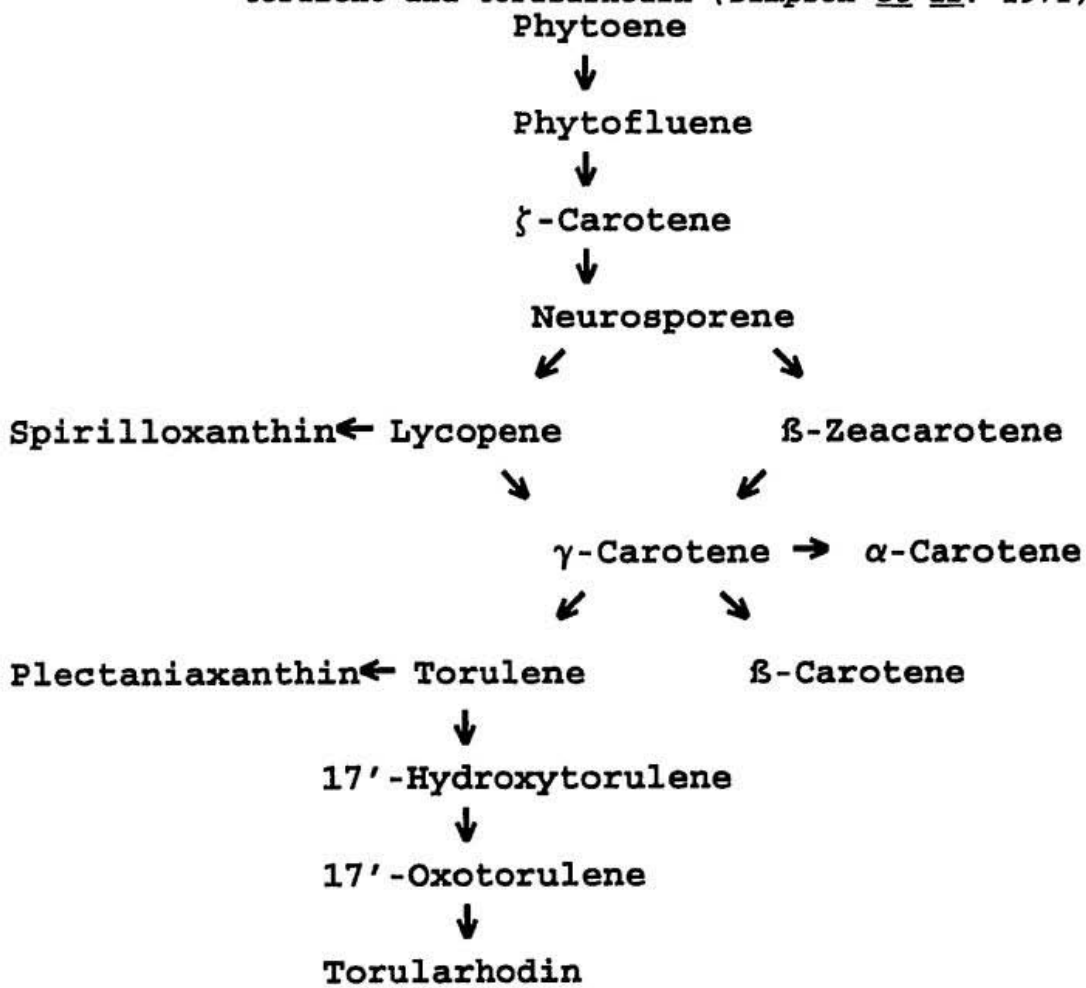
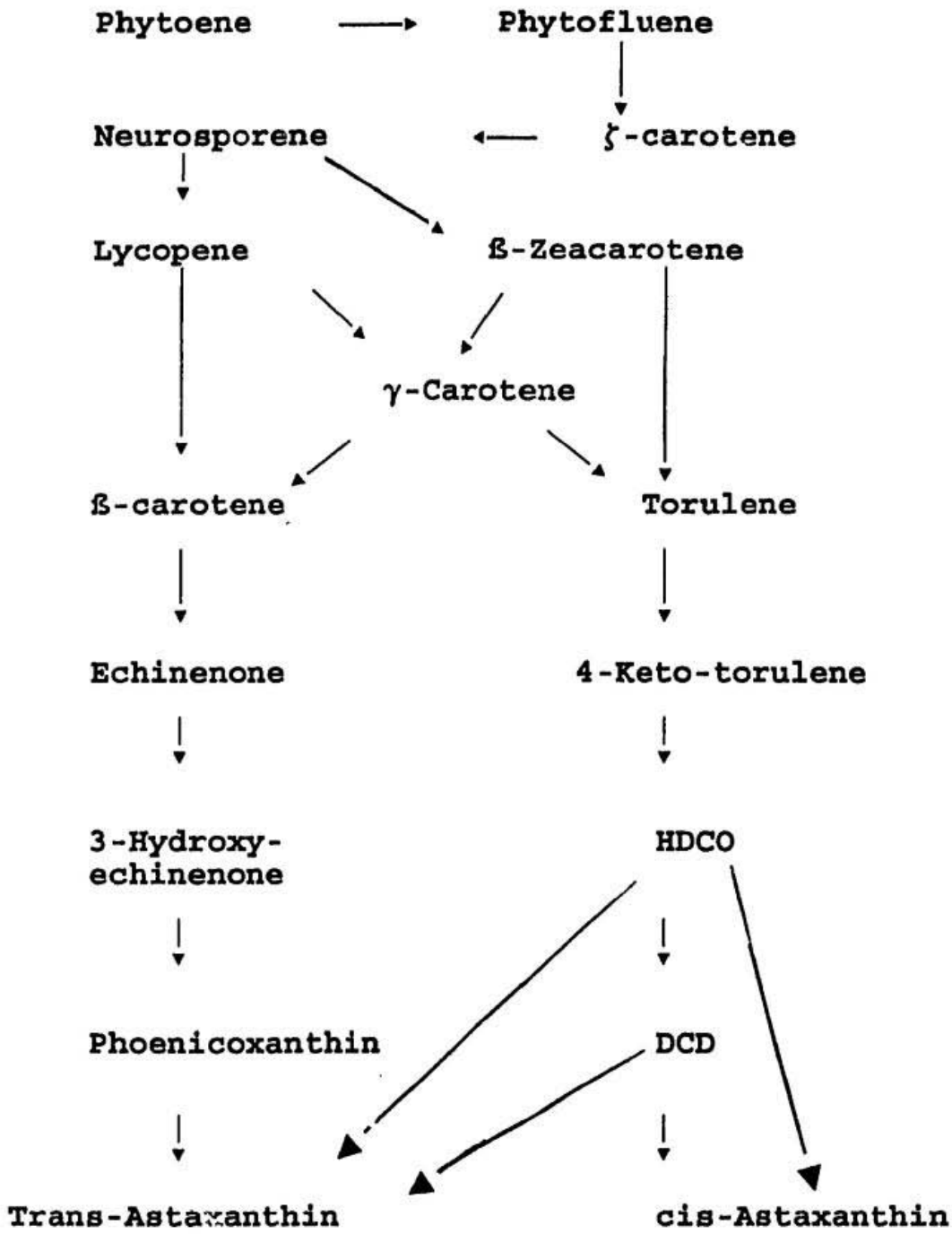


Fig. 1.8d. *Conversion of phytoene to astaxanthin (Andrewes et al., 1976; Johnson and An, 1991).*



RESEARCH OBJECTIVES

Due to the rising cost of the synthetic pigments added to aquaculture diets, an escalating concern among the general public regarding their safety in foods, and more stringent FDA regulations, more studies are being conducted that report the use of natural sources of these pigments. One of the areas where the present research was focused was to examine the possibility of using the new Rhodotorula rubra isolate reported in this thesis, as a carotenoid source for aquacultured fish, and to compare it's efficacy with synthetic pigments.

The objectives of my research were:

1. Identification of the unknown yeast strain that was isolated from yogurt.
2. Studies on the morphology and ultrastructure of the isolate.
3. Identification of the major pigments produced by the isolate.
4. Studies on the sexuality of the yeast isolate.
5. Studies on the growth parameters on cheaper substrates including industrial by-products.
6. Genetic studies involving mutagenesis with nitrosoguanidine.
7. Studies on the kinetics of growth and pigmentation.

8. Studies on the growth data of the batch cultures of the yeast grown in shake flask and fermenter.
9. Conducting a feeding trial on salmonid fish to determine the pigment uptake by the fish.

CHAPTER 2

Investigation of the new isolate from yogurt

2.1 INTRODUCTION

Due to the depletion of traditional fish species, aquaculture is developing worldwide to cope with the demands for additional protein foods. An important factor affecting the consumer acceptance of the many cultivated fish is the color of the flesh (Meyers, 1977; Ostrander et al. 1976). Since the use of synthetic feed colorants is fast declining due to stricter regulations and reluctance by consumers to accept their use, the possibility of using the red yeast, Phaffia rhodozyma as a substitute for salmonid and crustacean diets has been tested (Johnson et al. 1977; Johnson et al. 1980). This yeast produces a carotenoid, astaxanthin, as its major pigment (Andrewes et al. 1976; Haard, 1988; Johnson and Lewis, 1979). This could become an attractive alternative to the synthetic carotenoids.

However, three major drawbacks to commercialization are a rigid cell wall which limits the pigment extractability, a slow growth rate and poor digestibility of whole Phaffia cells by the fish (Johnson et al. 1980). The present study was thus undertaken to exploit the potential of a new yeast strain isolated from yogurt which has a faster growth rate and from

which a much higher amount of the carotenoid is readily extractable. The growth parameters of the two red yeasts on different media were compared along with other growth characteristics including generation time and biomass yield. The studies on the morphology of the red yeasts included surface topology as well as cell wall structure. The new isolate was assigned its present status as Rhodotorula rubra and studies were also conducted on the chemistry of the pigment.

2.2 MATERIALS AND METHODS

2.2.1 Organism: A red yeast contaminating a home - fermented yogurt was isolated and used in the present investigation. Phaffia rhodozyma ATCC 24202 was used as a control.

2.2.2 Culture conditions: Shake flasks of 2 L capacity containing 800 mL of Yeast extract/Malt extract medium (YM broth, Difco) were incubated at 20°C in a 'Psychrotherm' controlled environment incubator (New Brunswick, NJ, USA) in the presence of light and with a shaking rate of 150 rpm for 5 days.

2.2.3 Identification of the isolate: Using the API (Analytical Profile Index) 20C clinical yeast system (API analytical products, Plainview, New York), a ready-to-use

micromethod, 19 assimilation tests were performed and the biochemical reactions were recorded after 72 hours of incubation at 20°C.

2.2.4 Growth on different media: The new isolate and P.rhodozyma were compared for their growth parameters on different media like YM, malt agar, Sabouraud's dextrose agar (SDA), potato dextrose agar (PDA), Czapek Dox and tryptic Soy agar (TSA). All these media were supplied by Difco.

2.2.5 Nutritional and Growth characteristics: The growth experiments were performed in YM medium and the generation time, yield and cell-size were investigated.

2.2.6 Morphology and ultrastructure of the isolate: The morphology of the isolate was studied using a Zeiss EM 9A transmission electron microscope and a Hitachi S570 scanning electron microscope as well as a Zeiss 100X Phase Planachromat.

For the electron microscopy, the yeast cells were fixed for 1 hour at 4°C in Karnovsky's fixative in 0.1 M Sorensen's phosphate buffer(Karnovsky, 1965). After several rinses in buffer, the cells were post-fixed in 1% osmium tetroxide in Sorensen's buffer for 1 hour at 4°C (Dawes, 1971). The sample was then dehydrated through a graded series of ethanol.

For the transmission electron microscopy (TEM), the samples were embedded in Spurr's resin (Spurr, 1969). Thin sections were stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined with a Zeiss EM 9A

transmission electron microscope.

For scanning electron microscopy (SEM), the yeast cells were allowed to settle from drops of 100% ethanol on 0.1% poly-L-lysine (mol.wt. 70,000-150,000) coated glass coverslips (Mazia et al. 1975). The sample was then dried to its critical point from liquid carbon dioxide in a Polaron E 3000 critical point drying apparatus.

The coverslips were attached to aluminum stubs with silver paint, gold coated in an Edwards model 150A sputter coater and examined with a Hitachi S570 scanning electron microscope operated with an accelerating voltage of 20 KV.

For the phase-contrast microscopy, a wet mount of the culture was prepared.

2.2.7 Studies on pigment production by the isolate:

Pigment extraction: The pigment was extracted by rupturing the cells in a French press at an internal cell pressure of 32000 psi. The ruptured cells were extracted with acetone 4-5 times.

Pigment purification:

The pigment then was extracted from the acetone extract into petroleum ether (60-80, Analar) and cold 10% NaCl (10:90) (Jeffrey, 1974). The ether hyperphase was then removed and the pigment concentrated by rotavapor distillation and stored at -20°C (Fig. 2.1).

Absorption spectrum: The absorption spectrum of the pigment in acetone was taken using Shimadzu UV-Vis recording

spectrophotometer UV-260.

Thin-layer chromatography:

i) Analytical TLC: For the TLC of the purified pigment, a standard astaxanthin [mol. formula: $C_{40}H_{52}O_4$: mol. wt. 596.82: λmax: 475nm (acetone)] procured from Hoffman La Roche (Basle, Switzerland) was co-chromatographed using a silica gel G plate and developed with two solvent systems: (a) benzene : dioxane : acetic acid (60:36:04) for 90 minutes; (b) acetone: petroleum ether (20:80) for 60 minutes. Development was followed by staining with iodine and the Rf values were calculated.

ii) Preparative TLC: This was performed with the chromatotron using silica plate and solvent (a) as used in analytical TLC.

HPLC chromatography:

i) A reverse- phase high performance liquid chromatography with an octadecylsilylated (ODS) silica column (Brownlee, 260 X 4.6 mm; Applied Biosystems) was performed for the separation and analysis of pigments using a Beckman model 157 detector with varying wavelength of 345-510nm. A binary solvent system was used in the set-up; solvent A (80% methanol; 15% Milli-Q water; 5% ion pairing solution containing 7.5g ammonium acetate in 100mL water) and solvent B (Methanol: acetone; 70:30). A linear solvent gradient was run over six minutes during which the solvent flow was changed from solvent A to solvent B.

To 500μl of the purified acetone extract, 150μl of the

ion pairing solution was added and a 100µl of the mixture was run on HPLC for 30 minutes.

ii) Because the above method did not reveal much information about the identity of the pigment, a 10 µ partisil ODS-3 column (4.6mm i.d. X 250 mm length, Alltech Associates) equipped with a RP-18 spheri-10 guard column (4.6mm i.d. X 30 mm length), was used which eluted with a 15 min. concave gradient of 75% to 100% methanol in water at 2 mL/min. The eluate was scanned every second from 220 to 600nm using a HP 1040A diode array detector. Azobenzene was used as an internal standard.

Chemistry of the pigment: The pigment was characterized using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. For MS analysis, a Micro Mass 70.70 HS mass spectrometer with double-focusing beam operated at 70 eV was used.

HPLC - MS analysis: For the HPLC-MS, assistance was sought from Richard Taylor (Arthur D. Little Inc., Massachussetts, USA). A VG 70-250 SEQ mass spectrometer equipped with a moving belt interface was used. Full scan mass spectra were recorded over the mass range from 100 to 650 amu at a scan rate of 0.5 sec per scan. Source temperature was 280° C. Ionization was by electron impact with an electron energy of 40 eV and 1Ma emission current. A Waters 600 MSD pumping system was used which eluted the column (same as for HPLC above) with a 15 min concave program of 75% to 100% methanol in water at 1.5

mL/min.

Column chromatography: A 30 X 2 cm column was slurry filled with CF-11 cellulose in hexane and a portion of the pigment was applied. The column was eluted with 100 mL each of hexane, 1% acetone in hexane, 5% acetone in hexane, 10% acetone in hexane and 100% acetone. Fractions were collected from the column, bulked and reduced in volume. These were then further analyzed by HPLC and HPLC-MS.

2.3 RESULTS

2.3.1 Assimilation of carbohydrates: Using the API clinical yeast system, the isolate was found to utilize a series of carbohydrates including glycerol, trehalose, melezitose and galactose but failed to use complex carbohydrates like 2-keto-gluconate and methyl-glucoside (Table 2.1). The isolate has been identified as Rhodotorula rubra as the correlation index for R. rubra was the highest (1: 75). The identification results have been confirmed by Microcheck Inc. (Northfield, Vermont) using their technique involving cell wall fatty acid analysis. The similarity indices provided for R. rubra, R. minuta and Candida krussel are 0.305, 0.186 and 0.257.

The isolate, hereafter, has been named as R. rubra TP 1.

2.3.2 Growth on different media: Although P. rhodozyma grew on Czapek Dox, YM and TSA with slight pigmentation on the latter medium, it failed to grow on SDA, malt agar and

showed scant growth on PDA. The new isolate grew readily on all media but had little pigmentation on Czapek Dox.

2.3.3 Nutritional and growth characteristics: The nutritional and biochemical properties of the new strain are shown in Table 2.2 which again confirmed its status as R. rubra by comparing the results of the new strain with R. rubra ATCC 9449.

The new strain of R. rubra had a shorter generation time while the cell yield was higher than that of P. rhodozyma (Table 2.3).

2.3.4 Morphology and ultrastructure of the isolate:

The scanning electron and phase-contrast micrographs indicated circular and ellipsoidal cell shapes of this isolate and P.rhodozyma respectively (Fig. 2.2 & 2.3). From the micrographs it is apparent that the unknown yeast is considerably smaller in size compared to P. rhodozyma.

The transmission electron micrographs of P rhodozyma showed a lamellar or multilayered structure, typical of the cell walls of the basidiomycetous yeasts (Miller et al. 1976; Kreger-Van Rij and Veenhuis, 1971). The thin dark layers were observed which alternated with the grey broad layers (Fig. 2.4). However, the R. rubra cell wall structure was different from that of P. rhodozyma and the lamellae were not very well defined. Also, the cell walls of the older cells were considerably thicker than that of the young cells (Fig. 2.5).

2.3.5 Studies on pigment production:

Absorption spectrum: The absorption spectrum in acetone indicated that the pigment belonged to the family of carotenoids (Fig. 2.6). The initial spectral analysis demonstrated the presence of a major carbonyl containing carotenoid in the pigment extract from the new isolate. When the absorption spectra were taken in methanol, the pigment extracts from the new isolate and P. rhodozyma displayed the same absorption maximum, however, the fine structures of the spectra were different (Fig. 2.7).

TLC: Although the analytical TLC revealed Rf values very similar to those obtained for astaxanthin under two different solvent systems (Table 2.4), the TLC is of limited application in the characterization of carotenoids. The spots thus obtained were then subjected to preparative TLC. However, the contaminating impurities were not eliminated.

Mass spectroscopy: The mass spectrum analysis indicated that the pigment from the new yeast has a molecular weight of about 604 while astaxanthin has a molecular weight of 596 (Fig. 2.8 & 2.9, respectively).

HPLC:

i) Using the binary solvent system, when equivalent amounts of pigment from the new isolate and P. rhodozyma were analyzed, the HPLC profile demonstrated nearly eighty times more pigment in the sample (Fig. 2.10) compared to that of P.

rhodozyma. However, this method was not of much use in revealing the identity of the pigment.

ii) Using the second set of conditions as mentioned in the Materials and Methods section, the HPLC and diode array analysis of the pigment from the new isolate showed 4 major peaks with an erratic baseline indicating the presence of contaminants. Peak 1 was a non carotenoid while peak 2 had absorbance maxima at 483, 465 and 497nm indicating that the pigment was torularhodin (Fig. 2.11). The peak 3 had maxima at 427, 443, 460 and 480nm indicating the presence of α-carotene (Fig. 2.12). Peak 4 had maxima at 443 and 479nm (Fig. 2.13) suggesting that the pigment could be ß-carotene.

Two fractions obtained from the CF-11 cellulose column were analyzed by HPLC using the above conditions. Figure 2.14 illustrates the absorbance spectra of the carotene fraction #3 from the cellulose column while Figures 2.15 and 2.16 show the HPLC and the diode array analysis. The results suggested that this pigment could be ß-carotene.

Figure 2.17 shows the absorbance spectrum of the fraction #5 (xanthophyll) from the CF-11 column. The multiple peaks as well as the HPLC and diode array analysis (Fig. 2.18 and 2.19, respectively) indicate the presence of a cyclic carotene. The absorbance maximum at 485nm and the multiple peaks as seen in Figure 2.19 suggest its identity as torularhodin.

HPLC-MS analysis: The HPLC-MS was complemented with two methods: the extracted ion chromatogram wherein a specific ion was scanned as a function of elution time from the HPLC into the mass spectrometer, and the TIC (Total ion current) of the sample, a means of monitoring the total mass of material from a sample eluting from the HPLC into the MS. The TIC of the Phaffia pigment showed the presence of astaxanthin (Fig. 2.20, A, B and C). However, the TIC of the unknown pigment was more complex. When extracted ion chromatograms were generated at m/z 564 and m/z 534 (Fig. 2.21), significant peaks were obtained. These results indicated that torularhodin or its precursor torulene could be present.

Table 2.1. API 20C test for identification of the isolate

Substrate	Result
1. Negative control	-
2. Glucose *	++++
3. Glycerol	++
4. 2-Keto-D-gluconate	-
5. L-Arabinose	+++
6. Xylose	+++
7. Adonitol (Ribitol)	++++
8. Xylitol	+++
9. Galactose	++++
10. Inositol	-
11. Sorbitol (Glucitol)	+++
12. Methyl-D-glucoside	-
13. N-Acetyl-D-glucosamine	-
14. Cellobiose	-
15. Lactose	-
16. Maltose	++++
17. Sucrose	++++
18. Trehalose	++++
19. Melezitose	++++
20. Raffinose	+++

* Positive control

\- Negative assimilation, growth equal to negative control

++ Positive assimilation, growth equal to 50% of positive control

+++ Positive assimilation, growth equal to 75% of positive control

++++ Positive assimilation, growth equal to positive control

API profile: 6 672 073.

Correlation index:

Rhodotorula rubra : 1/75

Rhodotorula glutinis : 1/485

Candida guilliermondii : 1/1000,000

Table 2.2. Nutritional and biochemical properties of the new isolate compared with R. rubra ATCC 9449:

	TP 1	R. rubra
Fermentation of D-glucose	–	–
Assimilation of carbon compounds:		
Erythritol	–	–
Melezitose	+	+
Inositol	–	–
Melebiose	w	–
Maltose	+	+
Rhamnose	–	–
Mannitol	+	+
Trehalose	+	+
D-Ribose	+	+
Cellobiose	w	+
Ribitol	+	+
Raffinose	+	+
Citric acid	+	+
Sucrose	+	+
Arabinose	+	+
D-Xylose	+	+
Succinic acid	+	+
Soluble starch	+	+
Galactose	+	+
Lactose	–	-
KNO_3 test	+	+
Urease test	+	+
Glucose-yeast extract (50% w/w)	+	+
Starch formation	–	-
Growth on vitamin-free medium	+	+
Arbutin test	+	+
Gelatin liquefaction	+	+

- No growth or utilization
+ Growth or utilization
w Weak reaction (growth)

Table 2.3. Growth characteristics of the two red yeasts

Growth parameters	New isolate	*P. rhodozyma*
Cell-size	diameter: 3 ± 1μm	length: 7.5 ± 1.5μm width: 6.0 ± 1μm
Generation time (h)	10 ± 1	43 ± 1
Cell yield (g dry weight/L)	24 ± 1	10 ± 1

Table 2.4. Thin layer chromatography of the purified pigment

Solvent system	Rf-value	
	Astaxanthin[a]	Sample[b]
Benzene: dioxane: acetic acid (60:36:04)	1.0	1.0
Acetone: petroleum ether (20:80)	0.53	0.54
	0.3	0.31
	0.15	-

a Astaxanthin from Hoffman La Roche

b Purified pigment extract from the new isolate

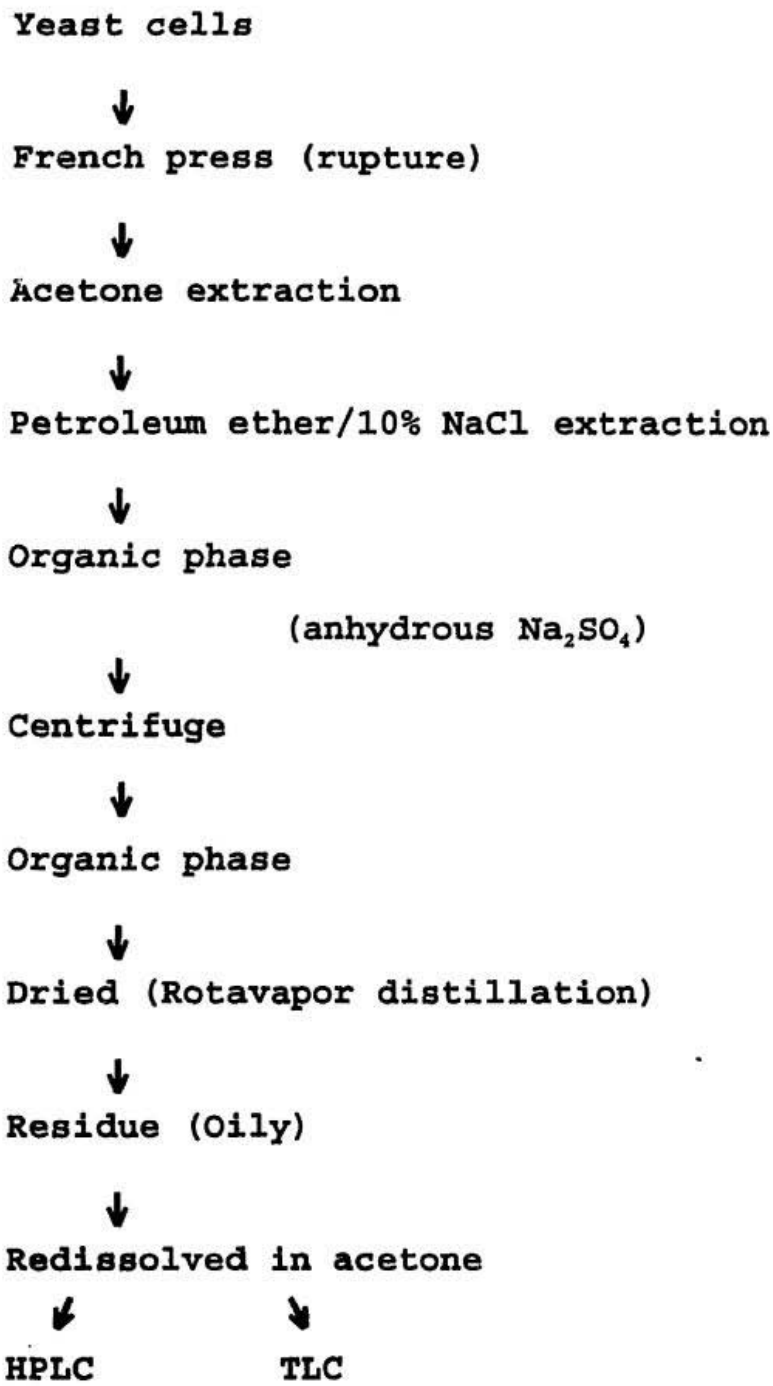


Fig. 2.1. Scheme of pigment extraction

Fig. 2.2. A scanning electron micrograph of R. rubra TP 1 showing a circular cell shape.

Fig. 2.3. A scanning electron micrograph of P. rhodozyma showing an ellipsoidal cell shape.

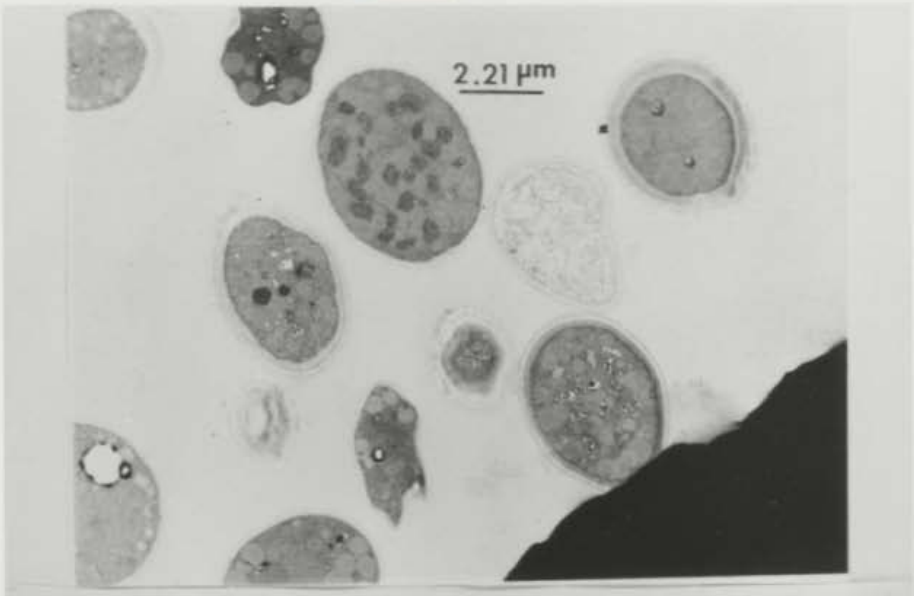


Fig. 2.4. A transmission electron micrograph of P. rhodozyma.

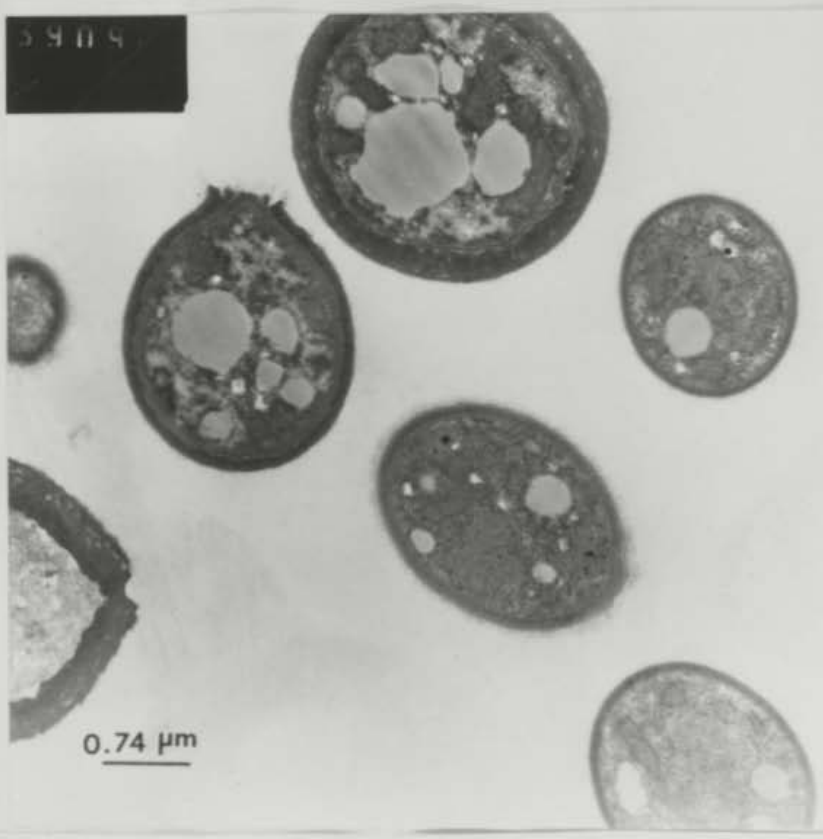


Fig. 2.5. A transmission electron micrograph of R. rubra TP 1.

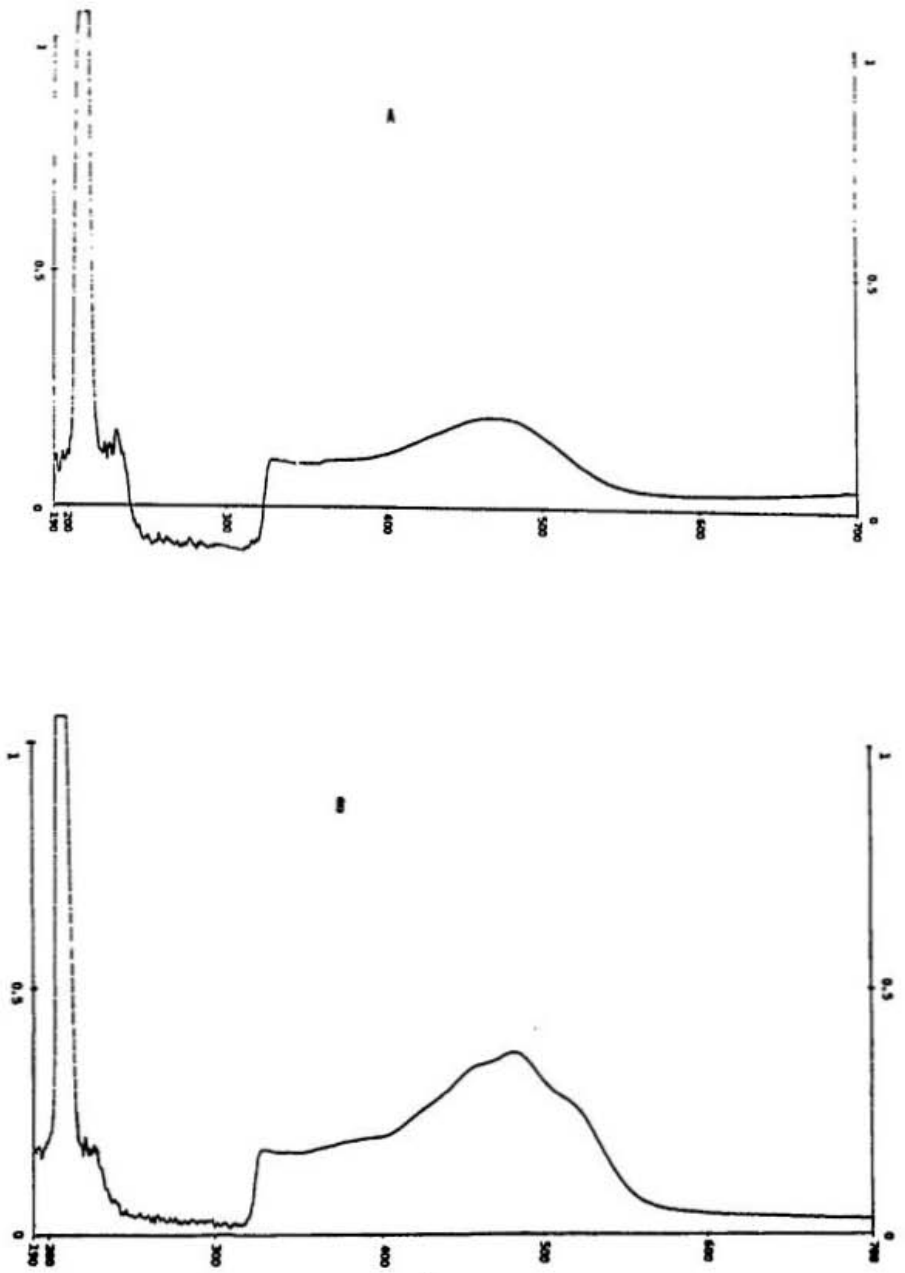


Fig. 2.6. Absorption spectrum in acetone.
(A) Pigment from Phaffia rhodozyma
(B) Pigment from Rhodotorula rubra TP 1

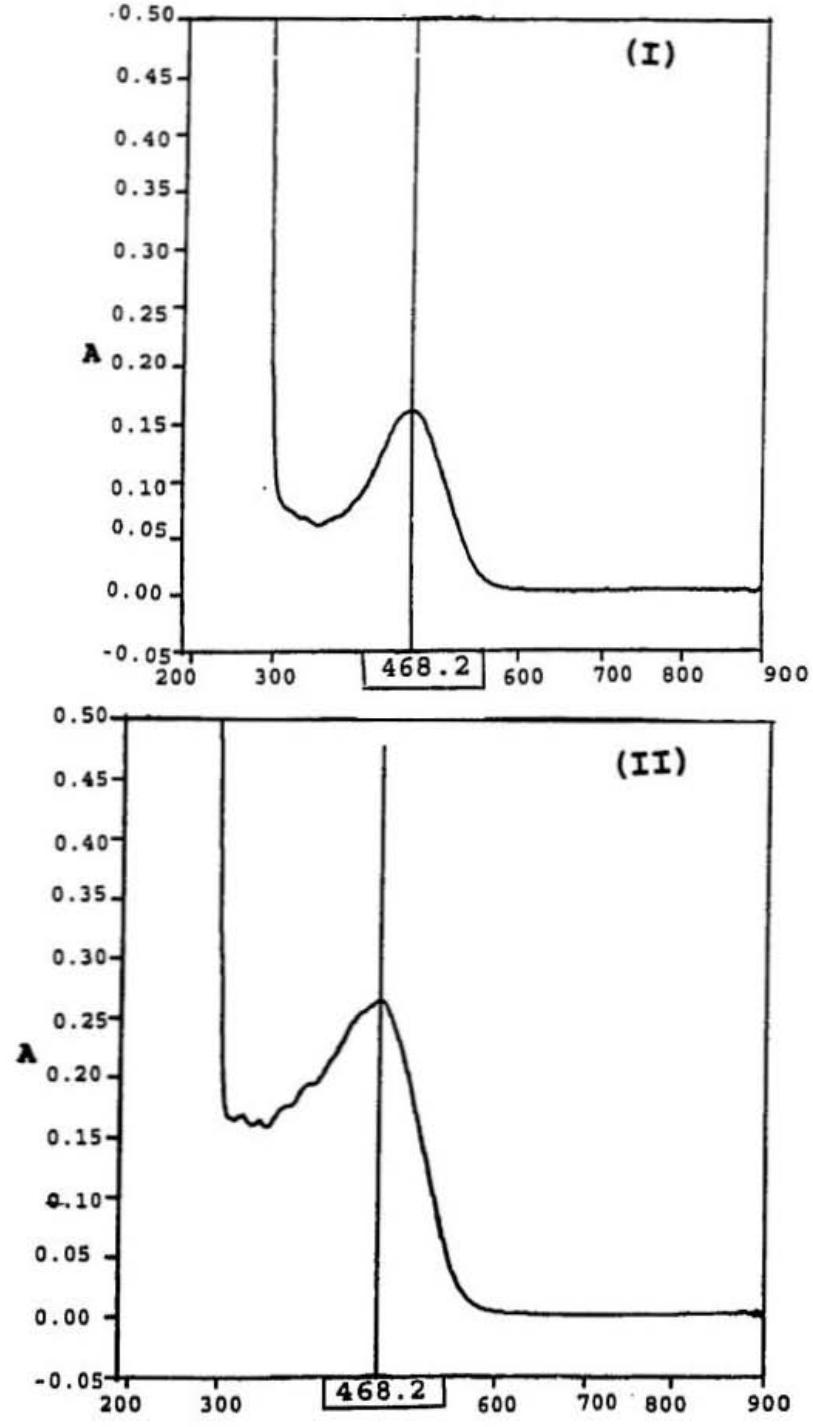


Fig. 2.7. Absorption spectrum in methanol.
(I) Pigment from Phaffia rhodozyma
(II) Pigment from Rhodotorula rubra TP 1

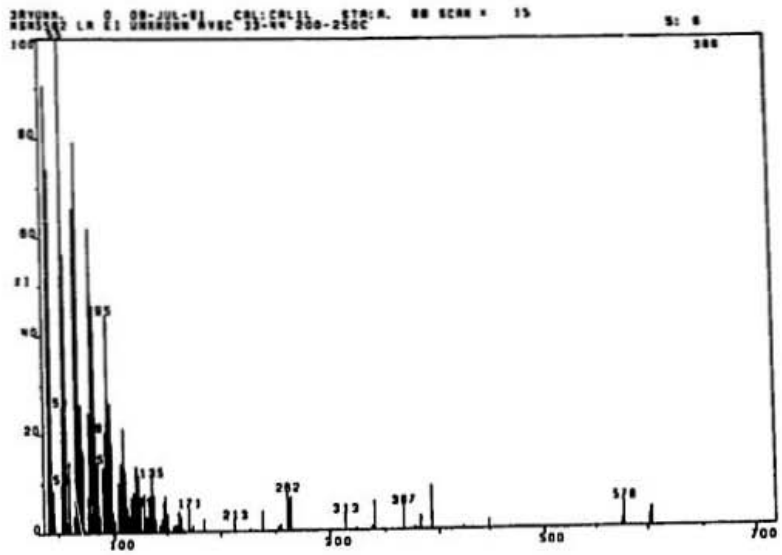


Fig. 2.8. The mass spectrum of the purified pigment from R. rubra TP 1.

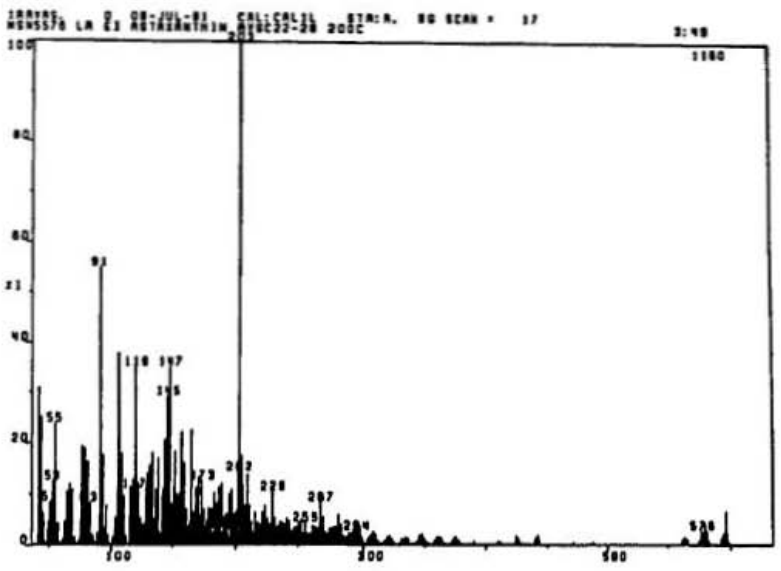


Fig. 2.9. The mass spectrum of astaxanthin.

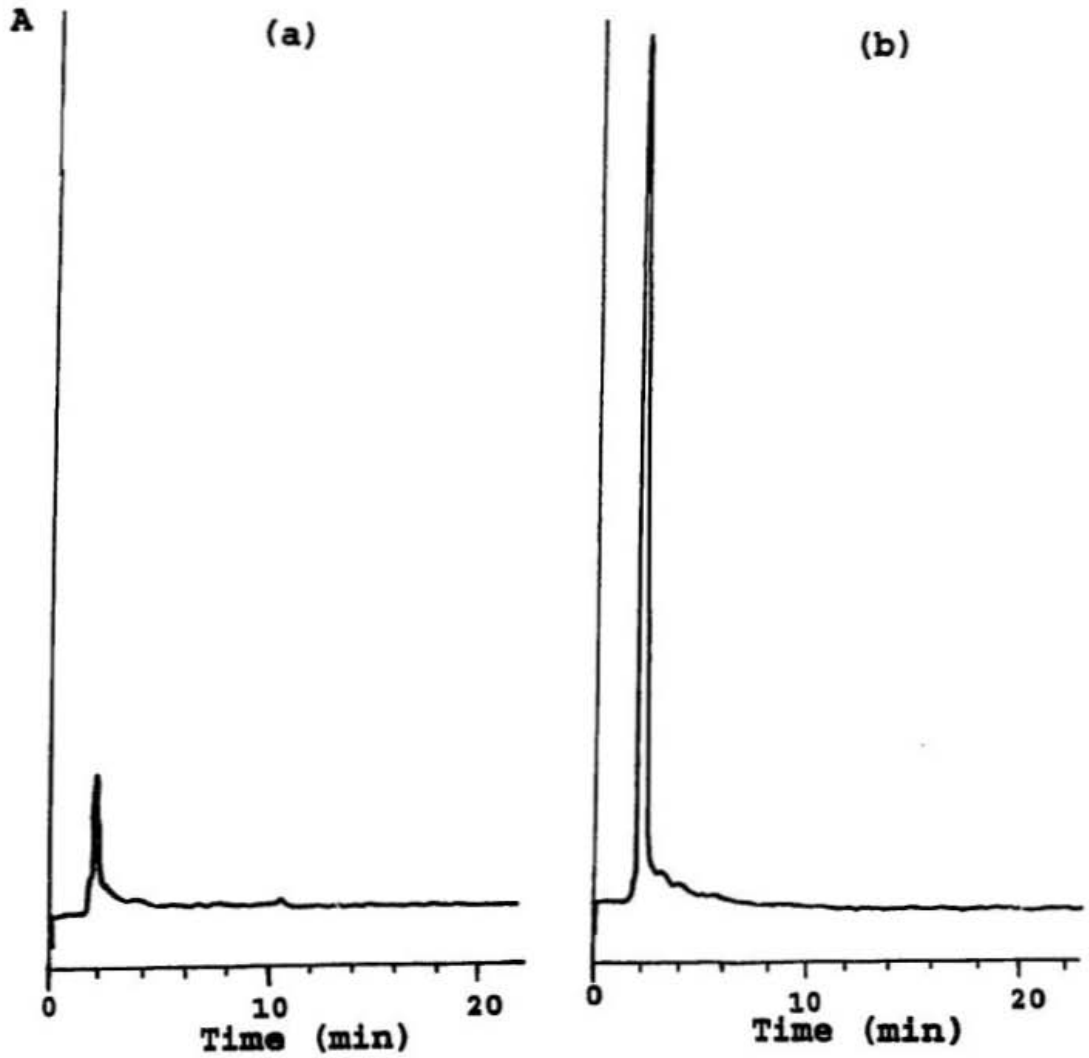


Fig. 2.10. Chromatographic behaviour using the binary solvent system (a) pigment from Phaffia rhodozyma (b) diluted pigment (1:10) from R.rubra TP 1 Sample: 100µl purified acetone extract containing 23% ion pairing solution. A : Absorption between 345-510nm.

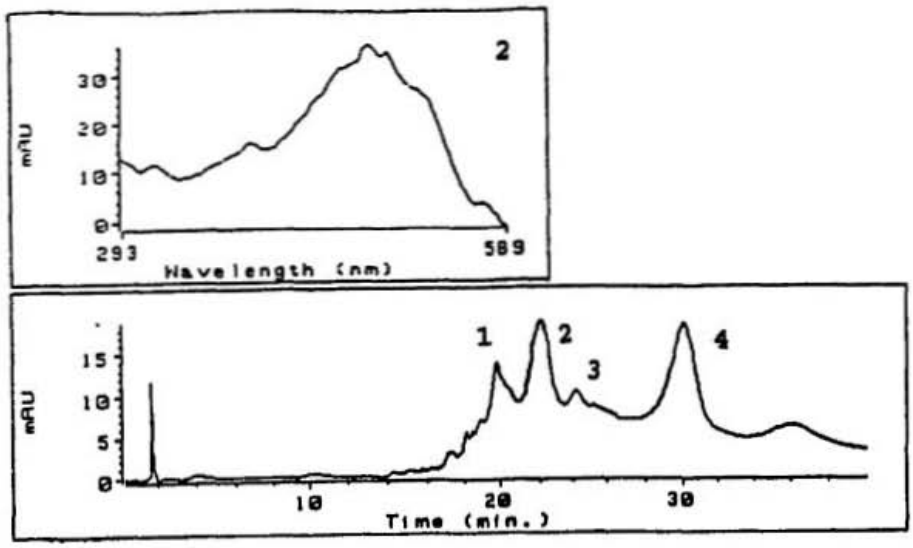


Fig. 2.11. Diode array analysis of peak 2 from the HPLC analysis (using one solvent) of the pigment extract from R. rubra TP 1.

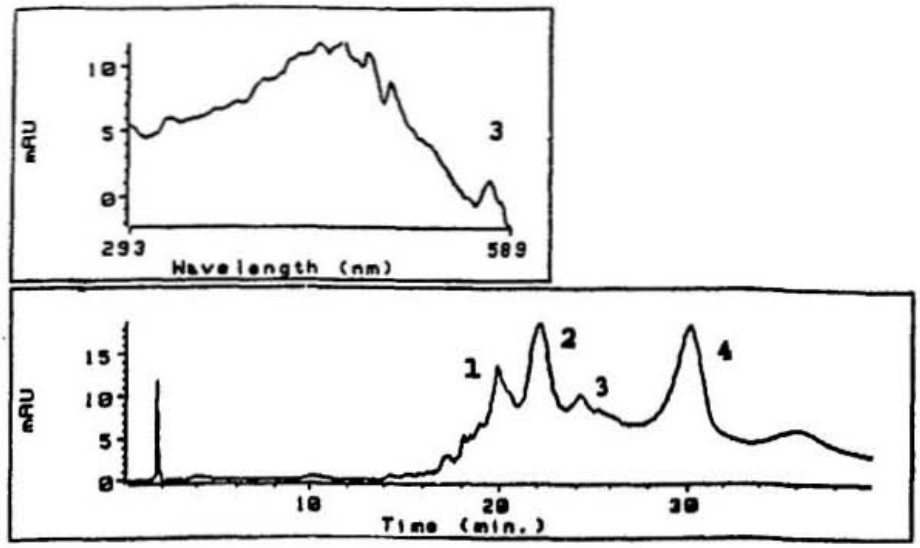


Fig. 2 12. Diode array analysis of peak 3.

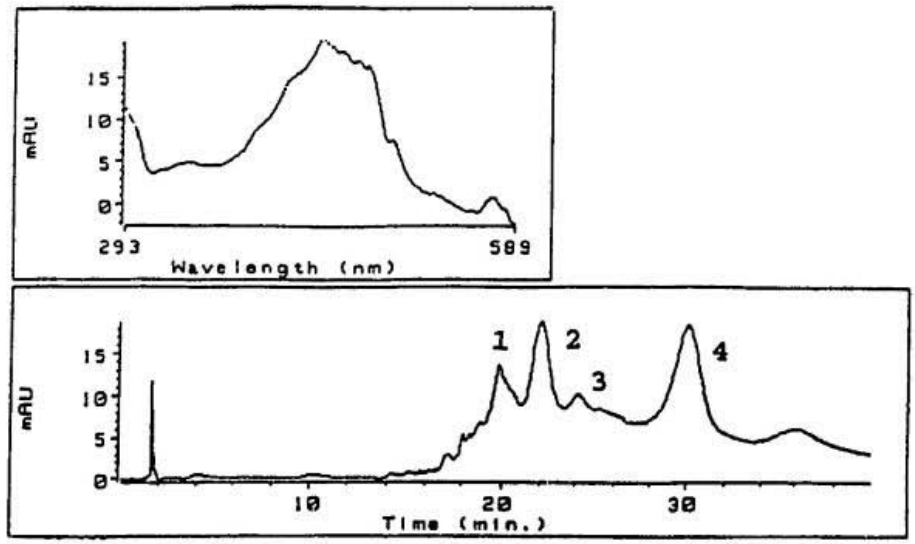


Fig. 2.13. Diode array analysis of peak 4.

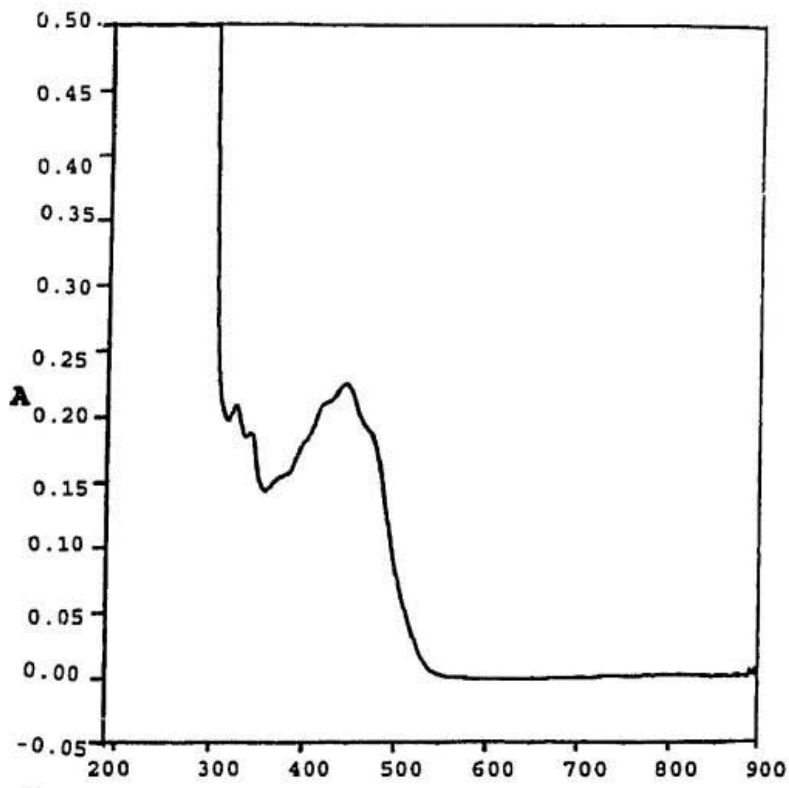


Fig. 2.14. Absorbance spectrum of fraction # 3 of the pigment extract from CF-11 cellulose column.

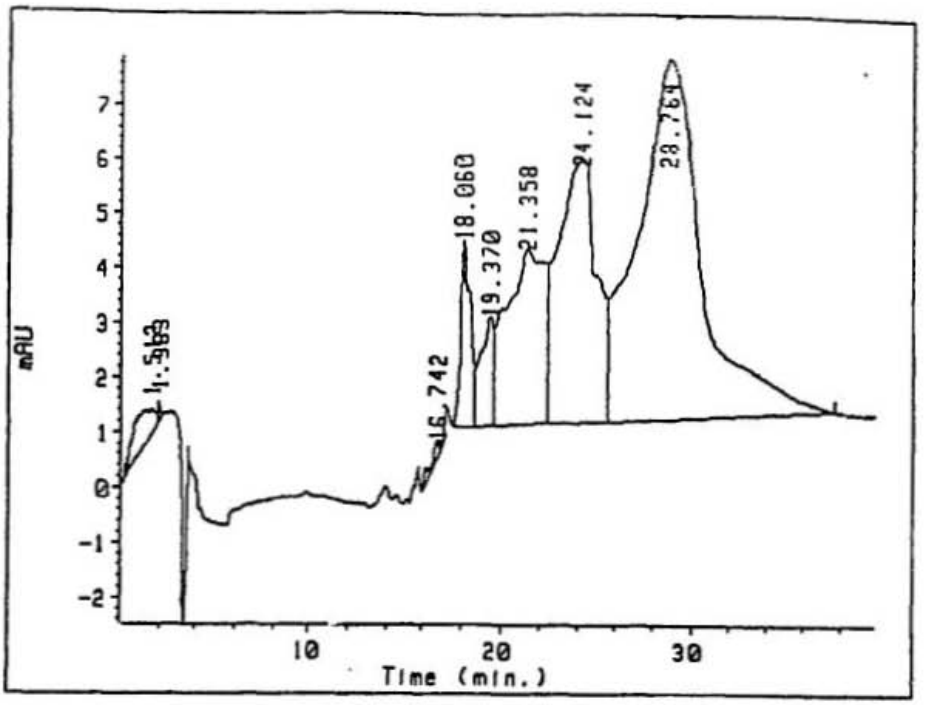


Fig. 2.15. HPLC analysis of fraction # 3 of the pigment extract.

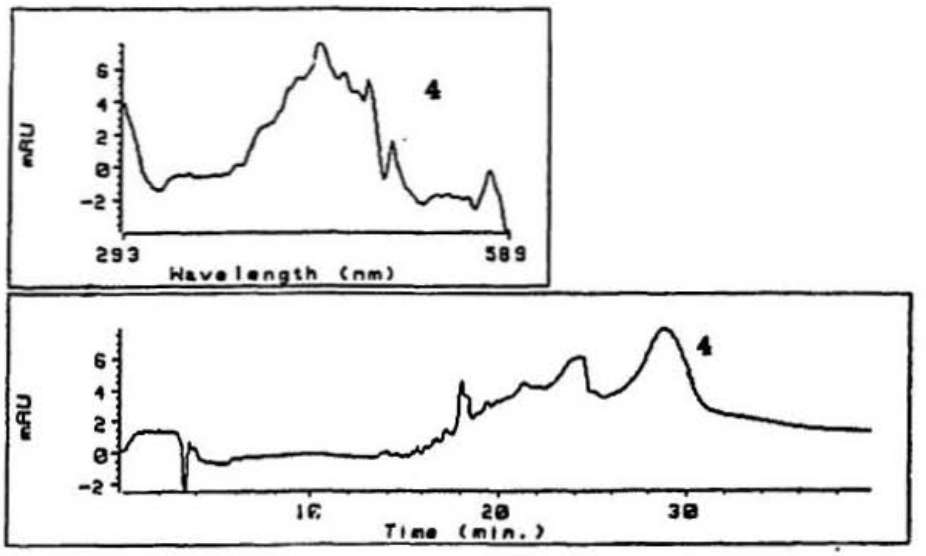


Fig. 2.16. Diode array analysis of fraction # 3 of the pigment extract.

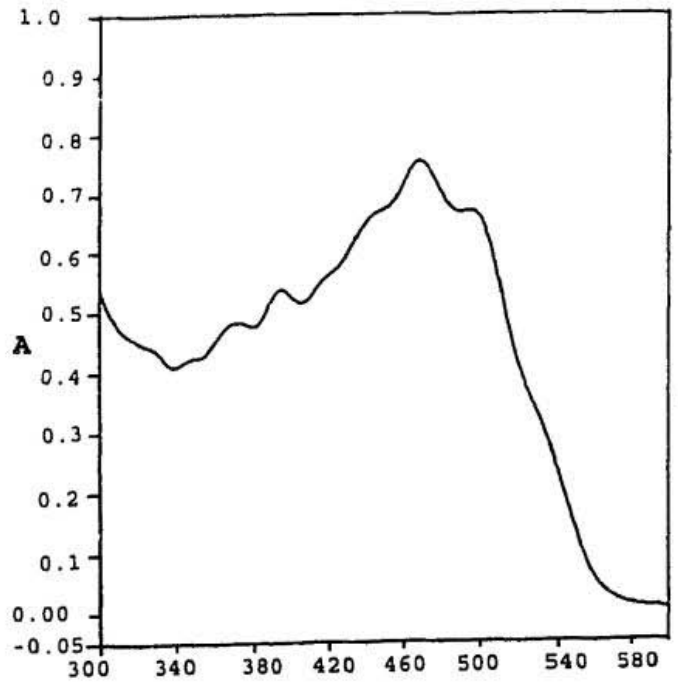


Fig. 2.17. Absorbance spectrum of fraction # 5 of the pigment extract from CF-11 cellulose column.

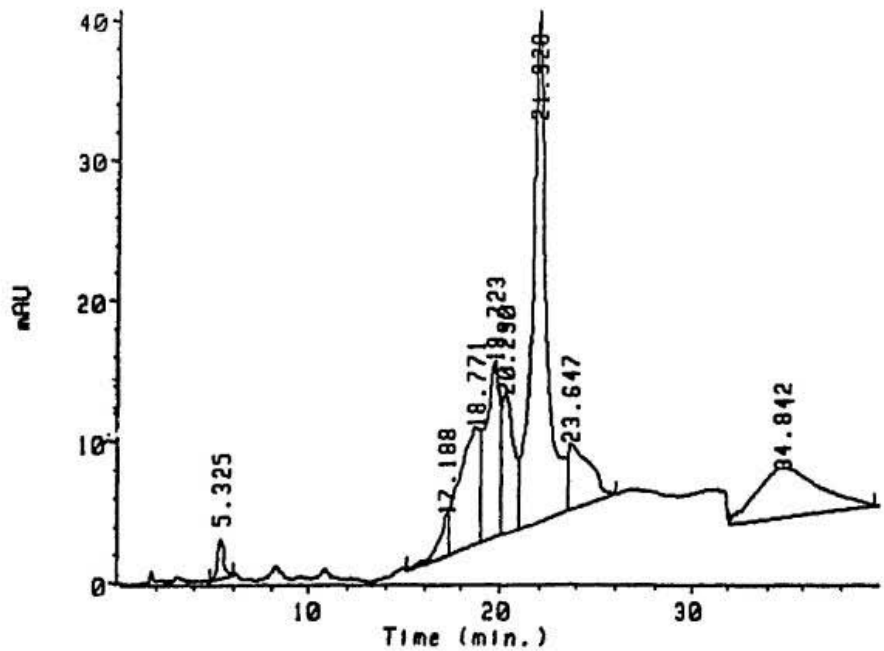


Fig. 2.18. HPLC analysis of fraction # 5 of the pigment extract.

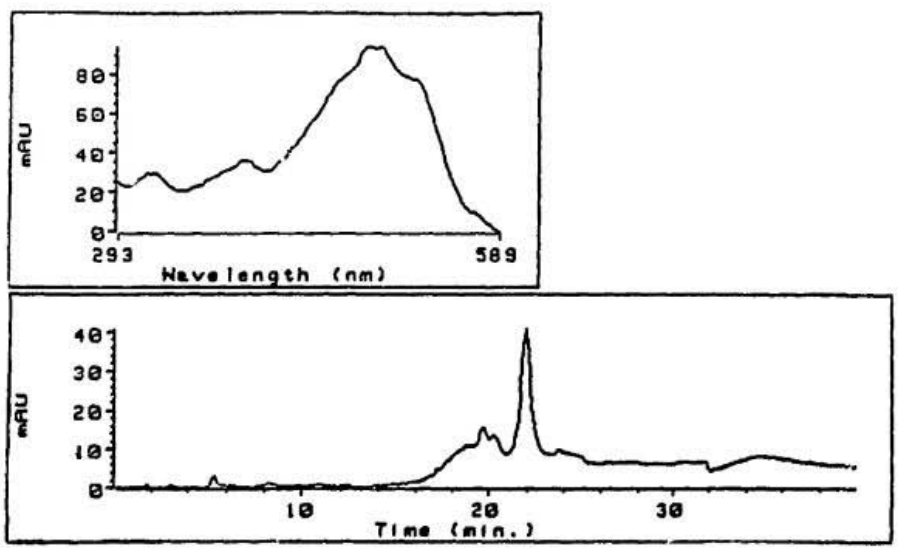


Fig. 2.19. Diode array analysis of fraction # 5 of the pigment extract.

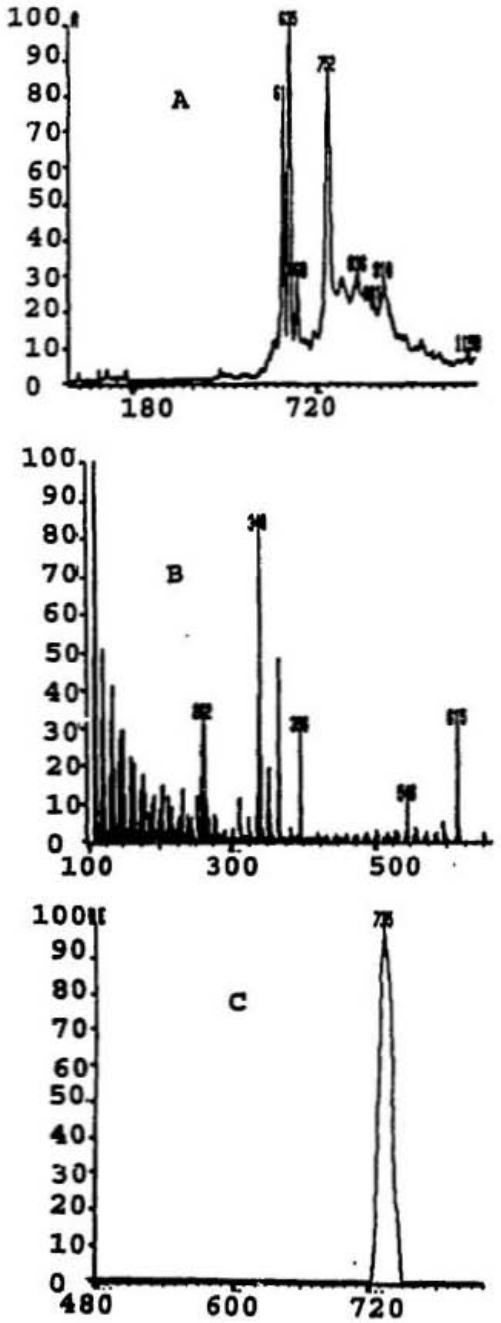


Fig. 2.20. Total ion current (A), mass spectrum (B) and extracted ion chromatogram at m/z 596 (C) of the pigment from P. rhodozyma.

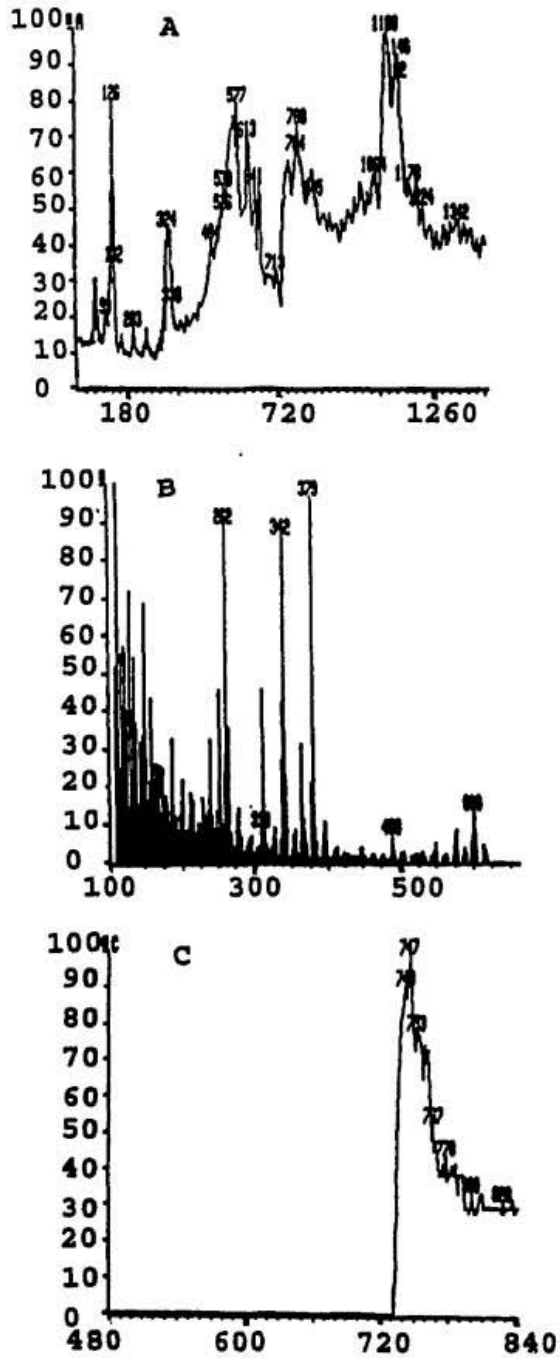


Fig. 2.21. Total ion current (A), mass spectrum (B) and extracted ion chromatogram at m/z 596 (C) of the pigment from R. rubra TP 1.

2.4 DISCUSSION

The new isolate demonstrated the best similarity with Rhodotorula rubra with a correlation index of 1:75 (API profile). Thus out of 75 R. rubra studied at random, there was an estimated chance that one out of 75 would have a profile identical to the unknown isolate. The cell wall fatty acid analysis also showed the best similarity index of 0.305 between the new isolate and R. rubra. In terms of nutritional and biochemical characteristics, the isolate demonstrated homology with the positive control R. rubra ATCC 9449. Except for melebiose and cellobiose that were weakly assimilated by the new isolate, the two strains showed identical assimilation patterns on a variety of carbon compounds. The positive control, however, did not assimilate melebiose while it grew on cellobiose. The new isolate, therefore, has been identified as Rhodotorula rubra TP 1.

The morphology of the new isolate was studied using scanning and transmission electron microscopy and differences were found in the cell wall structure of the two strains. While P. rhodozyma displayed the typical lamellar wall structure of the basidiomycetous yeasts, the lamellae were less distinct in the new isolate.

The new isolate of R. rubra TP 1 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. The new isolate also had a shorter generation time and higher cell yield than that of P. rhodozyma. A number of cheaper substrates including the industrial by-products have been tested for its growth as discussed later in the thesis. However, similar economical substrates to support the growth P. rhodozyma have not been reported. In recent study by Okagbue and Lewis (1984a), the potential of alfalfa residual juice as a substrate for the growth of P. rhodozyma was examined. However, limited success was achieved as the juice suppressed astaxanthin formation even though appreciable growth of the yeast was obtained. Thus the fastidious nutritional requirements of P. rhodozyma push the cost of the media used for its propagation much higher.

Another factor adversely affecting the commercial value of P. rhodozyma is its tough cell wall leading to difficult extraction of its pigment. Several methods have been tried to enhance the pigment extraction including mechanical breakage (Simpson et al. 1971) and chemical (acid or alkaline) hydrolysis, both of which have been found to be laborious and cumbersome. The latter method also denatured the carotenoids (Davies, 1976). The enzymatic digestion of the yeast cell walls was proposed by Phaff (1977) for the extraction of yeast protein employing microbial lytic enzymes. Thus, the breaking of P. rhodozyma cells by enzymatic digestion initially

appeared an attractive method for the extraction of the carotenoids. But due to technical difficulties, it was not successful. Johnson et al. (1978) utilized Bacillus circulans WL-12 for digesting the cell walls of P. rhodozyma wherein the lytic enzymes were induced by growth of the organism on heat-killed P. rhodozyma cells. Although astaxanthin was extractable by acetone-treatment of the cell mass, the process required heat-treatment to inactivate the yeast cells and the readjustment of the medium pH which was laborious. Also, the heat-treatment reduced the pigment concentration.

Thereafter, the two organisms were grown in a mixed culture (Okagbue and Lewis, 1985) to achieve yeast cell wall modification. The mixed culture method was found to be less laborious, less susceptible to contamination and less destructive to the carotenoids than the 2-step method of Johnson. The commercial attractiveness of the mixed culture was enhanced by the potential of re-use of cell-free culture fluid. The cell-free culture fluid in the Johnson method was similarly potent but the concentration of the enzyme by ultrafiltration was an expensive step. However, the mixed culture method of Okagbue and Lewis necessitated pH and temperature control as only a narrow range of the two factors supported good yield and extractability of astaxanthin. Also, an adequate bacterial inoculum for early yeast cell wall modification and a correct choice of time of harvest of

biomass were the limiting factors for maximum pigment extraction. Another drawback was the lower yields of astaxanthin as compared to the Johnson method.

Amongst all the known methods of astaxanthin extraction, no single method has been found to be most suitable for breaking the cell wall of Phaffia rhodozyma.

The most interesting feature of the new isolate was its much higher amount of pigment production when compared with P. rhodozyma. The enhanced pigment production observed in R. rubra may be explained by several factors: (a) easily breakable cells from which the pigment is readily released during extraction; (b) higher pigment production in R. rubra compared to P. rhodozyma; (c) greater biomass yield from R. rubra when grown under similar conditions; (d) higher extinction coefficient of the pigment in R. rubra. Although evidence thus far obtained points to higher pigment production per unit volume in the case of the new isolate, one or more factors may contribute to the observed results. Four pigments have so far been identified in the new isolate, namely, torulene, torularhodin, α-carotene and ß-carotene. The identification of the pigment extract from the new isolate was difficult due to the presence of non-carotenoid impurities. However, the possibility of astaxanthin or a similar xanthophyll in the new isolate is not ruled out due to similarities in terms of HPLC profile and absorption spectrum

between the pigment extracts from the two yeasts.

The present study shows that the new isolate does hold promise as a possible source of pigment for aquacultured animals, especially fish like trout and salmon. The rapid growth of the isolate and higher cell yields than P. rhodozyma together with its ability to grow on cheaper substrates are important considerations in this respect.

Feeding studies on fish have been undertaken to determine the potential of the new isolate as a source of protein, vitamin, minerals and carotenoid in aquaculture diets. These will be updated in a subsequent chapter.

CHAPTER 3

Studies on the sexuality in R.rubra TP1

3.1 INTRODUCTION

Harrison (1928) created the genus Rhodotorula for the red pigment producing asporogenous yeasts. Sexuality in R. glutinis Harrison was first reported by Banno (1967) which led to its transfer to the basidiomycetous genus Rhodosporidium toruloides. Since then several members of Cryptococcaceae (Rhodotorula, Cryptococcus and Candida) have been found to be related to basidiomycetous fungi (Newell and Hunter, 1970). The perfect form of another R. glutinis strain has been found to be related to a heterobasidiomycetous species Sporobolomyces roseus (Marchant and Smith, 1968). Strains of Candida showing heterobasidiomycetous life cycles were moved to Leucosporidium (Fell et al. 1969) while another strain of R. glutinis was moved to Rhodosporidium sphaerocarpum (Newell and Fell, 1970). Two other perfect forms of the yeast Rhodotorula were moved to Rhodosporidium malvinellum (Fell, 1970) and R. diobovatum sp. (Newell and Hunter, 1970).

Although most asexual yeasts are considered to be imperfect forms of ascomycetes, many of them have been found to have a basidiomycetous origin (Fell, 1970) and they have similar GC (Guanine-Cytosine) contents (Storck et al. 1969).

The present chapter deals with the sexual state in R.

rubra TP 1.

3.2 MATERIALS AND METHODS

3.2.1 Organisms: The organism used was a red yeast contaminating a home-fermented yogurt. Using the API Clinical Yeast System, cell wall fatty acid analysis and homology with a positive control, Rhodotorula rubra ATCC 9449, the isolate was identified as R. rubra TP 1 (Hari et al. 1992). Two controls, P. rhodozyma ATCC 24202 and Saccharomyces cerevisiae ATCC 9763 were also used.

3.2.2 Sporulation media: The following four media were tried to induce sporulation:

A. Fowell's acetate agar (Fowell, 1952) constituting 0.5% sodium acetate trihydrate and 2% agar.

B. Aqueous agar (Van der Walt and Yarrow, 1984) constituting 2% agar.

C. Yeast extract glucose agar (Van der Walt and Yarrow, 1984) constituting 0.5% powdered yeast extract, 2% glucose and 2% agar.

D. Malt extract agar (Van der Walt and Yarrow, 1984) constituting 5% powdered malt extract and 3% agar.

The last medium was found to be the best in inducing sporulation and was therefore used subsequently.

3.2.3 Microscopic examination:

A. Phase contrast and interference microscopy: The wet mounts of the yeast cells grown in the sporulation medium were prepared and were observed using a Zeiss 100X Phase Planachromat as well as a Zeiss photomicroscope I (Nomarsky interference).

B. Fluorescence microscopy: Two nuclear stains, mithramycin and DAPI (4, 6-Diamidino-2-phenyl-indole) were used. Both chemicals were purchased from Sigma Chemical Company. Mithramycin in a concentration of 0.6 mg/mL was used and the method of Slater (1976) was followed.

For the DAPI staining, a loopful of the cells was mixed with a few drops of DAPI solution (1 μg/mL) and smeared on a slide, air-dried and heat fixed. The last step was critical and hence a mild heat treatment was given so as to prevent early fading of the stain.

The slides stained by the above methods were then observed under a Carl Zeiss microscope (Oberkochen, West Germany).

3.2.4 Ultrastructure of the cell wall: This was observed by transmission electron microscopy. For details, refer to Chapter 2.

3.2.5 Urease test: Production of urease was determined by inoculating the culture on Christensen Urea agar (Difco) and the results were recorded after two days.

3.2.6 DBB (Diazonium blue B) test: The method of Hagler and Ahearn (1981) was followed.

The above three tests were performed to determine the affiliation of the isolate with ascomycetes or basidiomycetes.

3.3 RESULTS

3.3.1 Microscopic methods: Figure 3.1A and B apparently reveal the fusion of opposite mating types while zygote formation is evident in Fig. 3.2. The structure resembling asci with ascospores is seen in Figs. 3.3 to 3.5. The shapes of the putative asci as seen under a phase contrast microscope were as variable as the number of spores per ascus. Some of the asci were found to be sac-like with 4 spores each (Fig. 3.3A and B). The shapes and sizes of the spores were also found to be variable. Some asci had more than 4 ascospores as seen in Fig. 3.4, an ascus with seven ascospores. Some asci were cylindrical with four spores in them. Fig. 3.5 shows such an ascus with one of the spores being released.

Staining with mithramycin was not successful. The stain was found to fade too soon. On the other hand, DAPI was found to be quite effective in staining the nuclei of the ascospores although not all cells were found to be evenly stained. Fig. 3.6A shows two asci with five spores each, a circular ascus with spores arranged along the circumference and a funnel

shaped ascus with spores apparently piled up one on another. Figure 3.6B also shows an almost circular ascus.

S. cerevisiae stained with DAPI was used as a control (Fig. 3.7).

3.3.2 Ultra-structure of the cell wall: The cell walls of P. rhodozyma and the new isolate as seen by TEM (Fig. 2.4 and 2.5) were found to be lamellar with alternating light and dark layers, a wall characteristic of the basidiomycetous yeasts. However, the lamellae in the cell wall of Phaffia were found to be more distinctive and clearer than that of R. rubra.

In the case of S. cerevisiae, an ascomycetous yeast, the integrity of the cell wall was lost due to a problem in the fixation. Hence the typical ascomycetous characteristic of the cell wall, namely, a thin, dark outer layer and a broad, light inner layer, was not very clearly displayed by this yeast when observed by TEM (Fig. 3.8).

3.3.3 Urease test: The isolate was found to be urease positive.

3.3.4 DBB test: The isolate gave a very weak positive reaction with DBB; a faint violet color was observed only after 25 minutes of contact with DBB.

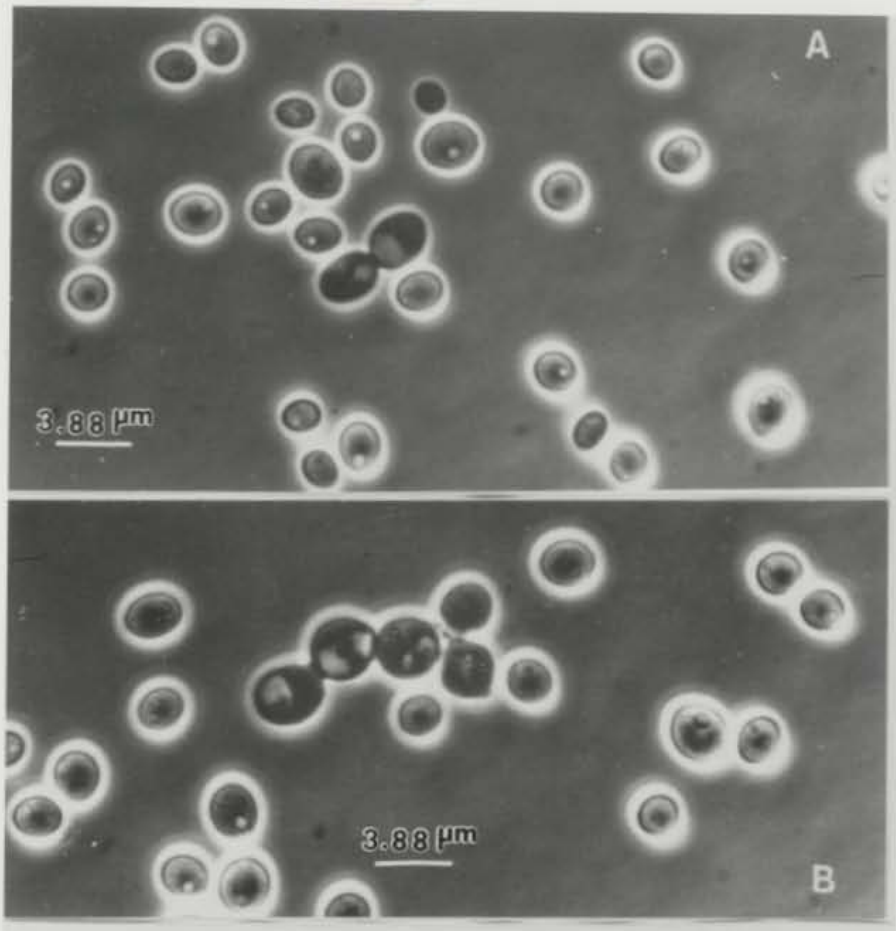


Fig. 3.1 (A & B). Fusion of opposite mating types of R. rubra TP 1.

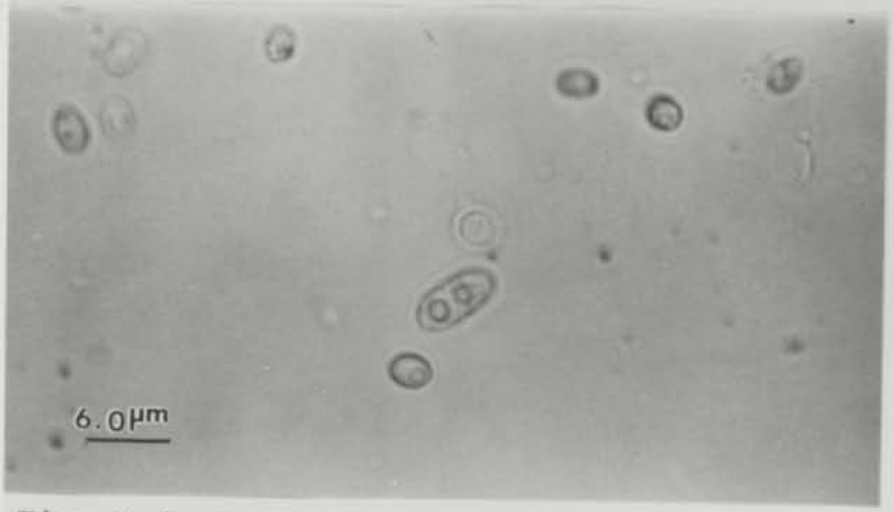


Fig. 3.2. Zygote formation in R. rubra TP 1.

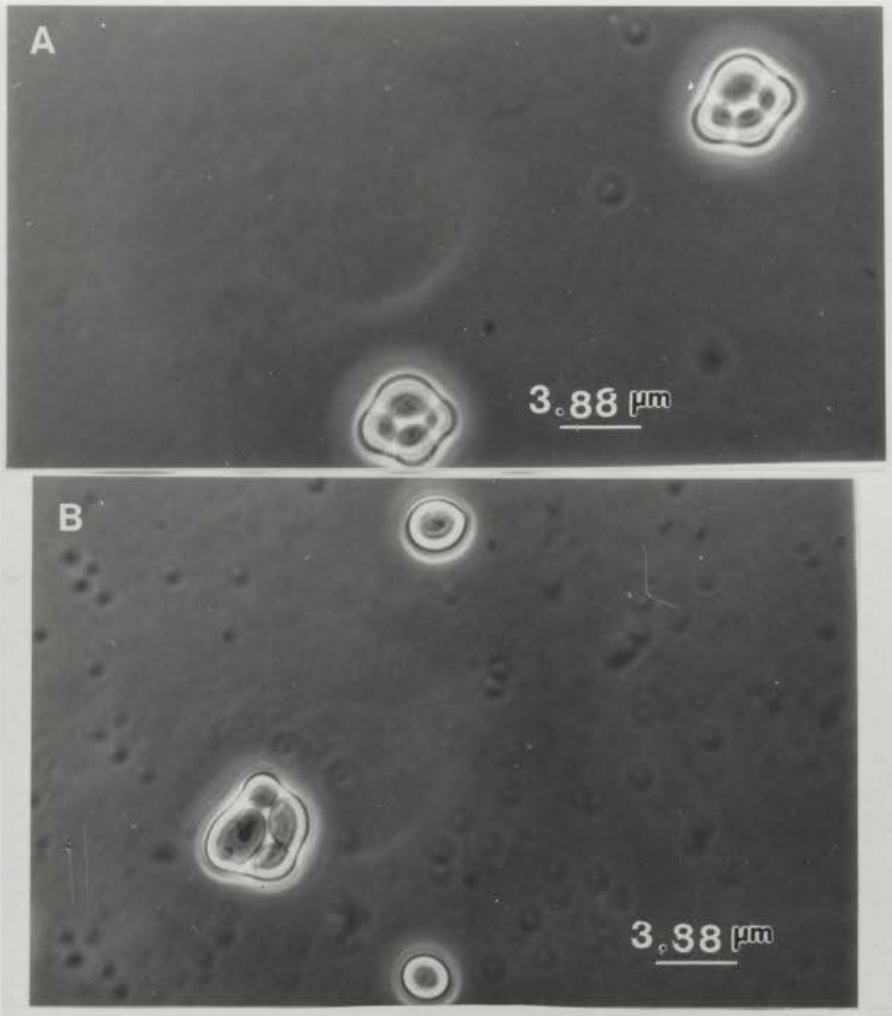


Fig. 3.3 (A & B). Sac-shaped ascus of R. rubra TP 1 with four spores.

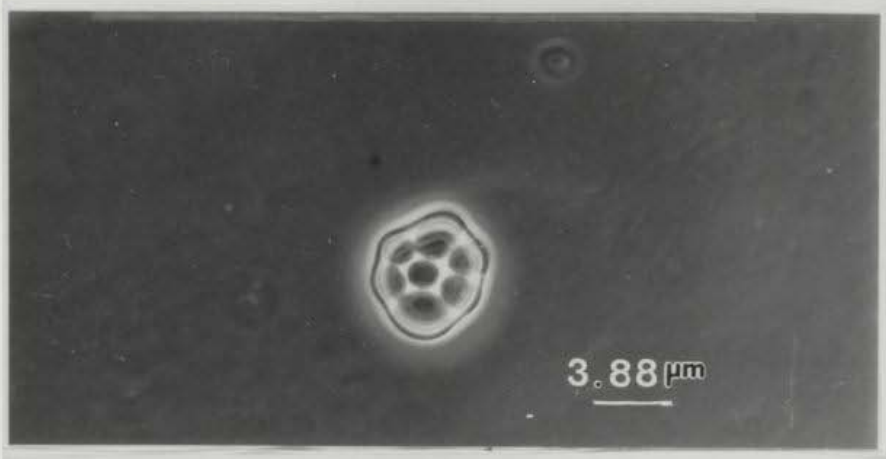


Fig. 3.4. Multi-spored ascus of R. rubra TP 1.

Fig. 3.5. Cylindrical ascus of R. rubra TP 1 with four spores.

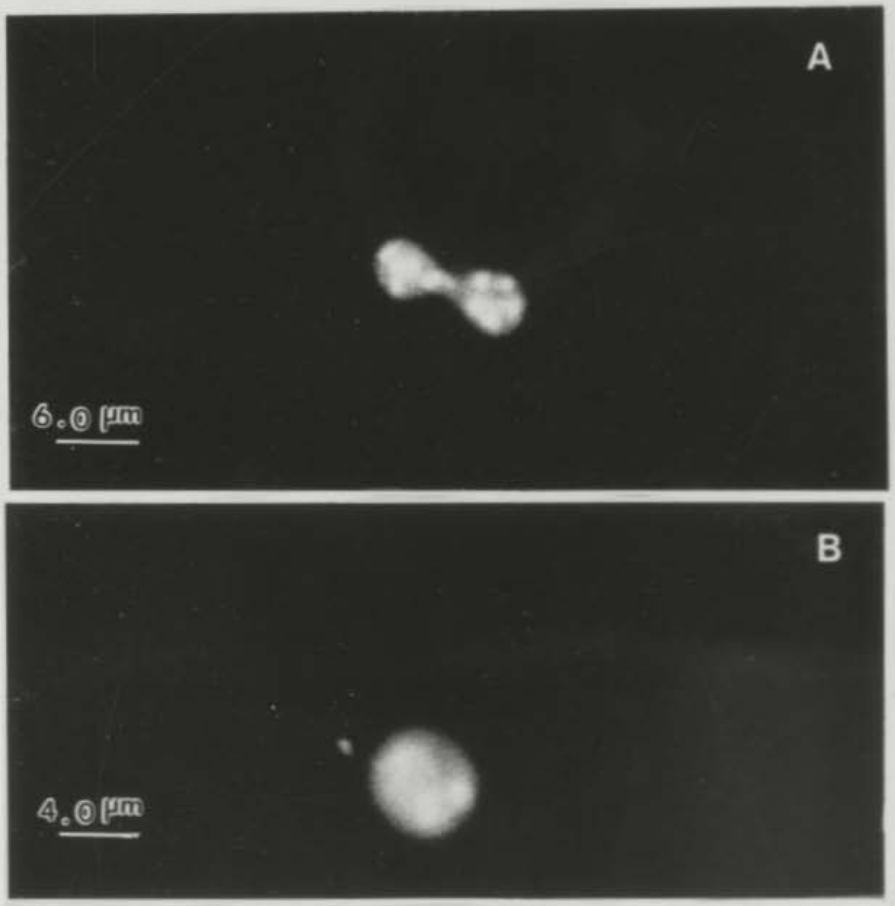
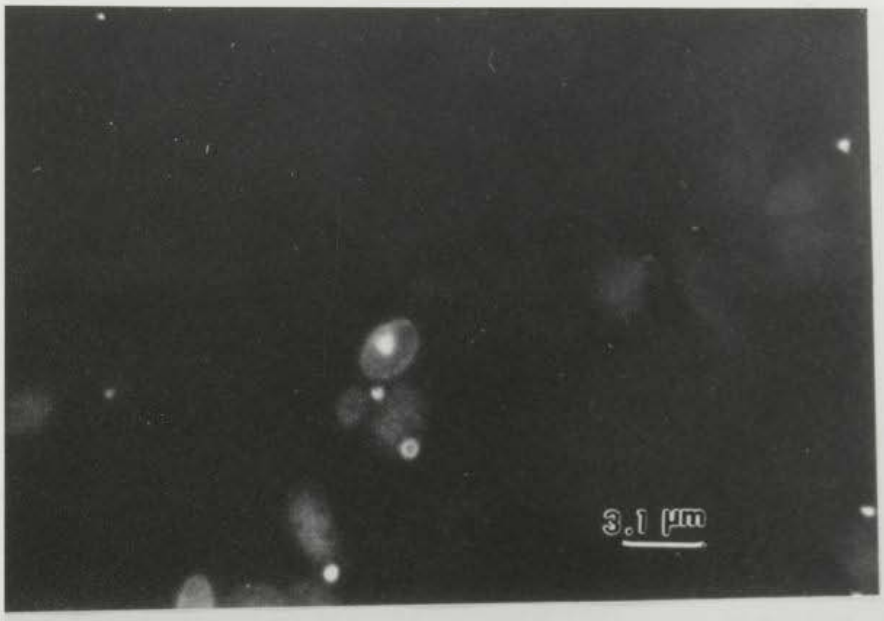


Fig. 3.6 (A & B). Ascospores of R. rubra TP 1 stained with DAPI.



ig. 3.7. S. cerevisiae stained with DAPI
howing a single nucleus.

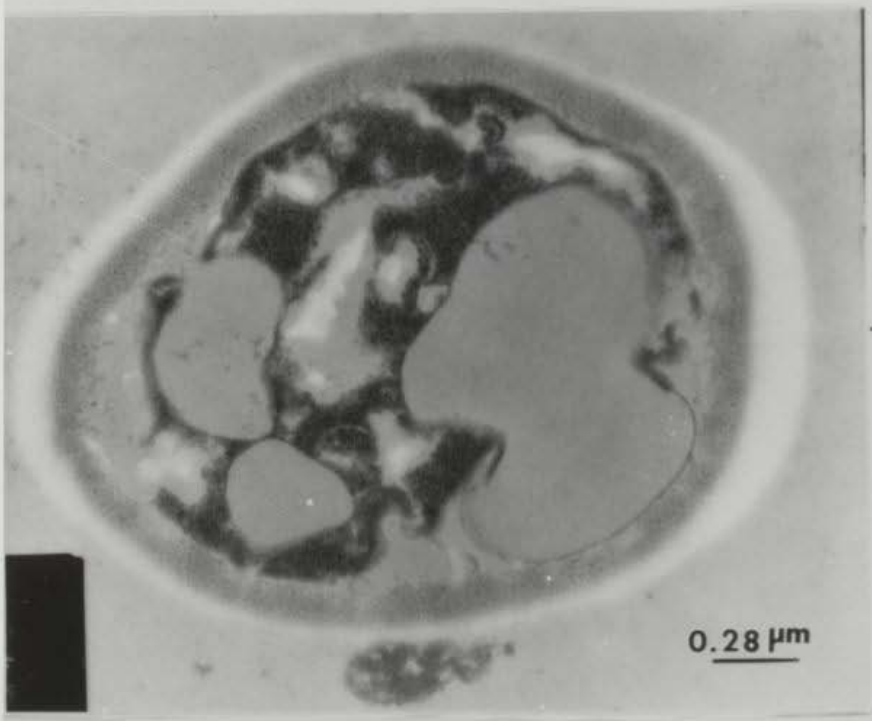


Fig. 3.8. A transmission electron micrograph of S. cerevisiae.

3.4 DISCUSSION

The exact phylogenetic affinity of the isolate R. rubra TP 1 is uncertain because of its ambiguous nature. The isolate displayed two characters of the Basidiomycetes, the cell wall structure and the urease test. The isolate showed a laminar cell wall structure, a characteristic basidiomycetous feature although the wall structure was not very well defined. The positive urease test demonstrated by the isolate, is not distinctive of basidiomycetous yeasts because a few ascomycetous yeasts have been found to be positive (Hagler and Ahearn, 1981). Nevertheless, the strongest evidence for its basidiomycetous affinity was the striking similarity of the putative ascospores reported in this thesis with the teliospores of Rhodosporidium toruloides (Newell and Fell, 1970). Considering the fact that R. toruloides is the sexual stage of Rhodotorula glutinis, and that the isolate R. rubra TP 1 differs from R. toruloides in a number of biochemical tests including the assimilation of succinic acid, soluble starch, galactose and methyl-D- glucoside, it is possible that R. rubra TP 1 could be a closely related and undescribed taxon of Rhodosporidium.

However, if the spores as observed by phase contrast microscopy and nuclear staining using DAPI are indeed ascospores, then the occurrence of these as well as weakly

positive DBB reaction (though the Ascomycetes show a negative DBB reaction) would indicate its ascomycetous affiliation. This is not surprising as Ascomycetes are known to have carotenoid producing yeasts, for example Saitoella complicata (Komagata et al. 1987) and some species of Taphrina and Protomyces (Eijk and Roeymans, 1982).

It is proposed that more definitive answers could be provided by the following studies that need to be undertaken:

a. Determination of DNA base composition and quinone system.
b. Chemical composition of the cell wall.
c. Cellular carbohydrate composition of whole cell hydrolysates.
d. Culturing and mating studies to isolate mating types.
e. Isolation of spores by micro-manipulator and detailed studies on the ascospores, e.g., examination of the internal structure of the spore wall by TEM and surface of the spores by SEM.
f. Quantitative nuclear staining using DAPI.

The occurrence of sexuality in R. rubra TP 1 is especially important considering the fact that the isolate shows enhanced pigmentation when compared with P. rhodozyma. It is a known fact that many members of Choanephoraceae, e.g., Choanephora cucurbitarum, C. conjuncta, Blakeslea circinans and B. trispora, the mated strains produced more ß-carotene in shaken flasks with synthetic media than either parent strain

grown separately (Hesseltine and Anderson, 1957). Similarly, the + and - cultures of C. cucurbitarum were found to produce 15 to 20 times more ß-carotene in liquid media than either mating type (Ciegler, 1965). Later, it was reported that mated cultures of + and - strains of Choanephoraceae produce substances which stimulated carotenogenesis by - strains only while + strains were not affected. These were named as ß- or BC factors. These factors were found to be poly-unsaturated C_{18} carboxylic acids.

These factors were later identified as trisporic acids A, B and C (Ciegler, 1965) and trisporic acid C was found to constitute 80% of total factors while B and A accounted for 15 and 1-2%, respectively.

Hence it is proposed that the occurrence of sexuality in this isolate could be responsible for the increased pigmentation shown by the isolate.

The process of sporulation is also important from the taxonomy and genetics point of view. As already discussed in the introduction, many asexual yeasts whose sexual stages were found, were moved to other genera.

The production of spores is also important for the hybridization of yeasts as this depends on the fusion of haploid spores or cells produced by the germination of spores. Thus improved strains of yeasts by hybridization could be used in industry, e.g., baking, brewing and spirit production

(Fowell, 1969).

The production of spores by yeasts in nature could probably be used as an important aid in adaptation and survival under changed environmental conditions. The fusion of spores could result in a variety of genotypes on which selective forces would operate so as to ensure that the fittest survive. Although the ascospores, unlike the bacterial spores, are only slightly more resistant than vegetative cells to heat, alcohol and other agents (Ingram, 1955), the differences in heat resistance have been reported to be a basis of a successful method of isolating spores from vegetative cells (Wickerham and Burton, 1954).

The results reported here are of importance in understanding the life-cycles of imperfect yeasts for which the sexual stage is not known yet.

CHAPTER 4

Growth kinetics of Rhodotorula rubra on different substrates

4.1 INTRODUCTION

The use of agricultural and industrial by-products for the growth of microorganisms is not an uncommon practice because these raw materials are readily available, can be assimilated and low in cost. Pure sugars like glucose and sucrose, however, are too expensive on an industrial scale.

Some inexpensive carbon sources that are routinely used for commercial purposes are, (1) carbohydrates like molasses, whey, sulfite waste liquor, starch derived from grains like corn, wheat, grain sorghum, barley, rice and oats and cellulosic materials, (2) hydrocarbons, (e.g., methane from natural gas, n-paraffins from crude oil and gas oil as well as refined alkanes), (3) alcohols, (e.g., methanol and ethanol). Other miscellaneous carbon sources used to a lesser extent include acetic acid, other organic acids and oils.

The nitrogen sources used on an industrial scale include both inorganic and organic sources. Gaseous ammonia, ammonium hydroxide and ammonium sulfate are examples of some typical inorganic nitrogen sources used while the organic nitrogen sources used in commercial fermentation processes are the by-products of agricultural and food processing industries, e.g.,

cottonseed, peanut, soybean and linseed meal, flour, corn steep liquor, dried distillers soluble, meat and bone meal, corn germ meal, etc. (Zabriskie et al. 1988).

Many agro-wastes and industrial by-products have been used for single cell protein (SCP) production. Some examples include olive oil mill effluent (Ercoli and Ertola, 1983), citrus wastes (Suhasukan and Yasin, 1986) as well as citric acid production waste effluents (Braun et al. 1979), vinasse, a by-product arising from ethanol and bakers yeast fermentation processes (Selim et al. 1991), orange peel (Curto et al. 1992), sugarcane bagasse pith (Rodriguez-Vazquez et al. 1992), waste leather chharri, a by-product of leather board factories (Mathur et al. 1993) and industrial mussel processing waste (Murado et al. 1993), lemonade-processing and sauerkraut-processing wastewaters (Hang, 1980). Various industrial by-products have also been used as substrates for the synthesis of important microbial products. Oxytetracycline has been produced by Streptomyces rimosus using date-coats (Abou-Zeid et al. 1991), date-seed lipid and hydrolysate (Abou-Zeid and Baeshin, 1992). Microbial enzyme production is possible on various substrates, e.g., ß-fructofuranosidase from Aspergillus japonicus grown on soybean residue, a by-product of the food industry (Hayashi et al. 199[illegible]), pectinase from citrus waste by A. foetidus (Hours and Garzon, 1992), yeast polygalacturonase from dairy wastes by Kluyveromyces

lactis (Murad and Foda, 1992), cellulase and ß-glucosidase from corn stover/sugarcane bagasse by Neurospora sitophila (Oguntimein et al. 1992). Substrates for citric acid production using A. niger include banana extract (Sassi et al. 1991) and crude beet molasses (El-Abyad et al. 1992). Xylitol, which is widely used in the food industry as a substitute for sucrose, can be produced from sugarcane bagasse using Candida guilliermondii (Mancilha et al. 1991) and xylose production is possible from xylan, a constituent of rice-straw, wheat bran, cotton-seeds, etc., by A. niger (Kawabata et al. 1992).

The yeast Rhodotorula has been grown on a wide variety of substrates as reported by different workers. Aligedy et al. (1977) grew Rhodotorula sp. in date extracts with nitrogen and phosphorus supplementation while Nishio and Nagai (1981) grew Rhodotorula in hydrolyzed mandarin orange peel.

The growth of Rhodotorula rubra TP 1 on various substrates is described in the present paper. This yeast produces carotenoids which have a potential as a colorant for aquacultured fish, an attractive commercial application. Using reverse-phase HPLC, the isolate has been shown to produce more pigment than Phaffia rhodozyma under similar cultural conditions (Hari et al. 1992). An effort was made to choose the best carbon and nitrogen sources for its propagation in a batch culture system. The effect of pH on the growth kinetics of the yeast was also examined.

4.2 MATERIALS AND METHODS

4.2.1 Organism: R. rubra TP 1 was used.

4.2.2 Substrates: The cane and beet molasses were procured from Lalle Nand Inc., Montreal; the brewer's wort from Labatts Brewery, St. John's, Newfoundland; the peat from Sundew Peat Bog, St. John's and the sulfite waste liquor from a pulp mill in Corner Brook, Newfoundland. The composition of these substrates are given in Tables 4.1, 4.2, 4.3 and 4.4. However, the substrate analysis was not done on the present preparations but the literature values were quoted.

4.2.3 Culture conditions:

1. Growth on molasses and peat hydrolysate: Crude molasses were diluted 1:10 and then used in the media as described below.

a. Effect of pre-treatment: Liquid medium (50 mL) in a Erlenmeyer flask (250 mL) contained 5 mL molasses (cane and beet in equal volumes) or peat hydrolysate, 0.25 g peptone and 0.15 g yeast extract. Two pre-treatments were used, namely, centrifugation at 2000 rpm for 45 min. (Treatment 1) and addition of potassium ferrocyanide, K_4FeCN_6 (0.01%) to the supernatant of the above treatment (Treatment 2).

b. Effect of different inorganic nitrogen sources:

i. Molasses: Culture medium (50 mL) contained 2.5 mL cane and 2.5 mL beet molasses, 0.25 g inorganic nitrogen source

[ammonium sulfate, ammonium hydrogen phosphate, ammonium hydroxide or urea] and 2 mL stock solution consisting of KH_2PO_4 (0.1%), $MgSO_4.7H_2O$ (0.05%) and $CaCl_2.2H_2O$ (0.01%). The stock solution containing minerals had the same composition in all the treatments and was used as it supported good growth of the yeast in the absence of peptone and yeast extract.

ii. Peat hydrolysate: The same medium as above with 5 mL of peat hydrolysate replacing molasses was used. However, the pH of the medium had to be adjusted to 6.0.

iii. Molasses and peat hydrolysate: A 50 mL aliqout of the culture medium was used which consisted of 1.25 mL cane molasses, 1.25 mL beet molasses, 2.5 mL peat hydrolysate, 0.25 g inorganic nitrogen source and 2 mL of stock solution. The pH was adjusted to 6.0.

2. Growth on molasses and wort: Three concentrations of wort (40%, 4% & 2%) were used. To 2.5 mL cane molasses and 2.5 mL beet molasses in each of the three sets, 20, 2 and 1 mL wort, respectively, were added. The final volume was made up to 50 mL with water.

3. Growth on sulfite waste liquor: The sulfite waste liquor in two concentrations (1 and 10%) was used with inorganic and organic nitrogen sources as shown in the legend of Fig. 4.4.

4. pH studies: For the pH studies, culture medium composed of dextrose (1%), peptone (0.5%) and yeast extract (0.3%) was used. Citrate, acetate and citrate-phosphate buffers were used

to maintain a pH of 5.0 while glycine buffer was used for pH 3.0. The growth medium of pH 2.0 was obtained by using 0.5N HCl while 1N NH_4OH was used for pH 7, 8, 9 and 10. A medium of pH 6.0 (unbuffered) was used as a control.

4.2.4 Biomass production: The yeast cell mass was expressed as dry weight (mg/mL). The culture samples were centrifuged and washed with physiological saline (0.85% aqueous NaCl solution). Cells were then suspended in sufficient water to transfer to aluminum pans and dried in an oven at 80°C until constant weight was obtained.

4.2.5 Proximate analysis:

a) *Ash*: A known amount of the wet yeast cell mass was taken in a porcelain crucible and dried in an oven at 640 °C for 2h.

b) *Total lipids*: The method of Bligh and Dyer (1959) was used for lipid estimation.

c) *Total protein*: The protein content was determined with the Folin-Ciocalteau reagent using the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

d) *Total nitrogen*: This was estimated by the Kjeldahl method (AOAC, 1970).

e) *Total carbohydrate*: The total carbohydrate content was obtained with the phenol sulfuric method of Dubois et al. (1956).

4.3 RESULTS

4.3.1 Growth on molasses and peat hydrolysate:

a. Effect of pre-treatment:

A maximum cell yield of 14.1 g/L was obtained with treatment 1 molasses and 8.07 g/L with treatment 2 peat hydrolysate (Fig. 4.1). The treated molasses and peat hydrolysate showed a statistically significant increase in the biomass yield when compared with the untreated substrate. The Anova analysis and multiple range test by the Duncan procedure was used in all the statistical determinations.

b. Effect of inorganic nitrogen source:

Peat hydrolysate alone suppressed the growth of the yeast while growth was enhanced when molasses alone was used (Fig. 4.2) Hence, some inhibitory components in the peat hydrolysate may be responsible for lower yields of the biomass. Ammonium sulfate and ammonium hydroxide were the best inorganic nitrogen sources while urea and diammonium hydrogen phosphate were poorly utilized. Ammonium hydroxide was the best nitrogen source when cane molasses alone or cane and beet molasses together were used as substrates. When beet molasses alone was used, ammonium sulfate gave a better yield than ammonium hydroxide. When peat hydrolysate together with molasses was used to grow the yeast, the yield was highest with diammonium hydrogen phosphate. A maximum cell yield of 10.1 g/L was

obtained with cane and beet molasses using ammonium hydroxide. All the increases in the yields were statistically significant.

4.3.2 Growth on molasses and wort: All the three concentrations of wort were found to be equally good in supporting growth of the yeast. Forty percent wort supported the best growth of the yeast (11.6 g/L) while the yields with 4 and 2% wort were not significantly different (Fig. 4.3). However, wort in a concentration more than 40% was not tried to see its effect on growth.

4.3.3 Growth on sulfite waste liquor: A maximum yield of only 3.5 g/L was obtained with 10% (v/v) sulfite waste liquor (SWL) containing 0.5% peptone and 0.3% yeast extract (Fig. 4.4). This declining growth was even further reduced when peptone was replaced by an inorganic nitrogen source. There was a significant difference in the biomass yield between 1 and 10% SWL when the latter was supplemented with peptone and yeast extract. However, when peptone and trace elements or ammonium sulfate and trace elements were used with SWL, there was no significant difference in the yield between 1 and 10% SWL.

4.3.4 Effect of pH :

An optimum growth was obtained at a pH of 5.0 and 6.0 (Fig. 4.5). Of the three buffers used, citrate buffer was the best followed by acetate and citrate-phosphate buffers

respectively (Fig. 4.6). The differences in the yeast yields with all the three buffers were statistically significant. The amount of growth decreased as the pH increased from 7 to 10. The least amount of growth was obtained at a pH of 3.0 using glycine buffer. However, the unbuffered treatment was better than either of the buffered treatments. Growth was observed in a broad pH range of 3-10.

4.3.5 Proximate analysis:

While P. rhodozyma has been tried as a source for astaxanthin in salmonid diets (Johnson et al. 1980), the new strain of R. rubra is currently being tested as a source of carotenoids, protein, vitamin and mineral in the pen-reared animals. Yeasts have been known to be a useful source of protein due to many desirable features like rapid growth, easy modification by genetic techniques to give useful variants, high yields with available raw materials, easy harvest and a high content of protein and other nutrients (Johnson et al. 1980). The protein content of R. rubra strain was lower than that of P. rhodozyma but its lipid content was higher as shown in Table 4.5.

Table 4.1. Composition of cane and beet molasses

Ingredient	Beet molasses (%)	Cane molasse (%)
Sucrose	48.5	33.4
Raffinose	1.0	-
Invert sugar	1.0	21.2
Organic matter	20.7	19.6
N	0.2 - 2.8	0.4 - 1.5
P_2O_5	0.02 - 0.07	0.6 - 2.0
CaO	0.15 - 0.7	0.1 - 1.1
MgO	0.01 - 0.1	0.03 - 0.1
Al_2O_3	0.005 - 0.06	-
Fe_2O_3	0.001 - 0.02	-
K_2O	2.2 - 4.5	2.6 - 5.0
SiO_2	0.1 - 0.5	-
Ash	4 - 8	7 - 11
Dry matter	78 - 85	77 - 84
	(µg/100g dry weight)	
Thiamine	130	830
Riboflavin	41	250
Pyridoxine	540	650
Niacinamide	5100	2100
Pantothenic acid	130	2140
Folic acid	21	3.8
Biotin	5.3	120

(Rhodes & Fletcher, 1966; Imrie, 1969)

Table 4.2. Composition of Brewer's wort

Ingredient	(mg/g)
Glucose	0.2 - 0.9
Fructose	0.3 - 1.6
Maltose	0 - 1.4
Sucrose	3.4 - 16.9
Raffinose	1.4 - 8.3
Ketose	0.7 - 4.3
Fructose	1.0 - 14.5
Total N	15.0

(Sikyta, 1983a)

Table 4.3. Composition of the peat extract

Ingredients	g/L
Total solids	62.26 ± 1.59
Dissolved solids	49.41 ± 0.77
Total carbohydrates	32.75 ± 1.23
Total reducing sugars	16.47 ± 0.51
Total lipids	0.99 ± 0.03
Total nitrogen	0.60 ± 0.01
Ash	4.51 ± 0.01
pH 1.0 ± 0.15	

The peat extract was prepared according to the method of Martin and Manu-Tawiah (1989a).

Table 4.4. Composition of sulfite waste liquor

Ingredients	Percent
Solids	52 - 60
Reducing sugars (Glucose)	48 - 50
Other carbohydrates	0.5 - 1.5
Non-carbohydrate organic compounds	6 - 8
Ash	2 - 3
Nitrogen	0.065
Volatile organic compounds	1 - 2

(Riviere, 1977)

Table 4.5. Proximate composition of Rhodotorula rubra and Phaffia rhodozyma grown on YM medium agar plates and incubated at 20°C for 3 days.

Constituents	Content (%)	
	P. rhodozyma	R. rubra
Ash	4.1	9.1
Total nitrogen	1.78	1.55
Protein (N X 6.25)	11.13	9.68
Protein (Folin reagent)	40.0	31.1
Total carbohydrate	32.2	33.0
Total lipids	10.0	15.0

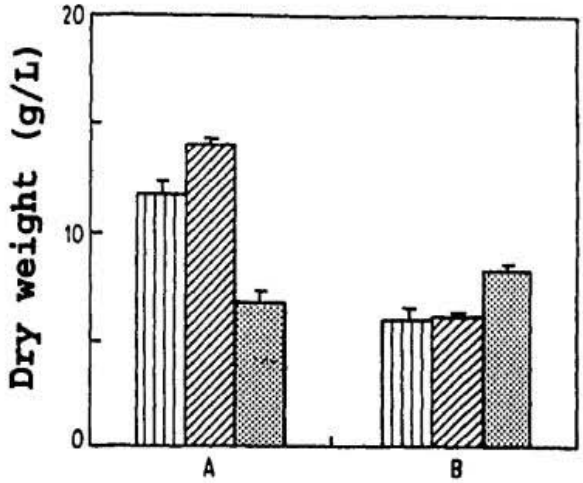


Fig. 4.1. Effect of pretreatment of molasses and peat extract on the growth of yeast
A Molasses; B Peat extract. Each data point represents mean and standard error of three determinations.
No pretreatment
Treatment 1: Centrifugation at 2000 rpm for 45 min.
Treatment 2: Addition of $K_4Fe(CN)_6$ (0.01%)

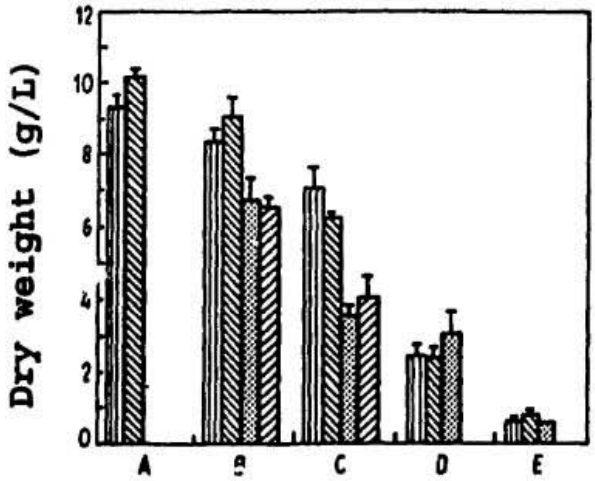


Fig. 4.2. Growth on molasses and peat extract using different inorganic nitrogen sources.
A Cane and beet molasses; B Cane molasses
C Beet molasses D Molasses and peat extract
E Peat extract. Each data point represents mean and standard error of three determinations.
Ammonium sulfate
Ammonium hydroxide
Diammonium hydrogen phosphate
Urea

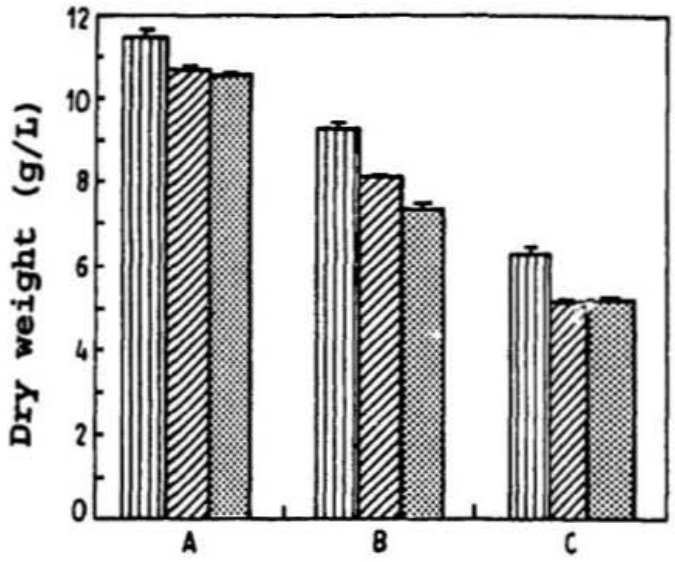


Fig. 4.3. Growth on molasses and wort
A Cane and beet molasses
B Cane molasses
C Beet molasses
Each data point represents mean and standard error of three determinations.
40% wort
4% wort
2% wort

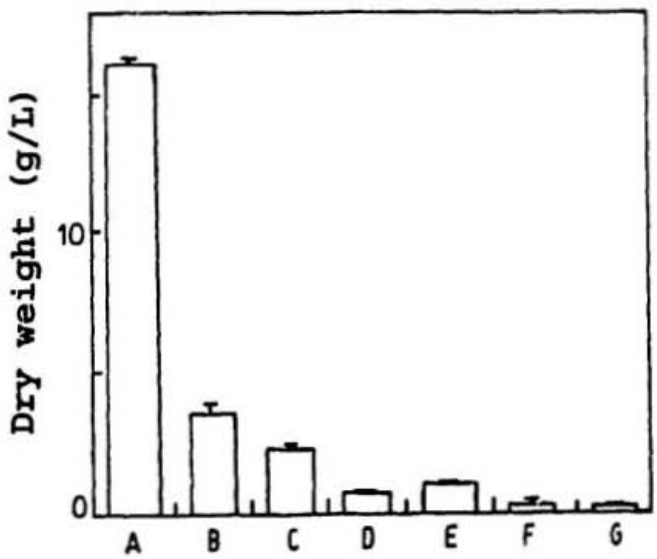


Fig. 4.4. Growth on sulfite waste liquor.
Different substrates for growth:
A Dextrose (1%); peptone (0.5%) and yeast extract (0.3%).
B SWL* (10%); peptone (0.5%) and yeast extract (0.3%).
C SWL (1%); peptone (0.5%) and yeast extract (0.3%).
D SWL (10%); peptone (0.5%) and salt solution[1].
E SWL (1%); peptone (0.5%) and salt solution.
F SWL (10%); ammonium sulfate (0.5%) and salt solution.
G SWL (1%); ammonium sulfate (0.5%) and salt solution.
Each data point represents mean and standard error of three determinations.
* Sulfite waste liquor
1 Salt solution: KH_2PO_4 (0.1%), $MgSO_4 . 7H_2O$ (0.05%) and $CaCl_2 . 2H_2O$ (0.01%)

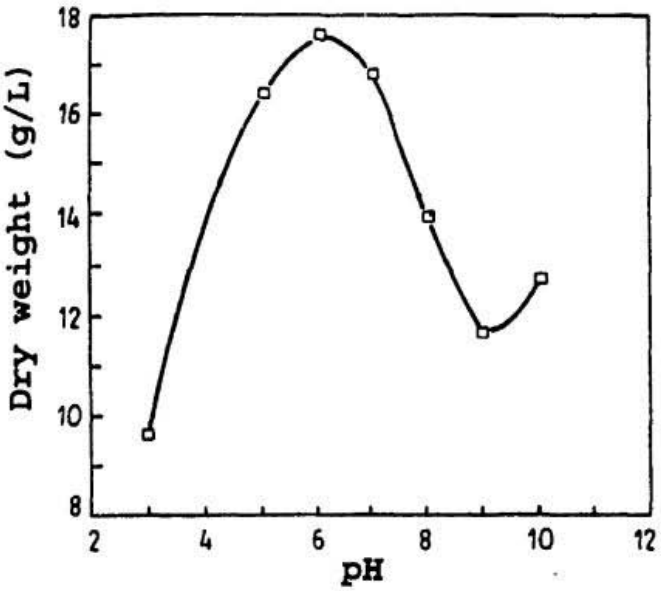


Fig. 4.5. Effect of pH on growth.

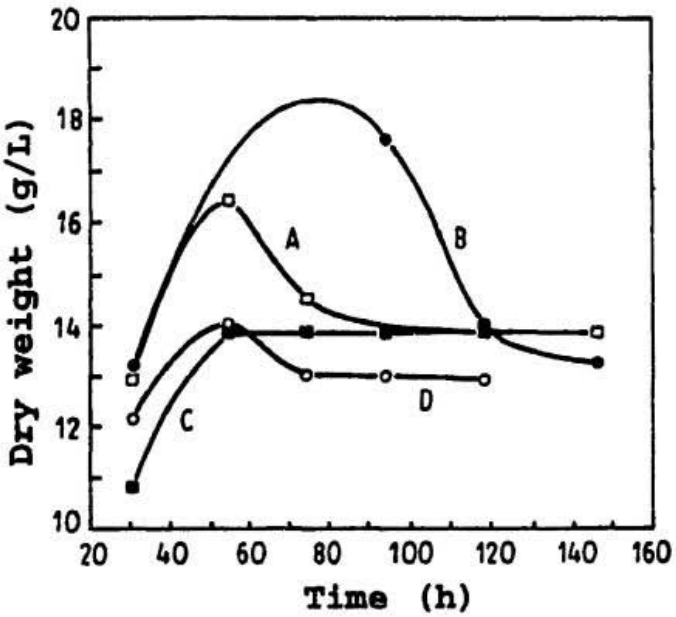


Fig. 4.6. Effect of different buffers on growth.

A	Citrate buffer
B	Control
C	Acetate buffer
D	Citrate phosphate buffer

4.4 DISCUSSION

The present study indicated that the yeast was able to utilize a wide variety of inorganic nitrogen sources with an optimum growth in the presence of ammonium sulphate and ammonium hydroxide. However, an organic nitrogen source like peptone was assimilated much better than an inorganic one.

With the use of molasses as a substrate for growth, the best yield was obtained with molasses from which particulate material was removed by centrifugation. Perhaps some inhibitory factors were eliminated from the mixture by this treatment.

With the use of peat hydrolysate, the best growth was obtained with the centrifuged and potassium ferrocyanide treated substrate. Abou-Zeid and Yousef (1972) also reported similar behaviour with Streptomyces caespitosus. Not surprisingly, the yeast preferred molasses over peat hydrolysate as a growth substrate. This could be due to the lower amount of reducing sugars in the peat hydrolysate.

Cane molasses were found to be better than beet molasses in supporting the growth of the yeast. It is postulated that the higher content of sugars (55-62% compared to 48% for beet molasses) in cane molasses may have a role in this. Cane molasses are also richer in biotin, pantothenic acid, thiamin, magnesium and calcium (Peppler, 1979). However, the content of

organic nitrogen and inositol is less (Prescott and Dunn, 1959) in cane molasses, assuming that the chemical composition of molasses is consistent from batch to batch. Although beet molasses has a 5-fold higher organic nitrogen content, half of it is betaine which is not assimilated by many yeast species (Peppler, 1979). The cane and beet molasses differed in the amount of sugars, organic nitrogen and growth factors. A combination of both in the growth medium was found to improve the biomass yields. Cane molasses has been used as a substrate for biomass production by different workers. Rolz (1984) reported a fed batch system with molasses to optimize between cell yield and substrate utilization using S. cereviceae while 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 .

The highest yield was obtained with molasses and wort. However, the biomass yield from this medium was much less compared to that from YM (Yeast extract/Malt extract medium, Difco) or a medium composed of dextrose, peptone and yeast extract. 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 have also been used to grow Phaffia rhodozyma (Okagbue

and Lewis 1984a) while wort has been used to boost astaxanthin production by the same yeast (Johnson and Lewis, 1979).

Sulfite waste liquor did not support a good growth of R. rubra. This reduced growth may be due to the lack of adequate nutrients or the presence of some inhibitory components. However, many yeasts have been grown successfully on sulfite waste liquor. Anderson (1979) grew Candida utilis on a commercial scale using sulfite waste liqour. Nitrogen supplementation of sulfite 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 by C. utilis (Simard and Cameron, 1974). Zhukova et al. (1976) reported a 20-40% increase in the yield by yeast extract addition. Continuous culture studies using sulfite waste liquor have been done by many researchers who reported a substrate utilization of 60 and 80% (Revah-Moiseev and Carroad, 1981).

The most interesting feature that emerged from this study was the ability of the yeast to grow in an unusually wide pH range of 3 to 10 with best growth achieved at a pH of 5 and 6. This suggested that the yeast was able to withstand fluctuations within a wide pH range. In fact, the addition of buffers was inhibitory to the yeast since the unbuffered treatment supported a much better growth of the yeast than the buffered one. This would improve the commercial value of the

organism. Although, on a laboratory scale, the cost of buffers used in the present investigation is negligible, on an industrial scale, this could reach prohibitive values.

The peat hydrolysates with various inorganic nitrogen sources did not support a good growth of the yeast; however, when supplemented with molasses, a satisfactory growth was achieved.

Because of the large peat reserves in Canada, USA and Europe, the peat hydrolysate is an inexpensive substrate and hence could be exploited for the growth of microorganisms.

Peat hydrolysates contain 70-90% of the reducing substance as monosaccharides; glucose, xylose, galactose, rhamnose, mannose and arabinose being the predominant sugars. Phosphorus, iron and amino-acids are also present. All of the above components are readily assimilable by microorganisms. Other components like vitamin B_1 and B_2, though initially present in peat, are destroyed. However, for the R. rubra isolate described in this paper, this loss is not significant because the yeast is auxotrophic in its vitamin requirements. This is not so in case of fermentation yeasts, like Saccharomyces, which require vitamins.

Peat hydrolysate has been used as a substrate for many microorganisms, like Brevibacterium flavum, Micrococcus glutamicus 95 and lipomyces lipoferus 3-6B (Fuchsman, 1980), Candida utilis (Martin et al. 1993a) and Phaffia rhodozyma

(Martin et al. 1993b).

The biomass yield obtained on peat hydrolysate was much lower than that obtained on molasses and wort. This could be due to hydroxymethyl furfural and humic substances present in peat hydrolysates which have been shown to have an inhibitory effect on the growth of yeasts. Interestingly, succinic acid, which is also present in peat hydrolysate, has been shown to be a stimulant for growth (Fuchsman, 1980). Hence the relative amounts of stimulants or inhibitory components would play a role in determining the biomass yields of an organism grown on this substrate.

To boost the biomass yields, the peat hydrolysate could be supplemented appropriately, such as by addition of molasses or wort, to upgrade its nutritional value. Some toxic elements present in peat hydrolysate could be lowered by the use of chelators like EDTA. The oxidation of unhydrolyzed peat residue to increase the amount of carbon available for yeasts could be another means to enhance the yeast yields. Kosonogova and Evdokinova (1977) used the oxidation method wherein the peat was first hydrolysed with acid catalyst to release sugars. The solid residue left, representing about one half of the original peat, was suspended in an alkaline medium and heated under pressure in the presence of atmospheric oxygen to yield a variety of potentially useful organic acids. The combined hydrolysate was used as a substrate. This method has

been found to increase the yield and protein content of the yeasts. Martin et al. (1990) diluted the sulfuric acid hydrolysates of peat extract with water in a 1:1 ratio and later supplemented it with yeast extract and $MgSO_4$.

Hence, the future areas of research could be standardisation of the peat hydrolysates to lower the inhibitory components and increase its stimulatory effect to achieve higher biomass yields and higher levels of pigment production.

CHAPTER 5

Nitrosoguanidine mutagenesis in a new strain of Rhodotorula rubra TP 1: Isolation and characterization of mutants

5.1 INTRODUCTION

To enhance the potential of a microorganism, the genotype can be manipulated by inducing mutations in the genome. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) is one of the most effective chemical mutagens used for this purpose. When used under optimum conditions, it induces a large proportion of mutants with a low killing rate, e.g., in Streptomycetes coelicolor, 8-10% of the survivors constitute auxotrophs and in E. coli, up to 50% of the survivors turn out to be mutants (Crueger and Crueger, 1989). Ninety percent of the total mutations induced by NTG are found to be GC -> AT transitions while a small number of deletions and frameshift mutations are also found due to deletion of GC pairs.

Nitrosoguanidine is easily decomposed *in vivo* and forms nitrous acid under acidic conditions, and diazomethane under alkaline conditions. The mode of action of NTG is by alkylation of non-replicating DNA. Besides this, the main

point of action is at replication point of DNA, through a change in DNA polymerase III. This causes incorrect duplication in a short segment of the DNA until the defective enzyme is replaced by an intact molecule (Crueger and Crueger, 1989).

The morphological and biochemical features were investigated in mutants of R. rubra TP 1 obtained using nitrosoguanidine.

5.2 MATERIALS AND METHODS

5.2.1 Microorganisms: R. rubra TP 1 was used in the present study. Other yeast strains used are shown in Table 5.1.

5.2.2 Nitrosoguanidine mutagenesis: Each of the 250 mL Erlenmeyer flasks containing 30 mL of Yeast extract/Malt extract medium (YM broth, Difco) was inoculated with 1 mL of the cell suspension in the logarithmic phase. The cell numbers were determined using a haemocytometer after staining with 0.4 % Trypan blue.

Nitrosoguanidine (4 mg) was dissolved in 4 mL of dimethyl sulfoxide and filter-sterilized using a Syrfil-MF nucleopore filter. Three concentrations of nitrosoguanidine were used, viz. 1.66, 10 and 30 µg/mL. A control with no mutagen added was also included.

The flasks were incubated in a 'Psychrotherm' controlled environment incubator (New Brunswick, NJ, USA) at 20°C and shaken at 200 rpm for 16 h.

Cells were harvested by centrifugation at 10,000 rpm for 20 min. and suspended in 10% sucrose solution after washing with 0.1 M potassium phosphate buffer (pH 7.0). The cells were plated onto YM medium after appropriate serial dilutions. The plates were incubated at 20°C for 3 days and screened for mutants.

5.2.3 Characterization of mutant strains:

Various phenotypic traits were utilized to characterize the mutants, including cell morphology using phase contrast microscopy, colony morphology and pigmentation, assimilation of various carbohydrates, fermentation of D-glucose, potassium nitrate utilization, urease test, growth on 50% glucose yeast extract and vitamin-free medium, starch formation, arbutin test and gelatin liquefaction.

5.2.4 Growth Kinetics:

The growth curves of all the organisms were plotted and expressed as dry weight (mg/L). For the measurement of dry weight, culture samples were centrifuged and washed with physiological saline (0.85% aqueous NaCl solution). The cells were then suspended in sufficient water to transfer to aluminum pans and dried in an oven at 80 °C until constant weight was obtained.

Other growth characteristics measured were:

Specific growth rate (μ) was determined by the following equation:

$$\mu = \frac{1}{X} \cdot \frac{dX}{dt} = \frac{\ln 2}{g}$$

where X is the biomass (g/L), t is time (h) and g is the generation time (h) (Paredes-Lopez et al. 1976).

Yield coefficient (Y) : biomass (g/L) dry weight per g sugar consumed (Sikyta, 1983b).

Generation time (g) was calculated as

$t_2 - t_1 / 3.32 \times (\log X_2 - \log X_1)$ where t_1= initial time, t_2= final time, X_1= initial biomass and X_2= final biomass (Rose, 1968).

Productivity (P) was calculated as $P=\mu X$ where μ is the specific growth rate and X is the biomass concentration (Sheehan and Johnson, 1971).

Economic coefficient (E) was defined as the biomass produced (g/L) divided by the total amount of introduced substrate (Sikyta, 1983b).

Molar growth yield (MG) was measured as the biomass produced (g/L) per mole of substrate consumed (Bolton et al. 1972).

5.2.5 Mutagenicity of NTG:

Survival ratio: This was calculated as the fraction of the initial number of cells able to survive and grow after the mutagenic treatment (Nieto et al. 1992).

5.3 RESULTS

5.3.1 Isolation of mutants:

Two mutants were isolated and designated as mutant A and B.

5.3.2 Colony morphology:

General morphology on all media:

The mutant A displayed a dry and wrinkled colony morphology on all the media used for cultivation (Fig 5.1). The mutant B showed a gummy or mucoid colony appearance (Fig 5.2). The parent culture had a shiny colony. The other red yeast, Phaffia rhodozyma formed a dry, orange-colored colony while all the remaining organisms formed red-colored colonies.

Typical cultural characteristics:

Czapek Dox agar supported the least growth of all organisms. The yeasts also did not grow very well on Sabouraud's dextrose agar, oat meal agar and Tryptic soy agar(TSA). TSA had an unusual effect on pigmentation in that all the cultures showed an orange color on this medium instead of a red color. Malt extract agar supported a good growth of all organisms. The most abundant growth, however, was obtained on potato dextrose agar, YPG (glucose, peptone and yeast extract), YM (Yeast extract/Malt extract agar) and Sabouraud's maltose agar.

5.3.3 Cell morphology:

The parent culture (R. rubra TP 1) showed spherical cells

of the same size as that of the mutants (Fig 5.3). Chains and budding forms, however, were found in the mutant A culture (Fig 5.4). The mutant B cells were oval (egg shaped) and occurred singly. (Fig 5.5). The cells of the other R. rubra strain (ATCC 9449) were twice the size of that of the new isolate (R. rubra TP 1) and extensive chain formation was found (Fig 5.6). The R. glutinis cells were elliptical, occurred singly with no chains and the average cell size was about half that of our isolate (Fig 5.7). Extensive chain formation, like the ATCC culture of R. rubra, was observed in Phaffia rhodozyma (Fig 5.8) and the cells were elliptical and twice the size of our isolate.

5.3.4 Nutritional and biochemical characteristics:

The nutritional and biochemical characteristics are listed in Table 5.2 and 5.3.

5.3.5 Growth kinetics:

Figures 5.9 and 5.10 depict the growth curves of the yeasts used in this investigation while Table 5.4 shows their growth characteristics. The mutant B exhibited the best yield constant, and the molar growth yield values. The overall productivity and economic coefficient were the highest for R. rubra (ATCC 9449). The other red yeast, R. glutinis had the lowest generation time and the highest specific growth rate.

The mutant B showed a higher value for the overall productivity, yield constant, economic coefficient and molar

growth yield than the parent strain. The generation time and specific growth rate, however, were almost similar for both strains.

5.3.6 Mutagenicity of NTG:

Table 5.5 shows the effect of nitrosoguanidine on cell viability in terms of the percentage kill. Almost 99% kill was obtained with all three concentrations used. The survival ratios of R. rubra were lower than those of P. rhodozyma (Fig. 5.11). As the NTG concentration increased, the former showed a more rapid drop in the survival ratios than P. rhodozyma.

Table 5.1. Yeast strains used in the study

Organism	Collection No.	Source
Rhodotorula rubra	TP 1	Own isolate
Phaffia rhodozyma	24202	ATCC
Rhodotorula rubra	9449	ATCC
Rhodotorula glutinis	-	Departmental culture collection

Table 5.2. Nutritional and biochemical characteristics of R. rubra TP 1 and the two mutants A and B:

Test	Growth			Pigmentation**		
	TP 1*	A	B	TP 1*	A	B
50% GYE	+	+	+	----------		
Urease	+	+	+	----------		
Arbutin	+	+	+	----------		
Sucrose	+	+	+	+	-	+
Arabinose	+	+	+	-	-	-
D-Ribose	+	+	+	-	-	+
Galactose	+	+	+	-	-	-
Cellobiose	+	-	+	w	-	w
Lactose	-	-	-	-	-	-
Melibiose	+	-	+	w	-	w
Citric acid	+	-	+	-	-	-
Maltose +++	+	+	+	-	-	
Rhamnose	-	-	-	-	-	-
KNO_3	+	-	+	-	-	-
Mannitol	+	w	+	-	-	-
Raffinose	+	w	+	-	-	-
Ribitol +++	+	+	+	++	-	
Gelatin liquefaction	+	+	+	-	-	-
Soluble starch	+	+	+	-	-	-
Erythritol	+	-	-	-	-	-
D(-)Xylose	+	-	+	++	-	-
Succinic acid +++	+	+	+	+++	-	
Trehalose +++	+	+	+	++	-	
Inositol	w	w	w	-	-	-

TP 1*: R. rubra TP 1
** Increased intensity of pigmentation is reported as +, ++, +++.
------- Pigmentation was not tested on these substrates

Table 5.3. Nutritional and biochemical characteristics of Phaffia rhodozyma (P. rd.), Rhodotorula rubra [ATCC 9449] (R. rb.) and Rhodotorula glutinis (R. gln.) :

Test	Growth			Pigmentation		
	P. rd	R. rb	R. gln	P. rd	R. rb	R. gln
50% GYE	+	+	+	----------		
Urease	+	+	+	----------		
Arbutin	+	+	+	----------		
Sucrose	+	+	+	+	++	-
Arabinose	+	+	+	++	-	-
D(-)Ribose	-	+	+	-	+	-
Galactose	+	+	+	w	-	-
Cellobiose	+	+	+	+++	-	-
Lactose	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-
Citric acid	-	+	+	-	+	-
Maltose	+	+	+	++	++	-
Rhamnose	-	-	+	-	-	-
KNO_3	+	+	w	_	-	-
Mannitol	+	+	+	++	-	-
Raffinose	+	+	+	-	-	-
Ribitol	-	+	-	-	-	-
Gelatin liquefaction	+	w	+	-	-	-
Soluble starch	+	+	+	++	-	-
Erythritol	-	+	-	-	-	-
D(-)Xylose	+	+	+	-	++	-
Succinic acid	+	+	+	-	w	-
Trehalose	+	+	+	+++	+++	-
Inositol	-	w	w	-	-	-

** Increased intensity of pigmentation is reported as +, ++, +++.

-------- Pigmentation was not tested on these substrates

Table 5.4. Growth kinetics of the yeasts cultivated on YM agar plates and incubated at 20°C for 3 days.

Organism	P	μ	Y	E	Mg	Gen
TP 1	0.055	0.076	0.77	0.65	138.6	9.1
P. rd	0.048	0.050	0.57	0.57	102.6	14.0
R. rb	0.067	0.063	0.84	0.79	151.2	11.0
R. gl	0.060	0.084	0.80	0.71	144.0	8.2
A	0.054	0.060	0.69	0.64	124.2	11.5
B	0.058	0.075	0.93	0.69	167.4	9.2

P: Overall productivity, g biomass/L/h = μ X biomass
μ: Specific growth rate (/h) = ln 2/ generation time
Y: Yield coefficient = g biomass formed/L/g sugar consumed
E: Economic coefficient = g biomass /g introduced substrate
Mg: Molar growth yield, g biomass/L/mole of glucose
Gen: Generation time (h) =
final time- initial time / 3.32 X (log final biomass - log initial biomass)

TP 1: R. rubra TP1 ; P. rd: P. rhodozyma;
R. rb: R. rubra (ATCC); R. gln: R. glutinis;
A: Mutant A; B: Mutant B

Table 5.5. Effect of nitrosoguanidine concentration on percentage kill.

	Percent kill		
	NTG (μg/mL)		
	1.66	10	30
R. rubra	99.48	99.92	99.98
P. rhodozyma	99.48	99.57	99.73

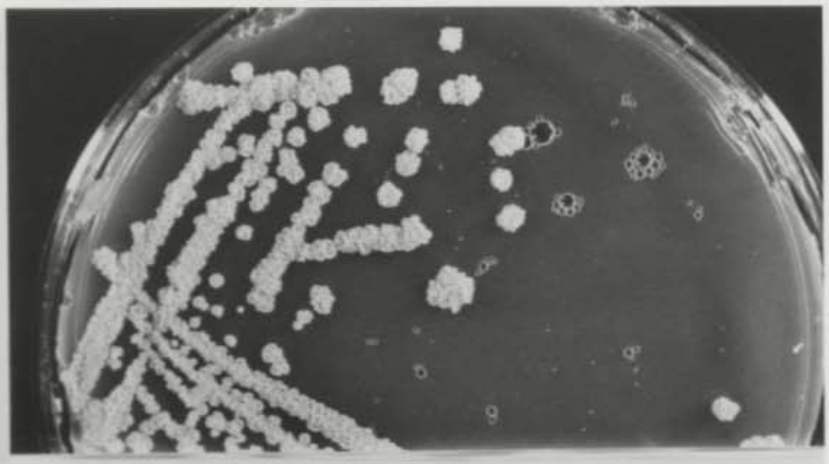


Fig. 5.1. Colony morphology of mutant A.

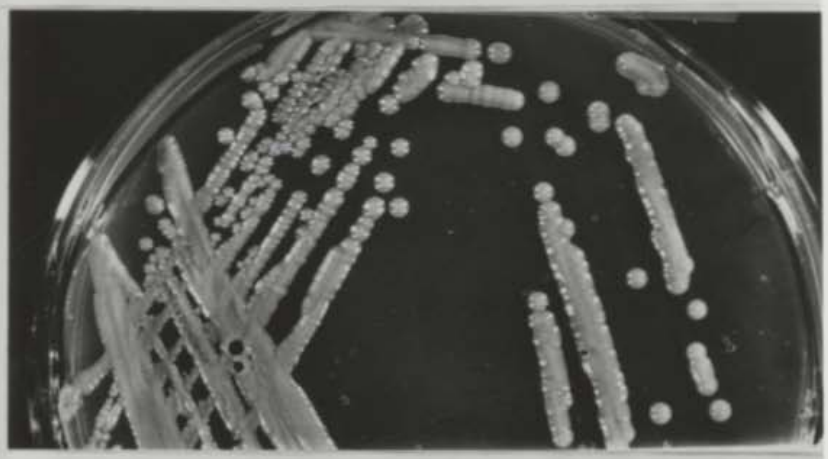


Fig. 5.2. Colony morphology of mutant B.

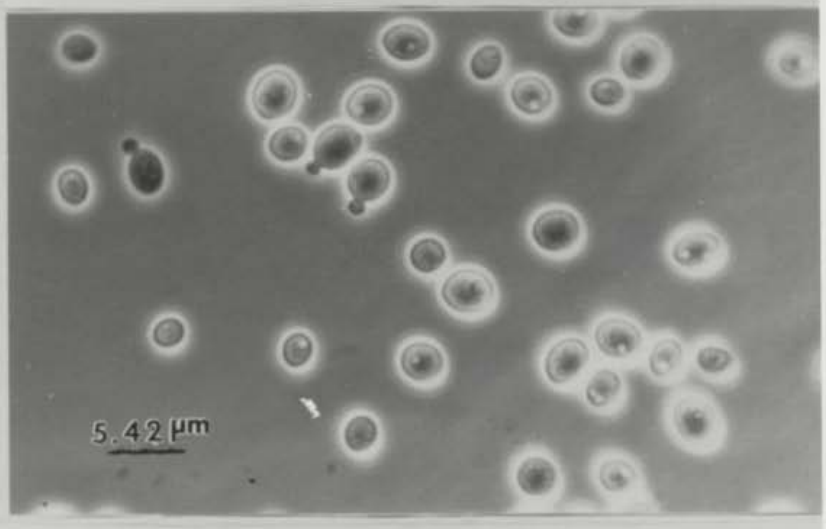


Fig. 5.3. Cell morphology of R. rubra TP 1.

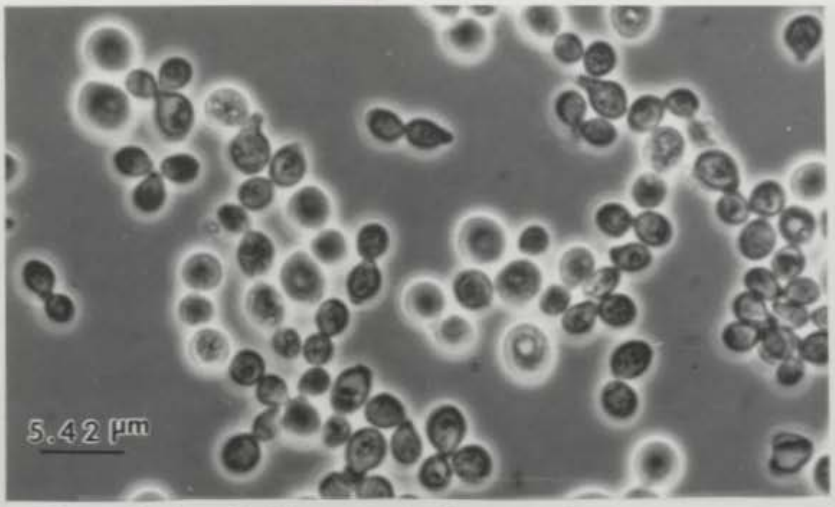


Fig. 5.4. Cell morphology of mutant A.

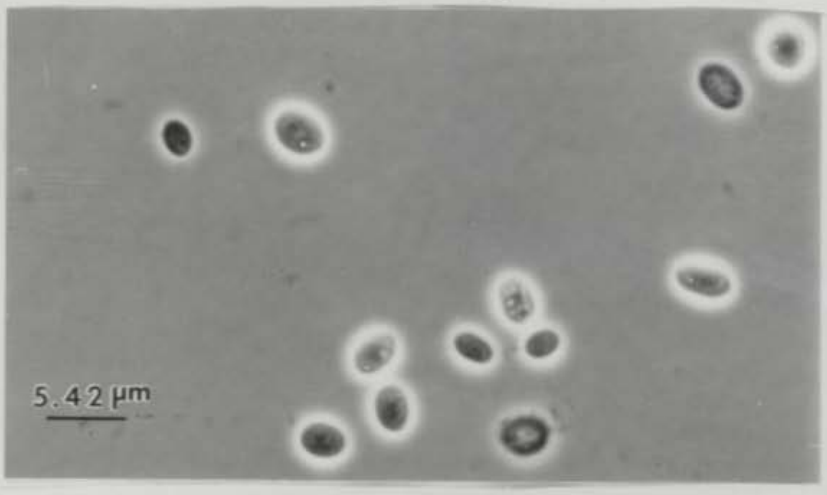


Fig. 5.5. Cell morphology of mutant B.

Fig. 5.6. Cell morphology of R. rubra ATCC 9449.

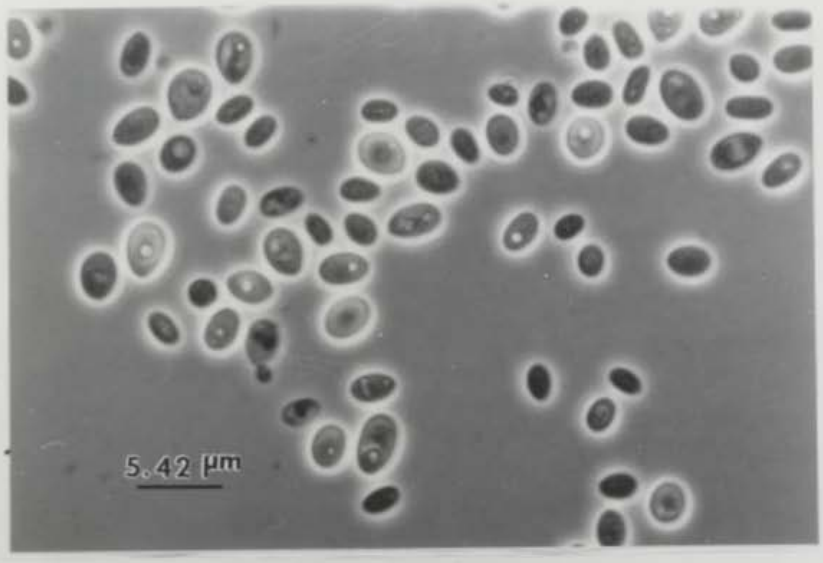


Fig. 5.7. Cell morphology of R. glutinis.

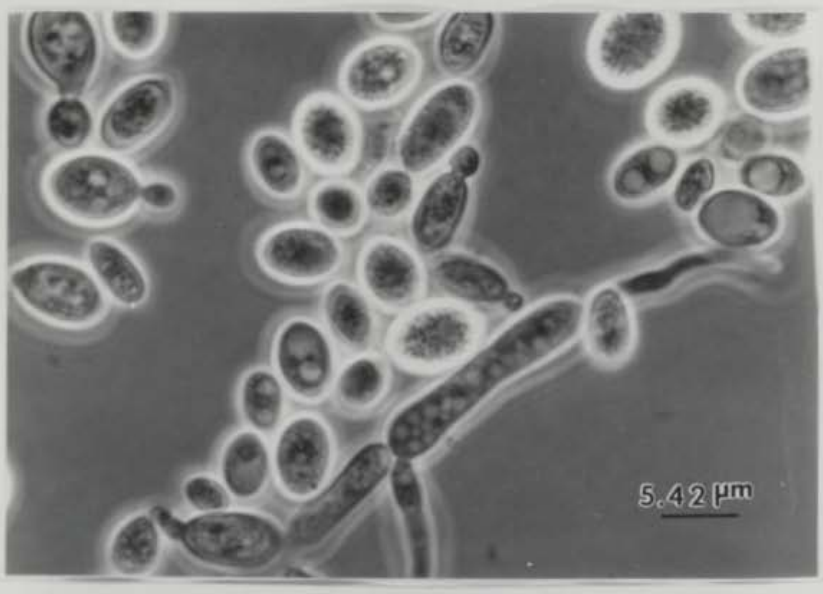


Fig. 5.8. Cell morphology of P. rhodozyma ATCC 24202.

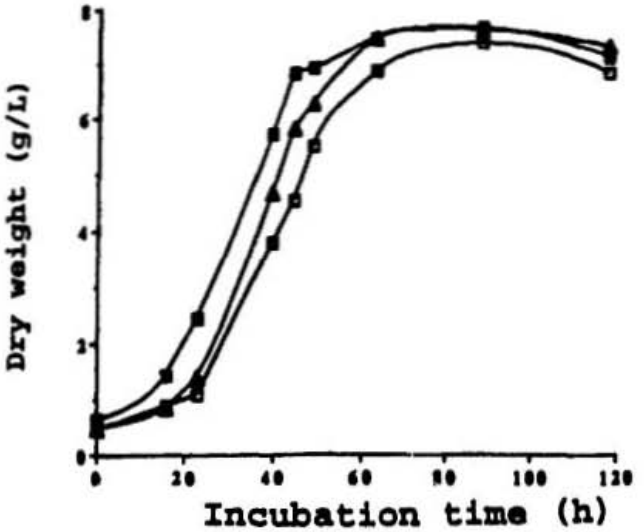


Fig. 5.9. The growth curves of parent and mutant yeast on YM medium.
- ■ Rhodotorula rubra TP 1.
- □ Mutant A
- ▲ Mutant B

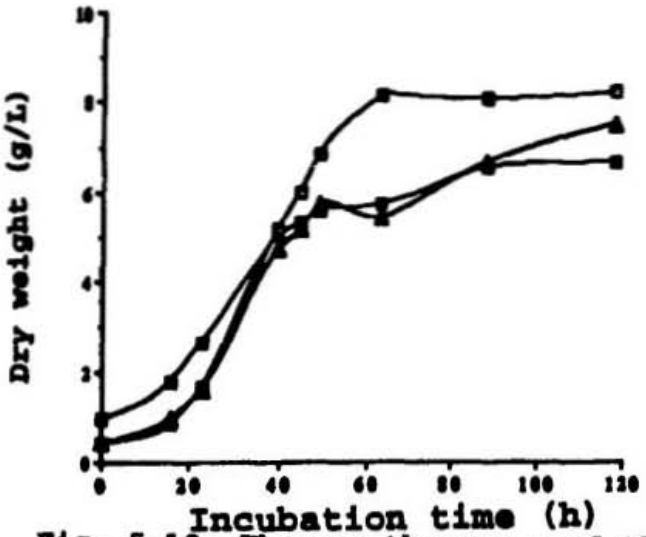


Fig. 5.10. The growth curves of three yeast species on YM medium.
- ■ Phaffia rhodozyma
- □ Rhodotorula rubra ATCC 9449
- ▲ Rhodotorula glutinis

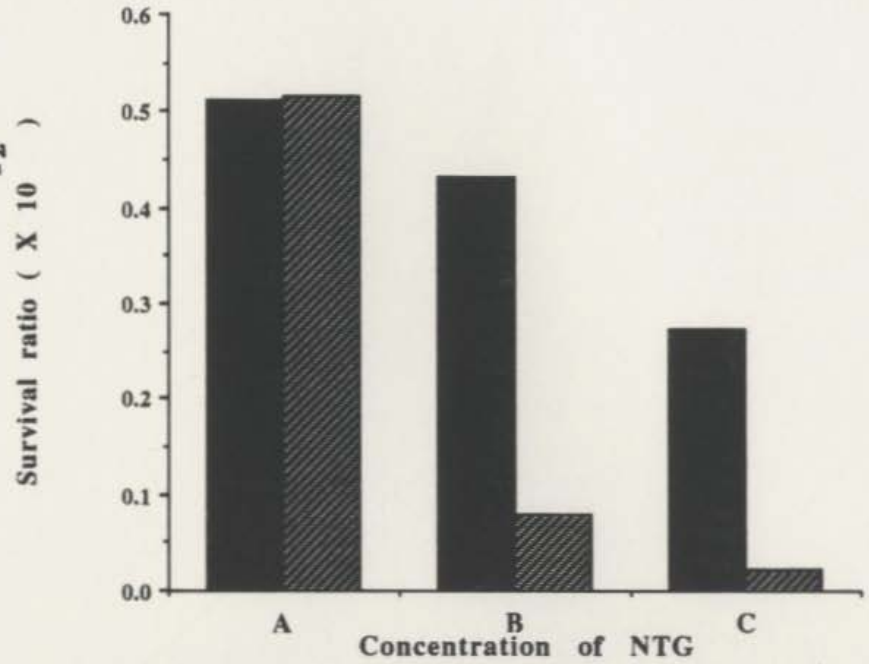


Fig. 5.11. The survival ratios of yeasts treated with nitrosoguanidine.
P. rhodozyma
R. rubra
Concentration of NTG:
A 1.66 μg/mL
B 10 μg/mL
C 30 μg/mL

5.4 DISCUSSION

This paper describes treatment of the parent culture, R. rubra TP 1 with NTG, obtaining two mutants that were studied for their morphological, biochemical and growth characteristics. NTG has been used by many workers for obtaining a wide variety of mutants, eg, methicillin-resistant mutants from Pediococcus cerevisiae (Widdowson and White, 1976), catalase deficient mutants of Salmonella typhimurium (Levine, 1977), pleiotropic mutants altered in carbon metabolism, from Aspergillus nidulans (Hynes and Kelly, 1977), mutants of E. coli defective in ribonucleoside and deoxyribonucleoside catabolism (Karlstrom, 1968), acetate non-utilizing mutants (Flavell and Fincham, 1968) as well as fluoroacetate-resistant mutants of Neurospora crassa (Owen et al. 1992), josamycin-resistant mutants of Halobacterium mediterranei (Bonelo et al. 1984), mutants of Clostridium saccharoperbutylacetonicum fermenting bagasse hydrolysate for acetone-butanol production (Lee et al. 1991) and an obligate osmophilic mutant of Zygosaccharomyces rouxii (Ushio et al. 1991).

Mutagenesis of red yeasts has been reported by different workers using various mutagens. Johnson et al. (1989) subjected P. rhodozyma to UV light, ethylmethanesulfonate and NTG to obtain hyper pigment producing mutants.

Nitrosoguanidine was reported to be the best mutagen. However, most of the mutants were unstable. Lewis et al. (1990) used NTG to mutagenize P. rhodozyma and then screened the astaxanthin-overproducers using ß-ionone. Bonner et al. (1946) subjected R. rubra to UV-radiation and obtained 7 mutants of which three produced higher amounts of total pigments than the parent strain. Elinov et al. (1988) subjected R. rubra to mutagenesis with N-nitroso-N-methylurea and obtained a set of mutants showing quantitative changes in the biosynthesis of mannan as well as an alteration in the composition and structure of these heteropolysaccharides. Nand et al. (1973) treated a strain of Rhodotorula sp. to UV radiation and used griseofulvin, an antifungal antibiotic, as a selective means to concentrate the mutant population. Their report dealt, more or less, with the efficacy of using griseofulvin to facilitate the isolation of mutants and no details on the morphological or biochemical characteristics of the mutants were given.

NTG has also been reported to have high mutagenicity (Fitt et al. 1989; Neale, 1976; Smith, 1978).

As seen from the survival ratio values in Fig 6, P. rhodozyma appeared to be more resilient to the lethality of NTG than R. rubra.

Mutant A did not show pigmentation on any of the media tested while the mutant B showed a higher amount of pigmentation than the parent strain on D-ribose, maltose,

ribitol and trehalose. Mutant A did not show growth on a number of substrates, including cellobiose, lactose, melibiose, citric acid, rhamnose, KNO_3, erythritol and D-xylose. Mutant B lost the ability to assimilate erythritol in contrast to the parent. Cellobiose and trehalose were the best substrates for pigmentation by P. rhodozyma. The latter also showed good pigmentation on arabinose, mannitol, sucrose and soluble starch.

Growth on some substrates could be economically attractive. Sucrose, a major constituent of molasses, a by-product of sugar production; maltose, the major carbohydrate in malt, an aqueous extract of malted barley, and xylose, a major constituent of the hemi-cellulose fraction of the agricultural wastes, can serve as cheap sources of carbohydrates and could be used for pigment production on a commercial scale.

The main focus of the work was to isolate mutants altered in carbon metabolism, i.e., mutants having a better capacity than the parent to utilize cheaper substrates for growth. One of the mutants also showed enhanced pigmentation on some substrates. Both the mutants were stable as neither of the two mutants reverted after repeated sub-culturing on YM agar slants.

CHAPTER 6

Growth and pigmentation in *Rhodotorula rubra* and *Phaffia rhodozyma*

6.1 INTRODUCTION

The kinetics of growth and pigmentation have been studied in *P. rhodozyma* and found to be growth-related wherein astaxanthin was found to be synthesized not only during growth of the organism but also after growth had stopped (Johnson and Lewis, 1979). This phenomenon is peculiar since pigmentation in other yeasts like *Sporobolomyces roseus* (Bobkova, 1965b), *Rhodotorula rubra* (Goodwin, 1959, 1972) and *Rhodotorula glutinis* (Vecher and Kulikova, 1968) is found to occur after the yeast growth has stopped. The pigmentation in the green micro-alga *Dunaliella salina* also follows the same pattern (Leenheer and Nelis, 1991).

In the present study, the kinetics of growth and pigment formation were studied in *P. rhodozyma* and *R. rubra* TP 1. The yeast cells were ruptured mechanically in a French Press and enzymatically with funcelase.

6.2 MATERIALS AND METHODS

6.2.1 Organisms: *Phaffia rhodozyma* ATCC 24202 and *Rhodotorula rubra* TP 1 were used in the present investigation.

6.2.2 Culture conditions: Shake flasks of 2 L capacity containing 800 mL of Yeast extract/Malt extract medium (YM broth, Difco) were incubated at 20°C in a 'Psychrotherm' controlled environment incubator (New Brunswick, NJ, USA) in the presence of light and with a shaking speed of 150 rpm. The flasks were inoculated with 9% (v/v) of a 24 h culture giving cell density reading of 0.71 at 600 nm. Aliquots of 20 mL were withdrawn at different time intervals and used for the determination of dry cell weight and pigment production.

6.2.3 Dry weight: For the yeast biomass production, culture samples were centrifuged and washed with physiological saline. The cells were then suspended in sufficient water to transfer to aluminum pans and dried in an oven at 80 °C until constant weight was obtained.

6.2.4 Mechanical rupture of cells by French press: The samples (in triplicate) were centrifuged and washed with physiological saline. The cells were suspended in sufficient water and ruptured in a French press at an internal cell pressure of 32000 psi (2000 psi gauge reading of a 40K cell; diameter of the piston being 1").

6.2.5 Pigment extraction: The ruptured cells were extracted with acetone 4 to 5 times until the supernatant was colorless. All the acetone extracts were pooled and filtered through glass wool to remove lipid globules and other impurities. The absorption spectrum of the pigment was then

taken using Shimadzu ultraviolet-visible recording spectrophotometer UV-260.

6.2.6 Enzymatic treatment for cell rupture: A commercial enzyme preparation (Funcelase, Yakult Honsha Co. Ltd., Japan) was used in the present study. To prepare a stock solution, 600 mg of the enzyme was added to 15 mL of water. Aliquots of the culture sample (5 mL) withdrawn at different time intervals (in triplicates) were centrifuged, washed with physiological saline and resuspended in 5 mL of distilled water. The pH was adjusted to 4.5 with acetic acid for the optimal enzyme activity. An enzyme preparation (0.6 mg/15 μL sample) was added to each sample and incubated at 30 °C for 24 h. However, because of the recalcitrant nature of R. rubra, a higher enzyme concentration of 1.6 mg was also used.

Total carotenoid amount: This was calculated by using the 1% extinction coefficient, $A^{1\%}_{1cm}$ of 2100 provided by Davies (1976) by this formula (modified from Johnson et al., 1989):

$$\text{Total carotenoids } (\mu g/g \text{ yeast}) = \frac{\text{Absorbance at } \lambda_{max} \times \text{volume of acetone extract} \times 100}{21 \times \text{dry weight of yeast}}$$

6.3 RESULTS

6.3.1 Pigment released by enzymatic method in P. rhodozyma: The pigment production was found to be growth

related (Fig. 6.1). The two curves can be demarcated into 3 phases. In the early log phase (Phase 1), no pigmentation was observed for the initial 40h. In the late log phase (Phase 2), the dry weight and pigment had a linear relationship. Thus maximum pigment production occurred in the exponential phase of the growth curve of this yeast. The onset of stationary phase (Phase 3) resulted in a plateau in pigment production.

6.3.2 Comparison of enzymatic and French pressure methods:

The pigment released from enzymatically treated cells was detectable earlier in the growth phase (40h), while pigment from mechanically ruptured cells was detectable only after 4 days (Fig. 6.2).

A higher amount of pigment was extractable from enzymatically treated cells than those of mechanically ruptured, a maximum of 347.9 µg/g of pigment by the former method while only 157.6 µg/g by the latter method. Hence the pigment from enzymatically treated cells was about two times greater than that of mechanically ruptured cells.

6.3.3 Pigment released by mechanical rupture in R. rubra:

As in the case of Phaffia, the pigment was growth related and three phases of pigmentation were observed. In the early exponential phase (Phase 1), there was no pigment during the first 2 1/2 days. Maximum pigment was produced during the late exponential and early stationary phases (Phase 2) of the growth curve (Fig. 6.3). The pigment production levelled off

in the late stationary phase (Phase 3).

6.3.4 The enzymatically released pigment in R. rubra:

It was interesting to note that the enzyme treatment was ineffective to render the pigment available from the new R. rubra isolate.

6.3.5 Comparison of pigment formation by P. rhodozyma and R. rubra : Apart from lower cell yields, Phaffia gave lower pigment yields than R. rubra in the French press rupture of the cells. The pigment yield was three and a half times higher in the R. rubra cells than P. rhodozyma (calculated on the basis of unit volume of the culture). The pigment in R. rubra was detectable 2.6 days after the inoculation while the P. rhodozyma pigment was detectable only after 4 days and the latter increased much more slowly than that of R. rubra. The maximum pigment yield in R. rubra and P. rhodozyma was obtained in about six and eight days, respectively.

Also seen are the curves for the pigment yields obtained by the French press rupture of the R. rubra cells and enzymatically treated P. rhodozyma cells.

The pigment yield from R. rubra was higher (2.07 mg/L of medium) than that from P. rhodozyma (1.21 mg/L of medium) (Table 1).

The pigment from R. rubra though was detected a day later than that of Phaffia (2.63 and 1.64d for R. rubra and P. rhodozyma, respectively), it increased more rapidly and was

about five-fold more than that from P. rhodozyma (after 4 days of incubation, 1678.98 μg and 346.63 μg of pigment/L of the medium from R. rubra and P. rhodozyma, respectively).

The R. rubra pigment reached a plateau in about six days while the pigment from P. rhodozyma levelled off in roughly eight days.

Table 6.1. The pigment yields of P. rhodozyma and R. rubra using the two methods of cell rupture.

Time of incubation (days)	P. rhodozyma				R. rubra	
	French press released pigment		Enzymatically released pigment		French press released pigment	
	µg/g dry weight	µg/L medium	µg/g dry weight	µg/L medium	µg/g dry weight	µg/L medium
0.64	-	-	-	-	-	-
1.64	-	-	-	-	-	-
2.63	-	-	-	-	-	-
4.0	-	-	131.30	346.63	277.06	1678.98
4.63	-	-	161.68	433.30	-	-
5.96	68.96	202.74	242.41	712.69	333.96	2043.84
7.0	87.91	237.36	299.98	809.95	336.64	2066.97
7.71	157.76	530.07	347.91	1168.98	255.69	1472.77
14.58	180.24	605.61	361.08	1213.23	330.00	1933.80

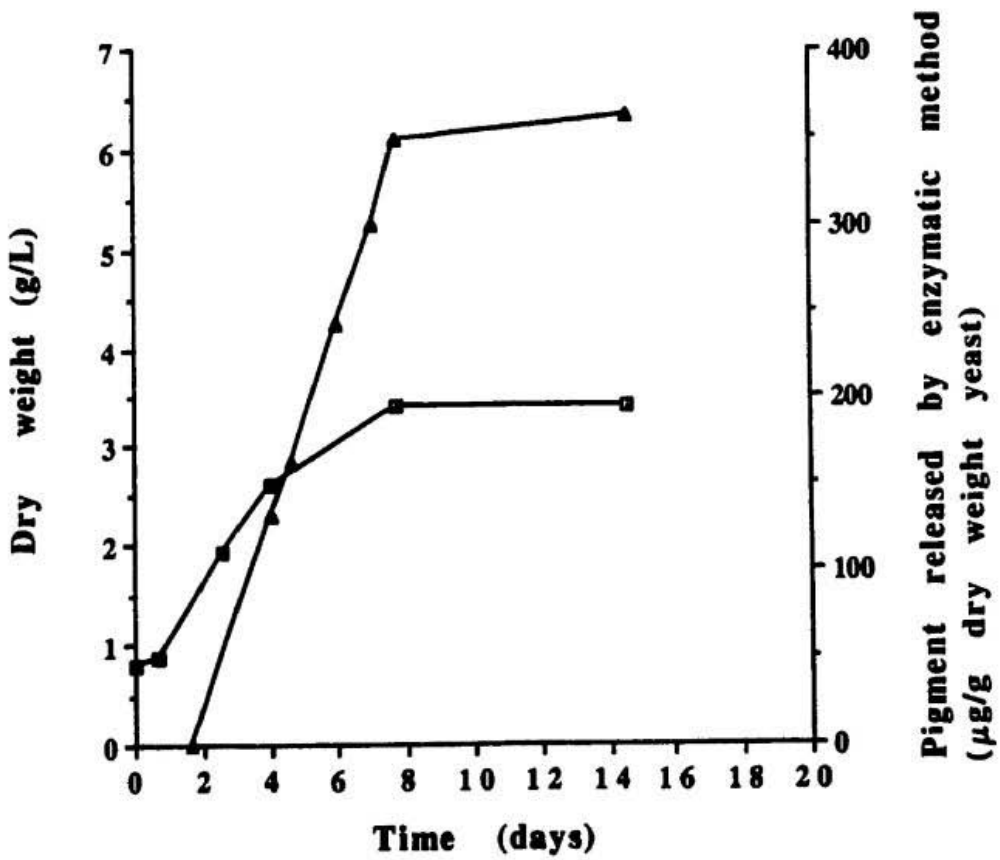
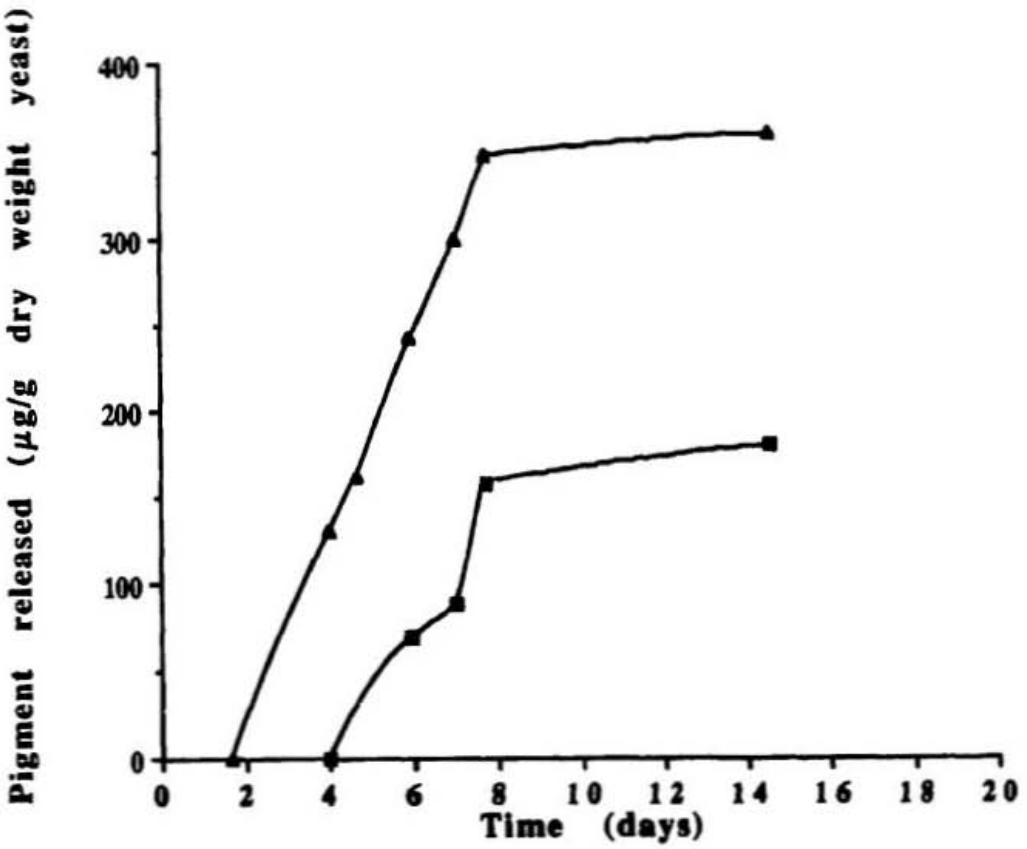


Fig. 6.1. Pigment released by enzymatic treatment of Phaffia rhodozyma cells.
▲ Pigment concentration (µg/ g dry weight yeast)
■ Growth (dry weight (g/L)



Fig, 6.2. Efficacy of enzymatic and mechanical rupture in the release of pigment of P. rhodozyma.
▲ Enzymatic treatment
■ French press treatment

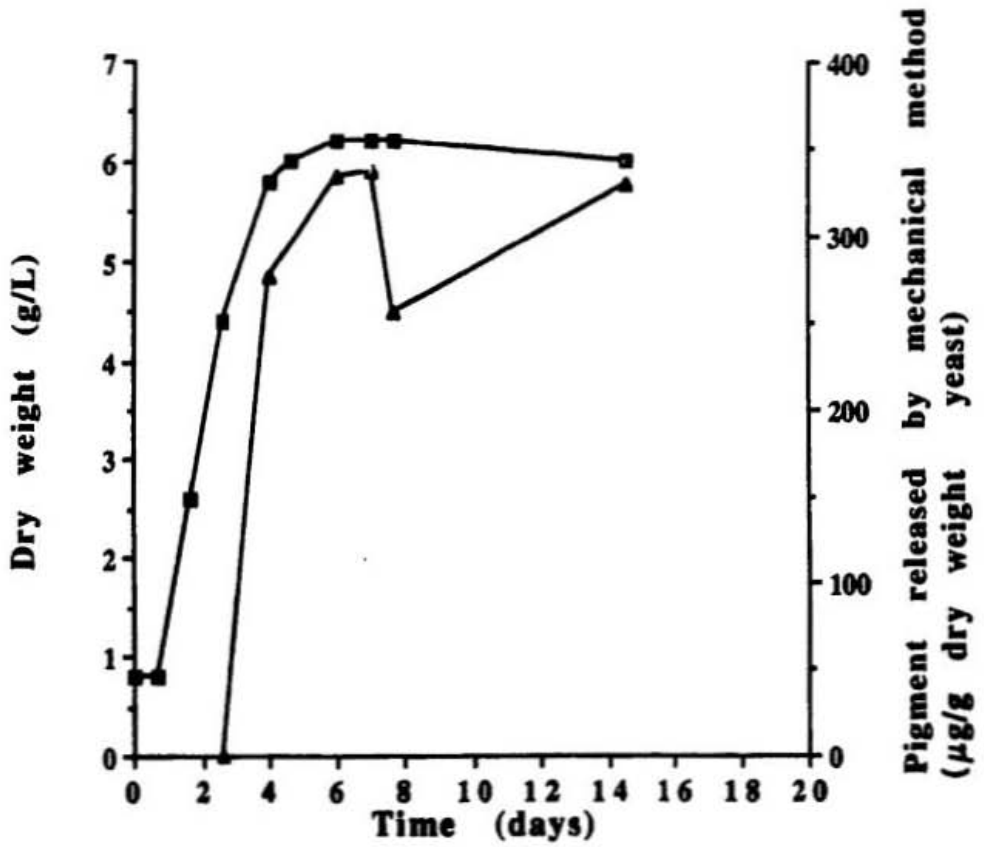


Fig. 6.3. Pigment released from Rhodotorula rubra by mechanical rupture.
■ Growth (dry weight (g/L)
▲ Pigment concentration (µg/ g dry weight yeast)

6.4 DISCUSSION

The pigmentation in P. rhodozyma has been shown by some workers to be growth-associated (Johnson and Lewis, 1979). However, a different pattern has been observed in other carotenogenic systems. In the case of R. rubra, three main phases of carotenoid synthesis have been shown to exist: a period of active synthesis leading to maximum concentrations of the pigment, a period of persistence where the pigment concentration remained constant and, finally a period of decline where the pigmentation was gradually lost (Goodwin, 1952). The same pattern was observed in R. gracilis 813/5 (Vecher and Kulikova, 1968). Bobkova (1965b) found that the accumulation of pigments in Sporobolomyces roseus reached a maximum on the 35th day of fermentation by which time the culture had finished development. Intensive carotene formation in Phycomyces blakesleeanus and Choanephora cucurbitarum was observed when the growth of the mycelium was completed and the intracellular lipids had been synthesized (Chu and Lilly, 1960). Pigmentation of the new strain of R. rubra, like P. rhodozyma, was found to be growth related.

Since the carotenoids in P. rhodozyma and R. rubra are produced in the exponentially growing cells and not in the stationary phase cells as in other red yeasts, it suggests (is possible to deduce) that the carotenoids in these two

organisms play a more vital and important role than those of other carotenogenic yeasts. Carotenoids are known to be effective anti-oxidants and quenchers of singlet oxygen (Krinsky, 1979; Krinsky and Deneke, 1982; Burton, 1989).

In an earlier study, carotenoids were shown to protect the red yeast R. mucilaginosa against oxygen radicals when duroquinone, a redox-cycling quinone known to generate intracellular superoxide, was added to the yeast culture (Morre et al., 1989). R. mucilaginosa had only one superoxide dismutase (SOD) compared to two found in Saccharomyces cereviseae. The authors postulated that the presence of only one SOD in R. mucilaginosa was balanced by the protective effect extended by the carotenoids. Chang (1990) also demonstrated increased astaxanthin formation by the addition of duroquinone to the yeast cultures.

It can be postulated that the two red yeasts studied in the present investigation deal with more oxygen radical stress leading to the synthesis of carotenoids in the exponential phase of their cycle to protect against oxidative damage. Thus a growth related pigmentation was exhibited by the two red yeasts.

As seen from the results obtained from Phaffia, much higher amounts (about two times) of pigment were extractable from the enzyme treated cells than the French-press ruptured cells. The latter treatment of cell rupture also resulted in

a delayed detection of pigment (pigment detected 2 days later than that of the former treatment). Hence P. rhodozyma cells are much more amenable to rupture by the funcelase than to mechanical rupture in a French press. It is to be noted, however, that the enzyme has a pH optimum of 4 to 5 and a temperature optimum of 30 °C. It appears 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 Gentles and Haard (1991). Thus the enzymatic technique is less commercially practical than the French press.

A different trend was noticed in the breakability of the R. rubra cell wall. As opposed to Phaffia, the 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 easy susceptibility of the Phaffia cell wall by the enzyme treatment. This also leads us to conclude that there are apparent differences in the structure of capsule in the two red yeasts. Incubation of the cells over longer periods of time to make the enzyme treatment effective, is one possibility which could be attempted.

The pigment production in the P. rhodozyma and R. rubra isolates started in the late exponential phase extending into the early stationary phase.

The mechanically ruptured cells of R. rubra in a French press yielded more pigment than those of P. rhodozyma ruptured by a similar method (about three and a half times more pigment from R. rubra cells than that from P. rhodozyma, calculated on the basis of unit volume of culture medium). This could be due to either higher pigment content or better breakability of R. rubra cells.

In addition to lower yields, the pigment released by the French press rupture method in the case of P. rhodozyma cells is also detected later than that of R. rubra. This is expected since the generation time for Phaffia is much longer than that of R. rubra (Hari et al. 1992).

Because of faster growth rate of R. rubra, the optimum pigment levels were achieved in shorter incubation time than that of P. rhodozyma. The shorter incubation time would translate into lower costs of pigment production when considering a commercial scale pilot-plant.

CHAPTER 7

Shake flask and fermenter culture studies of Rhodotorula rubra TP 1

7.1 INTRODUCTION

Molasses is one of the most widely used raw materials in industrial fermentations due to its nutritional characteristics, low cost and abundance. It is a good source of nitrogen, inorganic constituents, vitamins and carbohydrates. Molasses and brewer's wort were used as substrates in the present study using R. rubra TP 1 in a 1500 L fermenter. The biomass yield as well as pigment production were evaluated in context of the different kinetic parameters in a large scale fermenter.

One of the factors affecting the economic suitability of biomass production using a fermenter culture is the yield coefficient. The latter, in turn, depends on the microorganism used, type of substrate selected, the maintenance coefficient and the specific growth rate (μ) of the organism. The maintenance coefficient is a measure of the amount of substrate used which does not result in the production of new biomass. When the maintenance coefficient is small, the yield coefficient is affected minimally by the specific growth rate. However, in organisms with large maintenance coefficients, the yield coefficient declines rapidly as the μ decreases (Abbott

and Clamen, 1973).

7.2 MATERIALS AND METHODS

A growth medium composed of molasses and wort was used in this study as the organism R. rubra TP 1 has been shown to grow well on these substrates (Hari et al. 1993).

7.2.1 Conditions of growth in a shake-flask: Shake flasks of 2 L capacity containing the medium as shown in Table 7.1 were incubated at 20°C in a 'Psychrotherm' controlled environment incubator (New Brunswick, NJ, USA) in the presence of light and with a shaking rate of 150 rpm for 5 days.

7.2.2 Conditions of growth in the fermenter: The composition of the fermentation menstruum used is given in Table 7.1 while Table 7.2 describes some of the fermentation conditions.

The stainless steel fermenters were from Bio Engg. AG (Planning and production Microbiology plants, Switzerland). Table 7.3 illustrates the dimensions of various structural components of the two fermenters. Since the culture requires light for growth and pigmentation, two spot lights (150W each) were fitted on the lid of the large fermenter and one on the small fermenter. The addition of sterile anti-foam (33.3 ppm) was made every six hours. The cultures were aerated at the rate of 1.0 vol. of air/vol. of medium/min. The agitation

speed was 150 rpm.

Samples were withdrawn aseptically at different time intervals and the different growth parameters were measured as shown in Tables 7.4 and 7.5.

The productivity was calculated as $P=\mu X$ where μ is the specific growth rate and X is the biomass concentration (Sheehan and Johnson, 1971). The generation time was calculated as

$t_2 - t_1 / 3.32 \times (\log X_2 - \log X_1)$ where t_1= initial time, t_2= final time, X_1= initial biomass and X_2= final biomass (Rose, 1968).

In addition, the specific rate of substrate consumption was measured as the specific growth rate (μ) divided by yield coefficient (Y) (Abbott et al. 1974). The maintenance coefficient was measured as described in the discussion section.

7.3 RESULTS

7.3.1 Growth kinetics in a shake flask and fermenter:

The specific rate of substrate consumption, $(1/x)\ X\ (ds/dt)$ was calculated from the factor μ/Y where x is the biomass concentration, S is the substrate concentration, t is the time of incubation, Y is the yield coefficient (Abbott, 1973).

Thus (1/x) X (ds/dt) was calculated at each growth rate and plotted against μ (Fig 7.1).

Fig 7.2 and 7.3 show the effect of μ and the time of incubation on the yield coefficient.

Figures 7.4 and 7.5 show the growth curve of the yeast and the dissolved oxygen tension in the fermenter while Fig 7.6 illustrates the relationship between the amount of consumed substrate and the increase in biomass concentration.

7.3.2 Comparison of growth in a shake-flask and fermenter:

The different growth kinetics for shake-flask and fermenter culture are listed in Tables 7.4 and 7.5.

Figures 7.7 and 7.8 show the effect of time of cultivation on the specific growth rate of the yeast when grown in shake flasks and fermenter.

Table 7.1. Composition of medium for the growth of R. rubra in a fermenter.

Medium composition	Amount (%)
Cane and beet molasses (equal amount)	1.42
Wort	3.57
Peptone	0.30
Yeast extract	0.15
Malt extract	0.15

Table 7.2. Growth conditions of R. rubra in fermenters.

	Fermenter	
	150 L	1500 L
Total volume of the medium	70 L	500 L
Initial pH of the medium	5.49	6.21
Final pH of the medium	7.12	6.92
Initial dissolved oxygen in the medium	1.0 v/v	1.0 v/v
Final dissolved oxygen in the medium	0.38 v/v	0.97 v/v
Size of inoculum	14%*	14%
Age of the culture	18 h	18 h
Temperature of incubation	20°C	20°C
Length of run	18 h	44 h

* The inoculum used for the 150 L fermenter was prepared by growing the yeast in shake flasks of 2 L capacity containing the same medium as in Table 1. The flasks were incubated at 20°C in a 'Psychrotherm' controlled environment incubator in the presence of light and with a shaking rate of 150 rpm for 18 h.

Table 7.3. Dimensions (in meters) of various structural components of the fermenters.

Component	150 L fermenter	1500 L fermenter
Fermenter diameter (D_T)	0.42	0.88
Liquid height (H_L)	0.80	1.88
Impeller diameter (D_i)	0.19	0.36
Fermenter height (H_O)	1.15	2.60
Baffle height (H_b)	0.23	0.30
Baffle width (W_b)	0.04	0.10
Impeller length (L_i)	0.055	0.104
Impeller width (W_i)	0.04	0.075

Table 7.4. Growth parameters for the culture grown in a 1500 L fermenter using molasses and wort medium.

Time of cultivation (h)	Biomass (g/L)	Specific growth rate (μ)	Productivity (g/L/h)
0	1.26	0	0
8	2.13	0.066	0.141
8.6	2.3	0.128	0.294
10.17	2.9	0.15	0.44
12.6	3.65	0.095	0.35
13.7	4.1	0.11	0.45
15.5	4.75	0.08	0.38
16.3	5.0	0.064	0.32
18.08	5.55	0.059	0.33
19.0	5.92	0.07	0.41
23.0	7.32	0.053	0.39

Specific growth rate = ln 2/ generation time
Productivity = specific growth rate X biomass
1500 L fermenter used; working volume = 500 L
Initial pH = 6.2; Initial dissolved oxygen = 1.0 v/v
Rate of agitation = 150 rpm

Table 7.5. Growth data for cultures grown in shake flasks.

Time of cultivation (h)	Biomass (g/L)	Specific growth rate, μ (/h)	Productivity (g/L/h)
0	0.65	0	0
15.72	1.42	0.022	0.031
22.58	2.44	0.079	0.193
39.75	5.73	0.05	0.287
44.67	6.82	0.035	0.239
49.1	6.90	0.0026	0.018
63.58	7.48	0.0056	0.042
88.5	7.67	0.0002	0.002

Specific growth rate = ln 2/ generation time
Productivity = specific growth rate X biomass
2 L shaker flasks used; working volume = 1 L
Initial pH = 6.2; Rate of agitation = 150 rpm

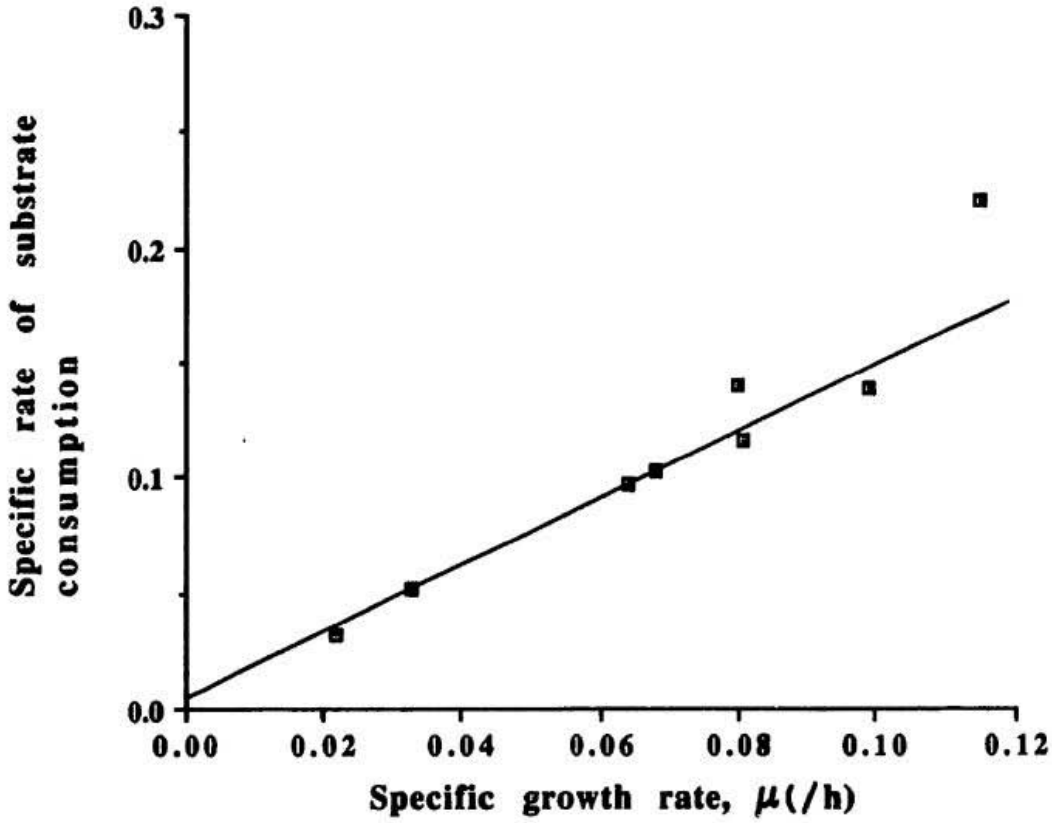


Fig. 7.1. Effect of specific growth rate on the specific rate of substrate consumption.
1500 L fermenter used; working volume = 500 L
initial ph = 6.2; initial dissolved oxygen = 1.0 v/v
rate of agitation = 150 rpm

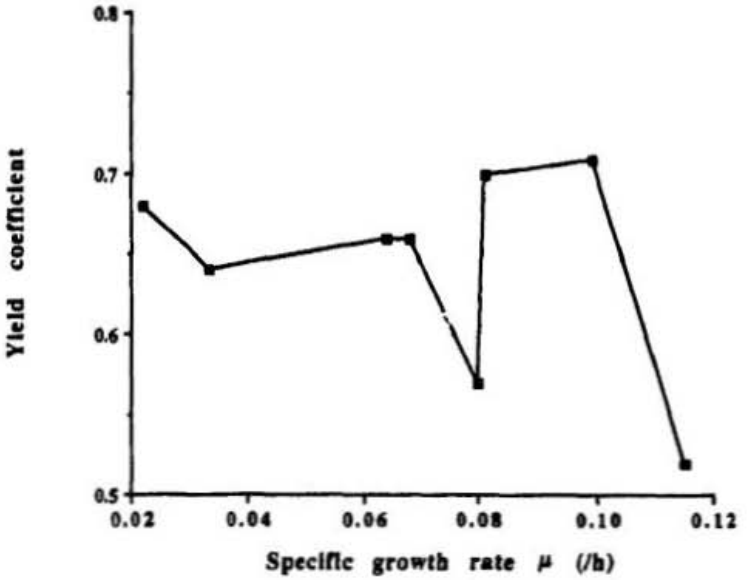


Fig. 7.2. Yield coefficient as a function of specific growth rate.

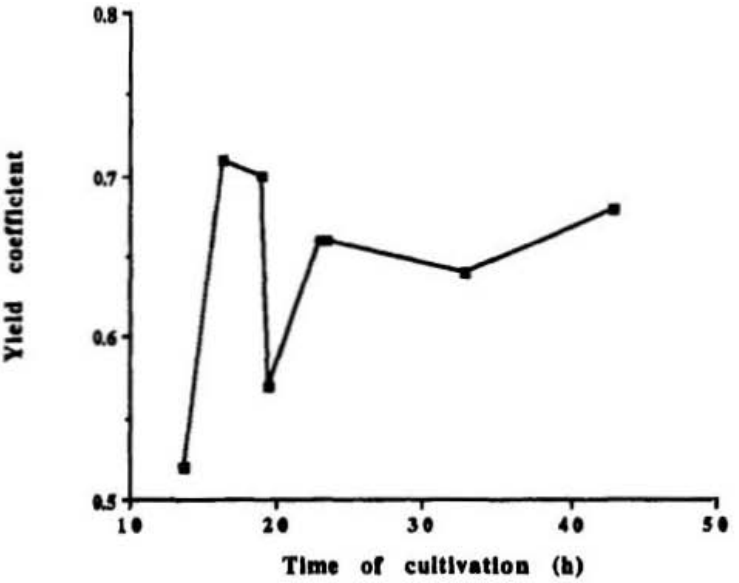


Fig. 7.3. Yield coefficient as a function of time of cultivation.
1500 L fermenter used; working volume = 500 L; initial pH = 6.2; initial dissolved oxygen = 1.0 v/v;
rate of agitation = 150 rpm.

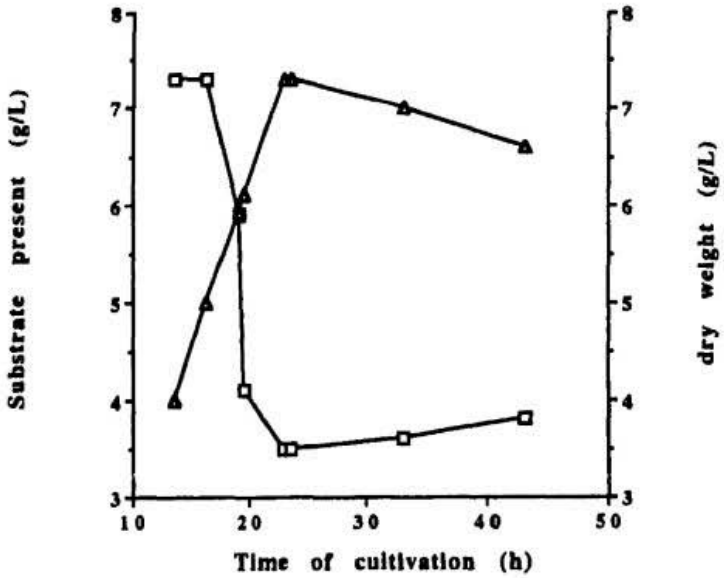


Fig. 7.4. Growth curve of the yeast grown in a 1500 L fermenter.

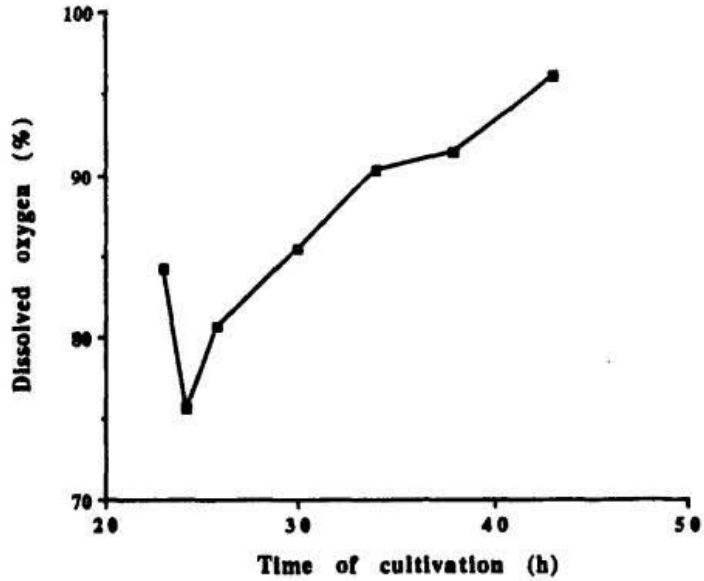


Fig. 7.5. Dissolved oxygen status of the fermenter.
1500 L fermenter used; working volume = 500 L; initial pH = 6.2; initial dissolved oxygen = 1.0 v/v;
rate of agitation = 150 rpm.

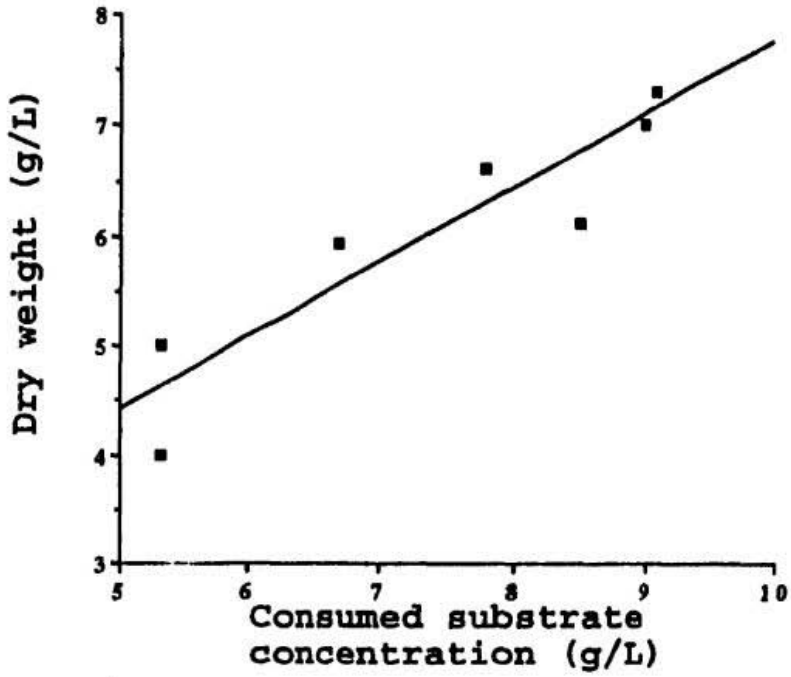


Fig. 7.6 Effect of consumed substrate concentration on the biomass.
1500L fermenter used; working volume= 500L; initial pH=6.2; initial dissolved oxygen=1.0v/v; rate of agitation=150rpm.

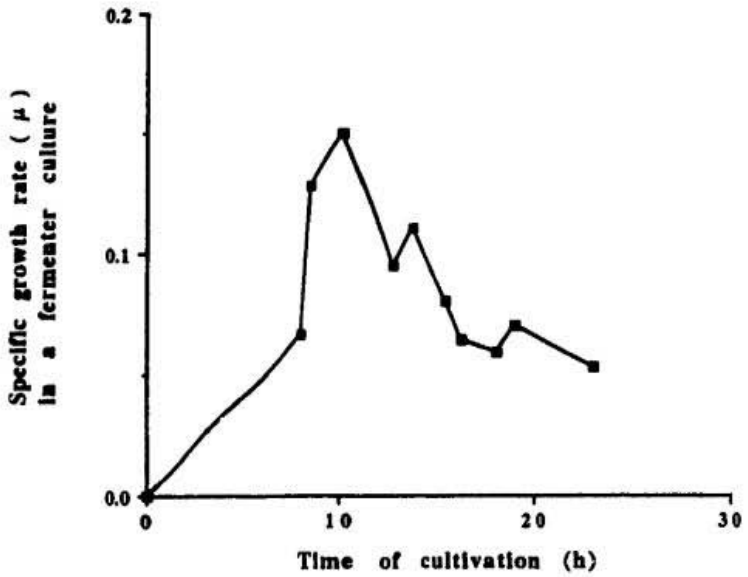


Fig. 7.7. Specific growth rate of the fermenter culture as a function of time of cultivation.

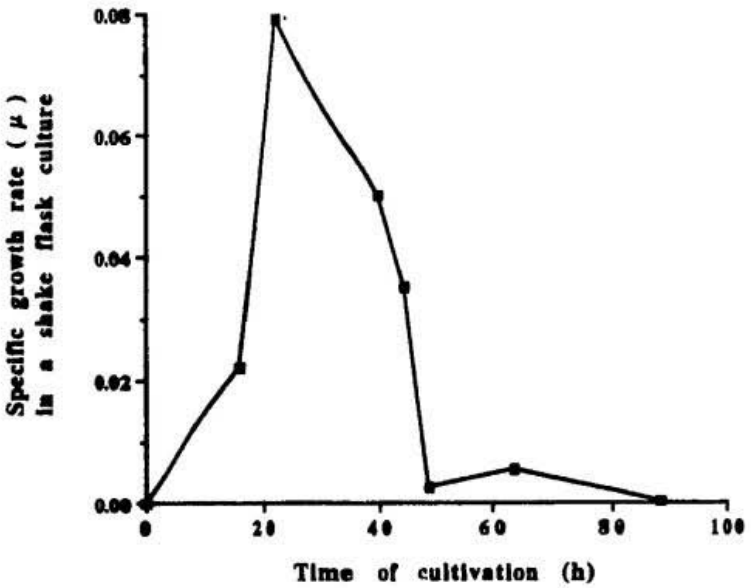


Fig. 7.8. Specific growth rate of the shake flask cultures as a function of time of cultivation.
2 L flasks used; working volume = 1 L;
initial pH = 6.2; rate of agitation = 150 rpm.

7.4 DISCUSSION

7.4.1 Measurement of maintenance coefficient:

A linear relationship was obtained between the specific rate of substrate consumption and μ (Fig. 7.1).

The value of maintenance coefficient (m), taken as the intersection of the straight line at the ordinate, was found to be 0.004 g sugar consumed/g biomass formed/h.

The R. rubra isolate grown in a fermenter, using a substrate which is low in cost, had a favourable m value when compared with other studies, e.g., Acinetobacter calcoaceticus grown on ethanol in a chemostat showed an m value of 0.11 g ethanol/g biomass/h (Abbott et al. 1974); Rogers and Stewart (1974) grew S. cerevisiae and Candida parapsilosis in a semi-synthetic medium composed of (g/L): glucose (5-10); $(NH_4)_2SO_4$ (3.0); KH_2PO_4 (2.0); $MgSO_4 . 7H_2O$ (2.0); $FeSO_4 . 7H_2O$ (0.005); $CaCl_2 . 2H_2O$ (0.1); NaCl (0.5) and yeast extract (5.0). They reported an m value of 0.13 g glucose/g biomass/h for the aerobic cultures of wild-type S. cerevisiae, 0.018 g glucose/g biomass/h for the micro-aerobic cultures of C. parapsilosis and 0.094 g glucose/g biomass/h for the petite S. cerevisiae. Parades-Lopez et al. (1976) reported a maintenance coefficient of 0.09 g sugar/g biomass/h for C. utilis grown on prickly pear juice.

As evident in Fig. 7.2, the yield coefficient rises

rapidly as the μ increases. At lower growth rates, a larger portion of the total substrate is used for maintenance, resulting in less available substrate for biomass synthesis. This leads to a lower yield coefficient.

A plot of yield coefficient versus time showed a rapid rise in the yield coefficient in the first 15 h after which it reached a plateau and declined (Fig. 7.3).

Figures 7.4 and 7.5 show the growth curve of the yeast as well as the dissolved oxygen tension in the fermenter. Figure 7.6 shows a linear relationship between the consumed substrate concentration and the increase in biomass.

7.4.2 Comparison of growth in shake-flask and fermenter:

When the time of cultivation was plotted against the specific growth rate (Fig. 7.7), the latter reached a certain value after which it dropped as the time of incubation progressed. The μ value obtained in a fermenter was almost double that of a shake flask grown culture (0.15/h as opposed to 0.08/h in a shake flask culture).

A higher value of productivity was obtained in a fermenter grown culture than that of the culture grown in a shake flask (a value of 0.45 g/L/h was obtained after 13 h of growth in a fermenter grown culture while the maximum productivity obtained for a shake flask culture was 0.29 g/L/h after 40 h of growth) (Tables 7.4 and 7.5).

CHAPTER 8

Rhodotorula rubra TP 1 as a pigment and nutrient source for rainbow trout (Onchorynchus mykiss)

8.1 INTRODUCTION

Carotenoids are widely distributed in living organisms and constitute the major pigments of most fish and crustaceans. As fish are unable to synthesize carotenoids *de novo*, these have to be added to their diets when reared in aquaculture farms, to maintain the redness of their flesh, an important quality criterion in consumer acceptance. Many different sources of carotenoids have been used to pigment the flesh of salmonid fish, e.g., (a) crustacean and crustacean by-products, like shrimp processing offal (Storebakken et al. 1985), red crab (Spinelli and Mahnken, 1978), krill meal (Kotik et al. 1979), shrimp waste (Torrissen, 1985), copepod (Lambertsen and Braekken, 1971), crab meals (Kuo et al. 1976) and fish oils (Lambertsen and Braekken, 1971); (b) plant and plant products, e.g., marigold and squash flowers (Lee et al. 1978) and green algae Spirulina (Choubert, 1979); (c) yeast, e.g, Rhodotorula sanneii (Savolainen and Gyllenberg, 1970) and Phaffia rhodozyma (Johnson et al. 1980); (d) synthetic sources, astaxanthin and canthaxanthin (Torrissen, 1986).

Because of the high expense of the synthetic carotenoids, the strict regulatory agencies concerned about the safety of the synthetics as food additives and the general instability of the artificially added carotenoids in the food mixture (Johnson et al., 1980; Savolainen and Gyllenberg, 1970), lately there has been a trend towards the use of biological sources of carotenoids.

In this study, R. rubra TP 1 was fed to rainbow trout as a source of carotenoids and other nutrients. The intact yeast cells, without any treatment to modify their cell wall, were added to the fish diets. Two other diets, one with synthetic astaxanthin added to it, and another with no pigment, whatsoever, were also included in the study. The proximate analysis, growth rate and specific growth rate of the fish as well as color measurements were then performed on fish fed with the three diets.

8.2 MATERIALS AND METHODS

8.2.1 Rearing and sampling of the fish:

Rainbow trout (Onchorynchus mykiss) were obtained from a salt-water cage site operated by S.C.B. Fisheries Ltd., St. Alban's, Newfoundland. These trout were then transferred to a nearby salmonid hatchery, where the experiment was conducted.

The tanks used were of the Swede variety (square with

rounded corners) with approximate dimensions of 1 M X 1 M X 0.5 M deep. The water volume in each tank was maintained at 0.47 M^3 with an incoming flow rate of 14L per minute.

Seventy eight rainbow trout were randomly assigned to each of the experimental conditions: control (no pigment) diet, commercial pigmented diet (Carophyll pink) and diet containing experimental yeast, R rubra TP 1 cells. Each of the three experimental conditions were conducted in triplicates (26 trout per tank).

During acclimation (two weeks) to experimental conditions these were fed to satiation using a 3.0 mm non-pigmented pellet (Corey Feed Mills Ltd., Fredericton, N.B., Canada).

Composition of the experimental feeds is shown in Table 8.1. All ingredients for feed formulation, except for the experimental yeast, were obtained from Corey Feed Mills Ltd., Fredericton, NB. The experimental yeast was prepared in a 1500L fermenter at the Food Research and Development Centre at St. Hyacinthe, Quebec. The medium composition as well as the conditions for propagation are discussed in detail in Chapter 7. The Carophyll pink used contained 6% astaxanthin. The commercial pigmented diet and the experimental yeast diet were formulated to contain 100 ppm red pigment. The feed ingredients were mixed and pelleted (6 mm) using a Hobart N50 laboratory mixer/pelleter. The trout were fed to satiation twice daily.

At the beginning of the experiment the average mass of the fish in each of the experimental diets were: control: 168g; commercial pigmented: 150g; pigment containing yeast: 156g. The age of the fish at the beginning of the feeding trial was one year.

All procedures, including husbandry and feeding regimes, were designed to result in near optimal growth of rainbow trout at temperatures between 10 - 19°C.

In order to determine growth rates and pigment levels, random sampling of the three experimental conditions was conducted at 0, 3, 6 and 9 weeks into experimentation. Four fish were sampled from each tank (12 per experimental condition) and were stunned by a blow to the head. They were then blotted dry and their mass determined. A gill arch was cut on each sampled fish and they were left to bleed in cool water. All internal organs, including the kidney, were removed.

All sampled fish was frozen at the time of sampling. At the conclusion of the experiment the relative level of pigmentation in the flesh of the frozen samples was evaluated using a colormet, a portable color analyzer from Metron Instruments Inc. (Hamilton, Canada). The following characteristics were determined:

8.2.2 Color parameters: The following color parameters were measured:

i. The L^*, a^* and b^* values, the Hunter scale (Hunter, 1975), the L^* value indicating the lightness, the a^* value indicating redness and the b^* value indicating yellowness.

ii. Hue: This was calculated as $H^\circ_{ab} = \tan^{-1} b^*/ a^*$, $H^\circ_{ab} = 0^\circ$ for red and 90° for yellow (Wyzecki and Stiles, 1967).

iii. Chroma: $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$ (Wyzecki and Stiles, 1967).

iv. a^*/b^* ratio (Schmidt and Cuthbert, 1969).

v. Total color difference, $E = (L^{*2} + a^{*2} + b^{*2})^{1/2}$ (Manual of Gardner XL-20 Tristimulus Colorimeter).

8.2.3 Growth rate: This was calculated as g weight gained/fish/day (Johnson et al. 1980).

8.2.4 Specific growth rate: This was calculated as:

Specific growth rate (Sgr) =

100 X (ln final weight _ ln initial weight)/ duration (days)

(Gomes et al. 1993; Cho, 1992).

8.2.5 Proximate analysis: The ash, total nitrogen, protein and lipids were determined as mentioned in Chapter 4. The quantitative analysis of total amino-acids was done at 110°C using 6N HCl with 0.05% phenol for 24h.

8.3 RESULTS

8.3.1 Visual inspection of the fish:

Figures 8.1, 8.2 and 8.3 show the color differences between the fish fed with the different diets. As seen from

the photographs, the diet 2 (containing the commercial pigment) induced the most intense pigmentation. The fish fed with this diet also showed the most rapid pigmentation in the first 6 weeks of feeding while the experimental yeast containing diet (diet 3) induced more color change from the 6 week to 9 week period than in the initial 6 week period.

8.3.2 Measurement of different color parameters:

The a* values of diet 1 fed fish showed no change while the fish fed diets 2 and 3 showed an increase in the a* score with the time of feeding. The b* scores of the fish in the three groups were found to increase at the end of nine weeks (Table 8.2). The a*/b* ratio of diet 1 fed fish declined after 9 weeks while that of diets 2 and 3 fed fish increased. The diet 2 fed fish, however, showed a much greater increase in the ratio. The L* values of the diet 1 fed fish flesh increased at the end of the experiment while those of the diet 2 fed fish remained the same. The diet 3 fed fish, however, showed a slight increase in the L* value (Table 8.3).

The hue of the flesh of diets 2 and 3 fed fish was found to decrease with the increasing feeding time. The lower hue values are associated with increasing redness (Wyzecki and Stiles, 1967). The diet 1 fed fish showed a higher value for hue. The chroma of the fish of all the groups was found to have a higher value with the duration of feeding, the increase being twice for the diet 2 fed fish, as that of the other

groups (Table 8.4).

The total color change from the beginning to 6 weeks was the highest for the diet 2 fed fish while that of the 6 weeks to 9 weeks period was the maximum for the diet 3 fed fish (Table 8.5). Thus the diet 2 fed fish displayed the maximum color change in the initial 6 weeks of feeding followed by a reduction in the total color difference. The diet 3 fed fish, on the other hand, showed an almost even color change during the entire period of feeding (Table 8.5). The values of total color change between the diets were found to be interesting. After 9 weeks of feeding, the color change between the diets 2 and 3 was reduced to almost one-third of that in the first 6 weeks of feeding (Table 8.6).

8.3.3 Relationship between duration of pigmentation and the different color parameters:

a) *The L*, a*, b* color scores*: The L* score was not related to the duration of pigmentation. The a* score of fish fed diet 1 also did not increase appreciably with the time of feeding. However, the a* and b* scores of those of diets 2 and 3 as well as the b* scores of diet 1 were found to significantly correlate and increased with the duration of feeding (Fig 8.4 and 8.5).

b) *Hue and chroma*: The measurement of hue revealed that the fish of diet 2 showed a strong negative correlation with the feeding time while those of diet 3 showed a weak negative

correlation with the feeding time. It is to be borne in mind that lower values of hue denote increasing redness of the sample while higher values denote increasing yellowness (Wyszecki and Stiles, 1967) (Fig. 8.6). The chroma of the fish flesh showed a very strong correlation with the feeding time, i.e., it showed a significant increase at the end of the feeding time and the trend was noticed in the fish of all the three diets (Fig. 8.7).

c) *a^*/b^* ratios*: The fish fed diet 1 showed a very strong negative correlation between the a/b ratios and the time of feeding while the diet 2 and 3 fed fish showed a strong positive and a weak positive correlation, respectively (Fig. 8.8).

8.3.4 Growth rate:

After 6 weeks of feeding, the maximum growth rate was observed for the diet 3 fed fish. The diet 2 fed fish in the initial 6 week period had the least growth rate but after 9 weeks it was almost equivalent to that of the diet 3 fed fish. The diet 1 fed fish showed a drop in the growth rate from 0.85 to 0.58 g/day at the end of the feeding period (Table 8.7).

8.3.5 Specific growth rate:

After 6 weeks of feeding, the diet 3 fed fish had the maximum specific growth rate. At the end of the feeding period, however, the specific growth rate was the highest for the diet 2 fed fish (Table 8.8).

8.3.6 Proximate composition:

The percent nitrogen and protein in the diet 2 fed fish flesh was reduced at the end the feeding period. However, the nitrogen and protein amounts in the diet 3 fed fish increased, and so did the diet 1 fed fish, after 9 weeks of feeding. The lipid levels of the fish of all groups decreased considerably after 9 weeks (Table 8.9).

Table 8.10 shows the amino-acid of diets 2 and 3 fed fish after 9 weeks of feeding. The diet 3 fed fish showed an increased level of all the amino-acids except hydroxy-proline and cystine while 3 methyl-histidine, though present at lower levels in the diet 2 fed fish, was absent from it.

Table 8.1. Composition of experimental diets for trout pigmentation; studyconducted at Bay d'Espoir.

Ingredients (g)	Diets 1 No pigment	2 Carophyll pink	3 test yeast
Fish meal	35.9	35.9	35.9
Blood meal	10.0	10.0	10.0
Corn Gluten	12.0	12.0	12.0
Soybean meal	8.65	8.65	8.65
Whey powder	12.25	12.25	12.25
Vitamin mix	1.5	1.5	1.5
Mineral mix	1.0	1.0	1.0
Guar gum	1.0	1.0	1.0
Fish oil	12.7	12.7	12.7
Brewer's yeast	5.0	4.83	0.43
Carophyll pink	-	0.17	-
Experimental yeast	-	-	4.57*
Total	100.0	100.0	100.0

* This value refers to the dry component of the experimental yeast. To achieve this amount of dry experimental yeast and the proper pigment concentration (100 ppm), 28.03g of the wet experimental yeast was added to the other ingredients in the experimental yeast diet. This adds a large amount of water to the above diet. To ensure that all the three diets have the same moisture content, 23.5g of water was added to each 100g preparation of diets 1 and 2.

Table 8.2. Relation of feeding time with the color parameters, a* and b*.

Weeks of feeding	Color parameters					
	a* value			b* value		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
0	3.18±0.25	5.4±0.44	4.05±0.14	10.3±0.51	13.5±0.66	14.55±1.18
6	3.24±0.25	14.35±0.22	4.38±0.25	12.35±1.07	20.28±0.06	16.65±0.76
9	2.25±0.44	13.1±2.15	7.35±0.43	15.35±0.48	19.65±1.06	19.55±0.38

a* and b* values are according to the Hunter's scale (Hunter, 1975). a* value denotes redness while b denotes yellowness.

Diet 1 control without pigment (negative control)

Diet 2 Carophyll pink,commercial (positive control)

Diet 3 Yeast TP 1 (experimental/test yeast)

Table 8.3. Relation of feeding time with the color parameters, a*/ b* and L*.

Weeks of feeding	Color parameters					
	a*/b* ratio			L* value		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
0	0.31±0.02	0.40±0.02	0.28±0.01	49.15±1.56	52.18±1.66	51.5±2.19
6	0.23±0.03	0.70±0.01	0.27±0.03	54.58±1.16	55.6±0.6	57.5±0.47
9	0.14±0.03	0.67±0.11	0.38±0.01	56.35±1.37	52.1±1.53	53.1±0.06

L * value indicates lightness in color of the fish flesh
a* represents redness and b* represents yellowness

Table 8.4. Relation of feeding time with the color parameters, hue and chroma

Weeks of feeding	Color parameters					
	Hue			Chroma		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
0	80.94	75.78	82.71	10.78	14.54	15.10
6	83.66	60.73	83.62	12.77	24.84	17.22
9	90.73	62.57	77.11	15.51	23.62	20.89

Hue = $H^{\circ}_{ab} = \tan^{-1} b^{*} / a^{*}$, $H^{\circ}_{ab} = 0$ for red and 90° for yellow (Wyzecki and Styles. 1967)

Chroma = $c^{*}_{ab} = (a^{*2} + b^{*2})^{1/2}$ (Wyzecki and Stiles, 1967)

Table 8.5. Total color difference between two feeding periods.

	Total color difference beginning to 6 weeks*	Total color difference from 6 weeks to 9 weeks**
Diet 1	6.66±1.0	5.73±2.13
Diet 2	11.44±0.08	-0.13±3.23
Diet 3	6.67±2.11	6.25±0.6

Total color difference, $E = [(L^*_f-L^*_i)^2 + (a^*_f- a^*_i)^2 + (b^*_f-b^*_i)^2]^{1/2}$ * L^*_f, a^*_f and b^*_f = Color parameters at 6 weeks
L^*_i, a^*_i and b^*_i = Color parameters at beginning of feeding trial

** L^*_f, a^*_f and b^*_f = Color parameters at 9 weeks
L^*_i, a^*_i and b^*_i = Color parameters at 6 weeks

Table 8.6. Total color difference between two diets

Feeding period	Total color difference between diets 1 and 3*	Total color difference between diets 2 and 3**
After 6 weeks	7.89±2.43	11.17±0.36
After 9 weeks	8.26±1.51	3.94±0.01

Total color difference, $E = [(L^*_f - L^*_i)^2 + (a^*_f - a^*_i)^2 + (b^*_f - b^*_i)^2]^{1/2}$

* L^*_f, a^*_f and b^*_f = Color parameters of diet 3
L^*_i, a^*_i and b^*_i = Color parameters of diet 1

** L^*_f, a^*_f and b^*_f = Color parameters of diet 2
L^*_i, a^*_i and b^*_i = Color parameters of diet 3

Table 8.7. The growth rates of rainbow trout fed with the three diets

Weeks of feeding	Growth rate (g weight gained/fish/day)		
	Diet 1	Diet 2	Diet 3
6	0.85±0.08	0.66±0.22	1.57±0.07
9	0.58±0.05	1.52±0.15	1.67±0.03

Growth rate = g weight gained /fish/day (Johnson et al., 1980).

Table 8.8. The specific growth rates of rainbow trout fed with the three diets

Weeks of feeding	Specific growth rate		
	Diet 1	Diet 2	Diet 3
6	0.63±0.04	0.67±0.23	0.98±0.07
9	0.43±0.03	1.19±0.14	0.93±0.04

Specific growth rate = 100 X (ln final weight- ln initial weight)/ duration (days) (Gomes et al., 1993; Cho 1992).

Table 8.9. The proximate composition of fish fed with the three diets

Feeding time	Nitrogen (%)	Protein (%)	Lipids (%)	Ash (%)
Baseline (o week)	10.81±0.60	67.54±3.78	29.15±4.20	4.24±0.18
6 weeks:				
Diet 1	9.65±0.15	60.32±0.32	18.17±0.50	5.78±0.15
Diet 2	10.9±0.66	68.13±4.13	22.0±1.3	5.66±0.06
Diet 3	11.09±0.004	69.34±0.03	21.78±0.81	4.44±0.14
9 weeks:				
Diet 1	11.02	68.86±7.03	19.73±0.09	4.62±0.02
Diet 2	9.21±0.15	72.00±0.92	19.56±0.74	3.61±0.17
Diet 3	12.04±0.35	75.25±2.17	22.31±0.77	4.58±0.23

Table 8.10. The amino-acid levels of fish fed with diets 2 and 3 at the end of feeding trial.

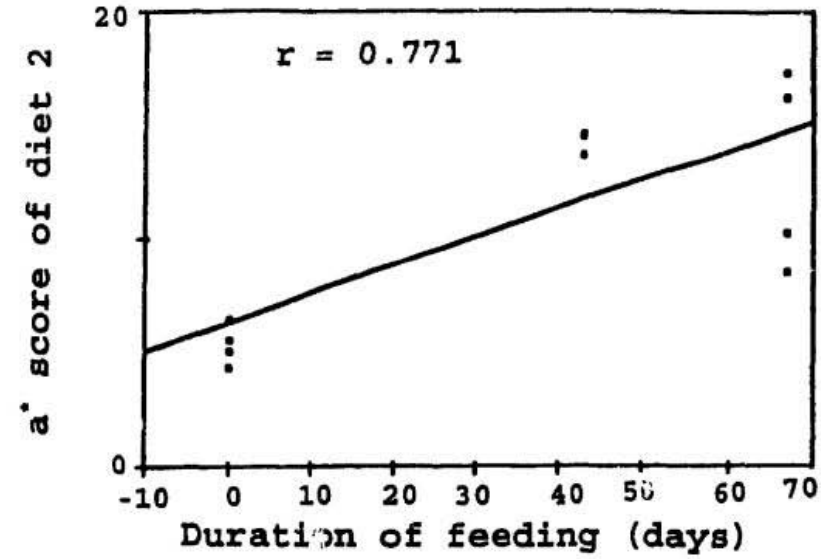
Amino-acid	Concentration (µmoles/g dry weight fish)	
	Diet 2	Diet 3
Cysteic acid	0.44	16.3
Taurine	16.53	25.2
Asp	547.21	673.7
Hydroxy-pro	9.35	6.9
Thr	275.5	340.3
Ser	238.71	303.5
Glu	654.94	815.1
Pro	208.1	242.7
Gly	426.4	535.6
Ala	436.7	533.2
Val	285.1	367.7
Cys	21.5	19.2
Met	129.7	167.1
Cysta-thionine	0.38	2.2
Ile	226.5	283.5
Leu	405.3	512.8
Tyr	114.1	158.3
Phe	166.9	207.2
ß-Ala	35.1	45.7
Ethanolamine	9.02	11.3
Lys	443.8	543.7
1-Methyl-his	34.4	45.4
His	140.1	170.7
3-Methyl-his	0.44	-
Arg	218.1	278.7

Fig. 8.1. Pigmentation of fish (Onchorynchus mykiss) at the beginning of the experiment.

Fig. 8.2. Pigmentation of fish (Onchorynchus mykiss) after 6 weeks of feeding.

Fig. 8.3. Pigmentation of fish (Onchorynchus mykiss) after 9 weeks of feeding.

Diet 1: Feed with no pigment (negative control)
Diet 2: Feed with Carophyll pink (positive control)
Diet 3: Feed with R. rubra TP 1 yeast cells



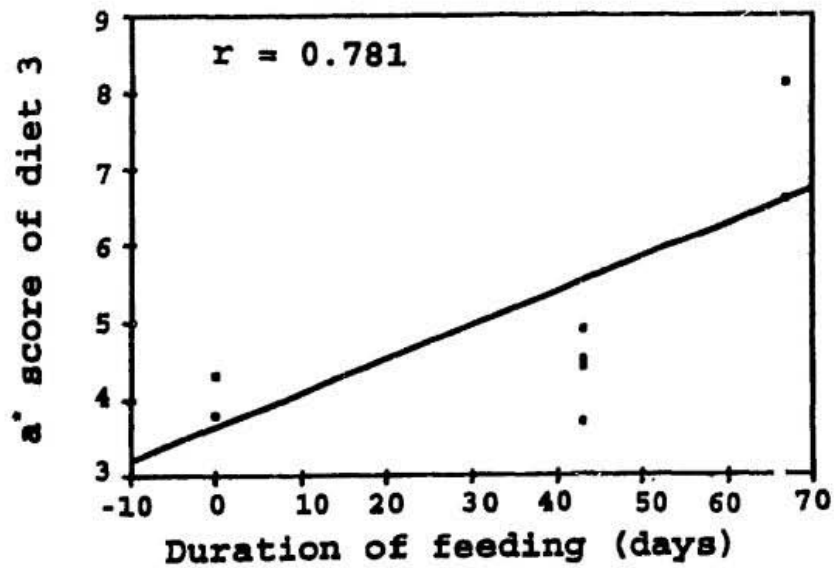


Fig. 8.4. Regression of a* scores of the fish flesh on duration of feeding.

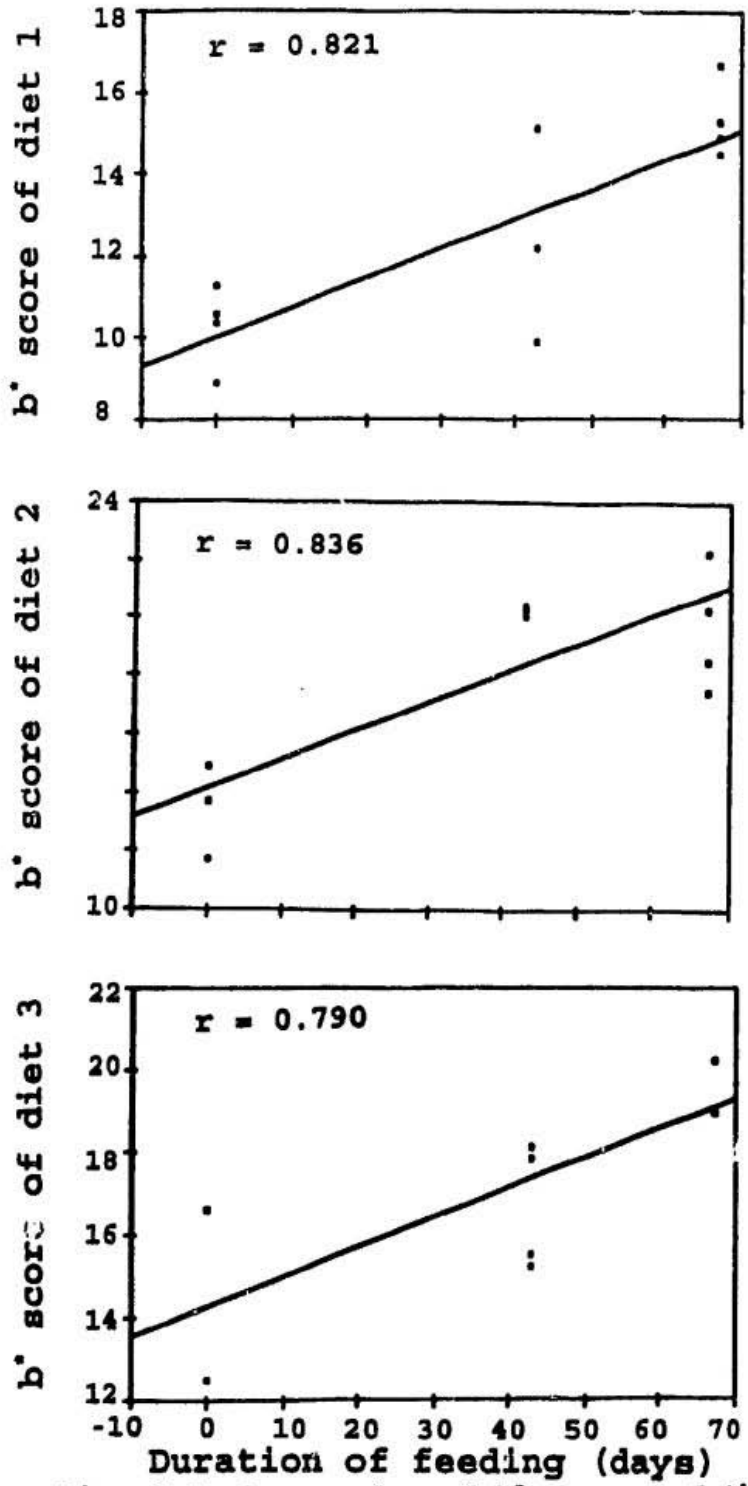


Fig. 8.5. Regression of b* scores of the fish flesh on duration of feeding.

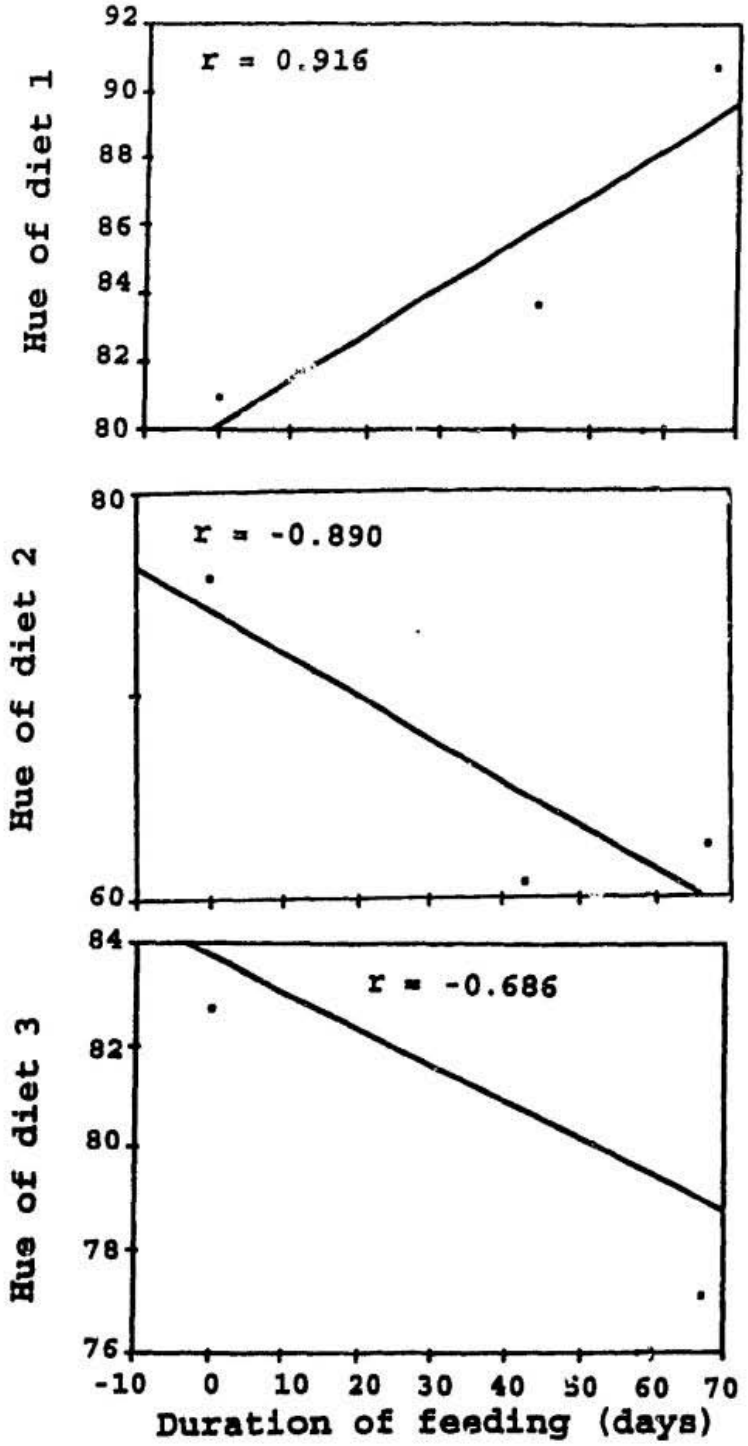


Fig. 8.6. Regression of hue of the fish flesh on duration of feeding.

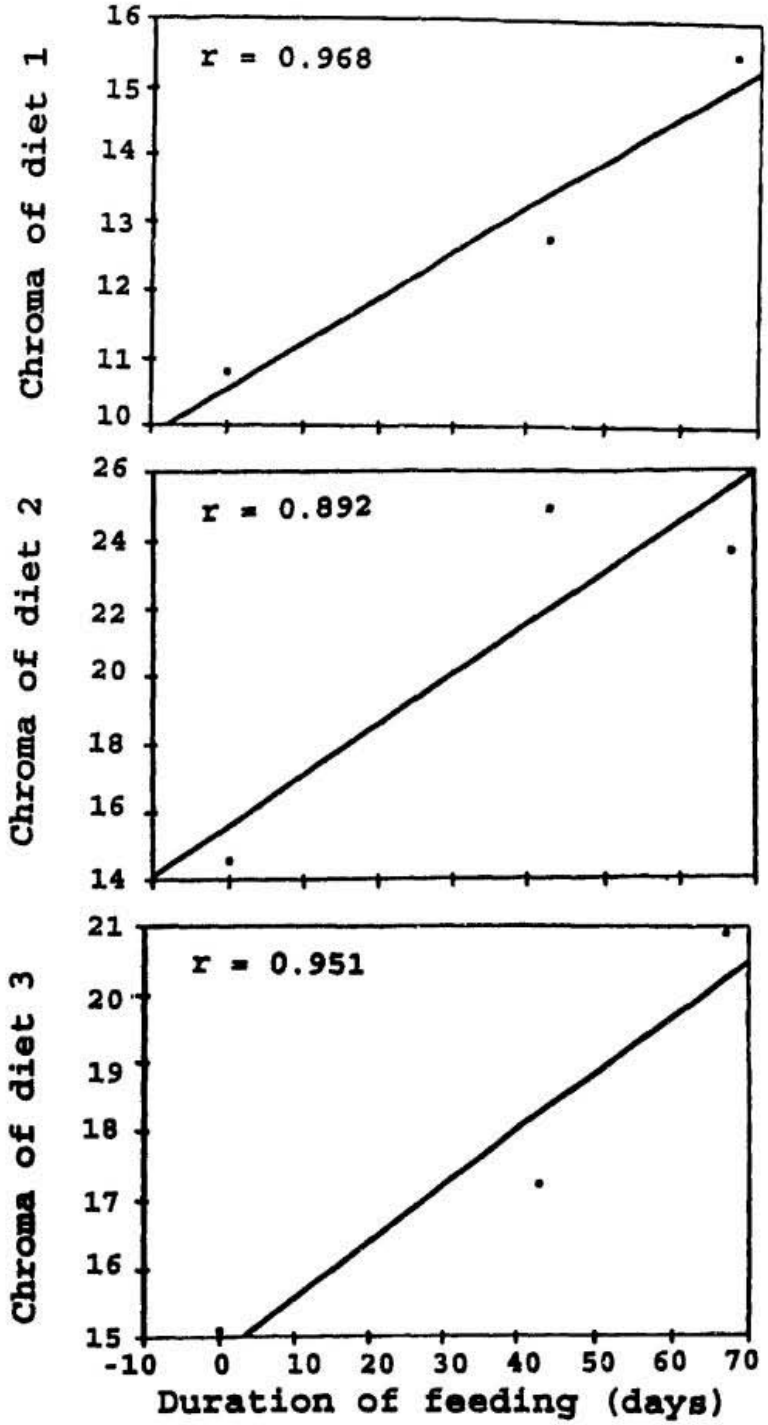


Fig. 8.7. Regression of chroma of the fish flesh on duration of feeding.

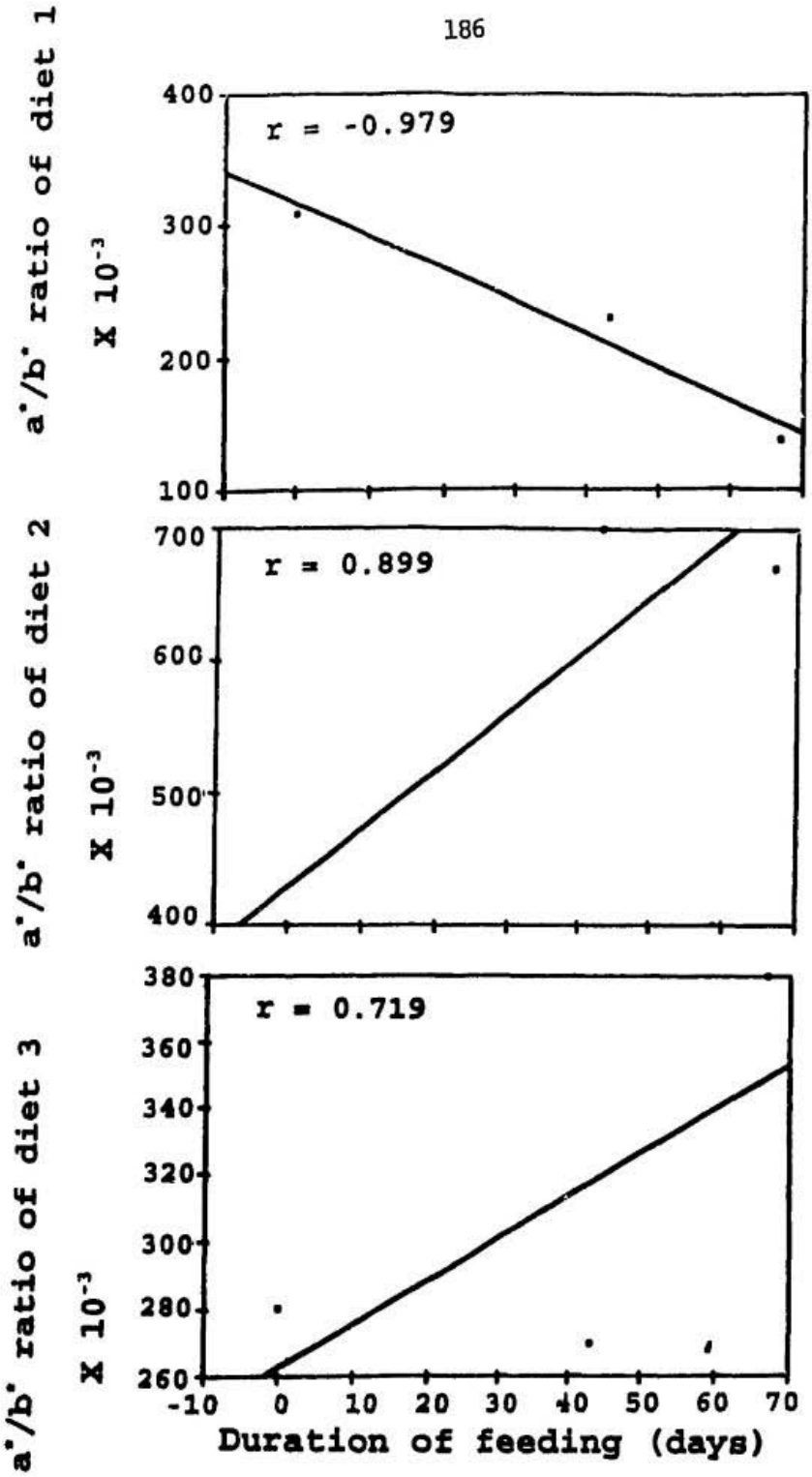


Fig. 8.8. Regression of a*/b* ratio of the fish flesh on duration of feeding.

8.4 DISCUSSION

Laine and Gyllenberg (1969) fed rainbow trout with R. sanneii and compared the results with those fed with a diet containing' Carophyll red' and a third group fed on a pigment free diet. An increase in the fat and decrease in the protein content was reported in the fish at the end of the trial in all the 3 groups. The protein and lipid amounts of the fish fed with the test yeast was higher in the present study (75.3% and 22.3%, respectively) than that reported by the above researchers (71.9% and 17.3%, respectively). The ash level of the test yeast fed fish in the present study (4.6%) was higher than that reported in the above investigation (3.8%). The gain in weight of the fish fed with the test yeast shown by Laine and Gyllenberg was less than that produced when the other 2 diets were fed to the fish. However, in the present study, the maximum growth rate was observed for the test yeast fed fish.

Varesmaa et al. (1969) reported only slight differences in the amino-acid content of fish proteins fed with different diets including the one containing the test yeast R. sanneii. In the present study, however, the fish fed with the test yeast were found to have an increased level of almost all the amino-acids studied.

Savolainen and Gyllenberg (1970) fed R. sanneii to rainbow trout and proposed that lutein, canthaxanthin and ß-

carotene may be synthesized by transformation of other carotenoids or from other precursors. However, the authors did not report higher amounts of R. sanneii carotenoids in the fish.

Johnson et al.(1980) fed rainbow trout with different preparations of the red yeast Phaffia rhodozyma. The authors reported that the yeast had a tough cell wall which was a major barrier to the uptake of carotenoids as well as other nutrients by the fish. The whole yeast cells were unable to pigment the bodies of trout, also leading to poor growth while mechanically broken yeast promoted rapid pigmentation and the fastest weight gain in the fish. The yeast cells that were partially and fully digested by using the extracellular enzymes of Bacillus circulans, led to poor and rapid pigmentation, respectively. Hence, the pigment and nutrient availability increased by breaking the cell wall of the yeast.

The data from the current study, however, clearly indicates the ability of the new yeast (unbroken) to induce pigmentation and good growth of the fish as seen from the results.

Thus, to sum up the results, the new yeast, R. rubra TP 1 was found to be a good source of pigments and nutrients for rainbow trout. The commercial astaxanthin containing diet, however, induced better pigmentation than that containing the test yeast, as seen from the higher a^*/b^* ratio, a higher value

of chroma and a lower value of hue of the flesh of fish fed with the former diet.

The total color difference comprising the values of lightness, yellowness and redness of the fish flesh, between the fish fed with the commercial astaxanthin and the test yeast, was more in the initial 6 weeks of feeding, and it declined to almost one-third at the end of the feeding trial. This leads us to suggest that probably the uptake of pigments from the whole yeast was less in the early period unto 6 weeks than in the later period. This was in contrast to that observed in the synthetic astaxanthin fed fish.

The test yeast supported better growth of the fish than the diet containing commercial pigment as seen from the higher growth rates. This is not surprising since yeasts have been found to be a good economical source of nutrients as they grow rapidly. Candida lipolytica and C. utilis have been used to feed rainbow trout (Matty and Smith, 1978) and bivalve molluscs (Epifanio, 1979), respectively, resulting in good growth of the animals.

The test yeast fed fish showed an increase in the protein amount at the end of the feeding trial while the synthetic astaxanthin fed fish demonstrated a decline in the protein amount. The level of almost all the amino-acids was found to be higher in the test yeast fed with fish than that of the synthetic astaxanthin fed fish.

CHAPTER 9

General discussion and conclusions

The finding of a sexual stage is the first report in Rhodotorula rubra. However, the exact phylogeny of the isolate remains unclear. A weakly positive DBB reaction would indicate its ascomycetous affiliation. More evidence, however, needs to be collected in favour of this. In the light of a laminar cell wall structure and a positive urease reaction, its affinity with Basidiomycetes is possible. The resemblance of the spores of the isolate R. rubra TP 1 with the teliospores of Rhodosporidium toruloides would indicate it is related to the genus Rhodosporidium. As it differs from R. toruloides and other species included in the genus Rhodosporidium in many phenotypic characters, there is a good possibility that the isolate could be an undescribed species of Rhodosporidium. A number of tests as mentioned in chapter 3 could confirm this.

It is proposed that the occurrence of sexuality in this isolate is linked with enhanced pigmentation since in many carotenogenic fungi, especially members of family Choanephoraceae, the mated strains have been reported to produce more pigment than either parent alone (Hesseltine and Anderson, 1957). The occurrence of sexuality is important to the taxonomist for the better understanding of the life cycles of imperfect yeasts with no sexual stage known to date. The economic value of the isolate is also favourably affected

since the production of spores aids in the hybridization of yeasts thus resulting in improved strains. Since members of R. rubra are ubiquitous in nature and have been isolated from a wide variety of sources, the production of spores could probably aid in the adaptation and survival under adverse environmental conditions.

Another important aspect of this study was the possible use of the yeast as a source of carotenoid pigment and nutrients for salmonid fish. Comparing this study with the two previous studies, namely, Laine and Gyllenberg (1969) and Varesmaa et al. (1969), the protein and lipid amounts as well as the growth rate of the fish fed with the test yeast were higher in the present study as were the amino acid levels of the fish protein. Both the earlier studies used R. sanneii preparations. Johnson et al. (1980) fed P. rhodozyma to rainbow trout and reported that whole cells were not taken up by fish, also leading to poor growth. However, the present study clearly showed the uptake of pigments from whole yeast cells by the fish.

Commercial astaxanthin induced better pigmentation than the test yeast, as seen from the higher a*/ b* ratios and chroma. However, it is to be noted that the two pigment sources were added to the diets in different preparations. While the commercial pigment was supplied in an oil form, the test yeast was added as intact whole cells to the diet. The

total color difference between the fish flesh fed with the commercial pigment and the test yeast was more in the first 6 weeks and dropped to one-third at the end of the feeding trial. It is speculated that the uptake of pigments from the whole yeast cells is slower. Two approaches could be tried to counteract this; (a) a longer feeding trial conducted to see if the pigmentation increased with the feeding time. It was interesting to note that the differences in the a^*/b^* scores of the fish fed with the commercial pigment at 6 and 9 weeks, were statistically insignificant while the a^*/b^* scores of the fish fed with the test yeast at 6 and 9 weeks showed significant differences; (b) use of mechanically ruptured yeast cells to see their effect on pigmentation. Another important finding was the increase in the amount of protein at the end of the feeding trial in fish fed with the test yeast while those of fish fed with the commercial pigment declined.

Many significant findings have been shown by different workers, namely, (a) two main carotenoids found to be readily deposited in salmonids are astaxanthin and canthaxanthin (Torrissen et al. 1989), (b) the absorption of carotenoids is reported to be enhanced by incorporation of hydroxyl groups in carotene skeleton (Torrissen, 1986; Foss et al. 1984; Tidemann et al. 1984), (c) ß-carotene is found to be poorly absorbed in salmonids (Schiedt et al. 1985), (d) salmonids are shown to preferentially absorb 4, 4'-keto carotenoids (Torrissen et al.

1989), (e) in the astaxanthin producers, P. rhodozyma and microalgae Haematococcus pluvialis, used to feed the salmonid fish, though other carotenoids are found along with astaxanthin, the only carotenoid found to be assimilated by salmonid fish was astaxanthin (Seabright Corporation, personal communication). Therefore, since the pigments in the new isolate have been readily taken up by the fish, this suggests that the unidentified pigments could be related to astaxanthin or canthaxanthin.

As opposed to carotenogenesis in the stationary phase as shown by the members of Rhodotorula, a growth related pigmentation in the exponential phase of the growth curve was shown by the new isolate. This trend was similar to P. rhodozyma. Hence it is proposed that carotenoids play a more vital and important role in the above two yeasts, like the primary products which are vital for the organism's existence. The cell wall of the new isolate was resistant to the action of funcelase enzyme, contrary to P. rhodozyma, but was more amenable to mechanical rupture by French press.

The cell physiology of the new isolate was also found to be unique. The analysis of fatty acids revealed that about 51% of the fatty acids were of uncertain nature and 21% of these had short carbon chains (carbon number less than 14). Among the known fatty acids, the highest amount was contributed by 14:0 fatty acids (Lu, 1993). However, the major fatty acids of

Rhodotorula are found to be 18:1, 16:0, 18:0, 18:2, 18:3 and 16:1, in that order (Ratledge, 1981). Hence the fatty acid profile of the new isolate is definitely different from other Rhodotorula species.

Considering the fact that the economic factors play an important role in the commercialization of any process, the yeast isolate, R. rubra TP 1, was found to grow satisfactorily on numerous inexpensive raw materials. The isolate grew on cane and beet molasses supplemented with wort, peat hydrolysate and sulfite waste liquor though the growth on the last medium was not very good. Many inorganic nitrogen sources were utilized for growth, the best being ammonium sulfate as well as ammonium hydroxide, and growth occurred in a wide pH range of 3 to 10. The growth on peat hydrolysates could be further exploited considering the vast reserves of peat in Newfoundland. Interestingly, pigmented yeasts like Sporobolomyces pararoseus T and Rhodotorula glutinis T-2, have been grown satisfactorily on the peat hydrolysates and high levels of carotene production have been attained (Raitsina and Evdokimova, 1977). Also, P. rhodozyma has been grown successfully on this substrate (Martin et al. 1992) as well as the edible mushroom Pleurotus ostreatus (Martin and Manu-Tawiah, 1989b) and the fungus Scytalidium acidophilum (Martin and White, 1986).

The growth characteristics of the new isolate were

compared with P. rhodozyma and R. rubra TP 1 was found to have many advantages over P. rhodozyma, namely, rapid growth resulting in higher biomass yields, growth on common laboratory media due to simple nutritional requirements, ability to grow on many less expensive raw materials, easily breakable cells thus facilitating the pigment extractability and finally, feeding trials on rainbow trout showing its ability to pigment the fish when fed as intact cells.

The morphological and biochemical characters were studied in one of the mutants of the new isolate, obtained by mutagenesis with nitrosoguanidine and the mutant was found to show a higher pigmentation on D-ribose, maltose, ribitol and trehalose while it lost the ability to assimilate erythritol. The isolate was also found to be less resistant to the lethality of NTG.

Finally, using molasses and wort as a substrate, the fermenter grown culture was compared with that of the shake flask and the former showed a higher specific growth rate and productivity. The generation time of the fermenter grown culture was less and a lower cultivation time was needed to harvest an equivalent biomass. The maintenance coefficient of the fermenter grown culture was found to be low. Table 9.1 shows the maintenance energy requirements of different organisms. As reported by Abbott and Clamen (1973), organisms with low maintenance coefficients are very important from the

economic viewpoint. Zamenhof and Eichhorn (1967) also suggested that low maintenance energy requirement represented a competitive advantage to the particular cell type. They demonstrated that auxotrophic strains of Bacillus subtilis outgrow the parental wild type strains when grown in continuous cultures that were limited for energy source. The workers proposed that in these strains, the redundant synthesis of metabolites or macromolecules was prevented and so these were able to outgrow the parental cells.

Final conclusions drawn from this study are:

1. The discovery of a perfect form of the isolate could affect the technological potential through hybridization. The study is also important from the taxonomy point of view.
2. In many ways, the isolate was found to be very different from the genus Rhodotorula as well as other carotenogenic yeasts. Its more detailed study could aid in basic research.
3. As the feeding trials on rainbow trout using this isolate as a source of pigment have shown the uptake of pigment by the fish, the isolate shows promise in aquaculture.
4. Since the isolate grows on many inexpensive raw materials, SCP production using these substrates could be contemplated.

Table 9.1. The maintenance energy requirements for different organisms.

Organism	Limiting factor	Growth conditions	m (g substrate/ g cells/h)
Klebsiella aerogenes	glucose	aerobic	0.042
	glucose	anaerobic	0.50
	tryptophan	anaerobic	3.69
Saccharomyces cerevisiae	glucose	anaerobic	0.036
Azotobacter vinelandii	nitrogen	nitrogen-fixing high oxygen	1.5
	nitrogen	nitrogen-fixing low oxygen	0.15
R rubra TP 1	sugars	aerobic	0.004

Harrison (1976); Pirt (1975); Hari (present study)

REFERENCES

Abbott BJ, Laskin AI and McCoy CJ (1974). Effect of growth rate and nutrient limitation on the composition and biomass yield of Acinetobacter calcoaceticus. Appl. Microbiol. **28(1)**: 58.

Abbott BJ (1973). Ethanol inhibition of a bacterium (Acinetobacter calcoaceticus) in chemostat culture. J. Gen. Microbiol. **75**: 383.

Abbott BJ and Clamen A (1973). The relationship of substrate, growth rate and maintenance coefficient to single cell protein production. Biotech. BioEngg. **XV**, 117.

Abou-Zeid A and Baeshin N (1992). Utilization of date-seed lipid and hydrolysate in the fermentative formation of oxytetracycline by Streptomyces rimosus. Bioresource Technology. **41**: 41.

Abou-Zeid A, Baeshin N and Baghlaf A (1991). The formation of oxytetracycline in a date-coat medium. Bioresource Technology., **37**, 179.

Abou-Zeid A and Yousef A (1972). Fermentative production of mitomycins by Streptomyces caespitosus. Appl. Chem. Biotechnol., **22**, 933.

Aligedi KH, Bechkov M and Roshkova Z (1977). Nauchni Tr. Vissch, Inst. Khranit., Vkusova Prum-St Plovdiv. **24(1)**: 275.

Altman LJ, Ash L, Kowerski RC, Epstein WW, Larson BR, Rilling HC, Muscio F and Gregonis DE (1972). Prephytoene pyrophosphate, a new intermediate in the biosynthesis of carotenoids. J. Amer. Chem. Soc. **94**: 3257.

Anderson RA (1979). Production of food yeast from spent sulfite liquor. Pulp & Paper Canada. **80(4)**: 43.

Anderson DW, Tang C-S and Ross E (1991). The xanthophylls of Spirulina and their effect on egg yolk pigmentation. Poultry Science. **70**, 115.

Andrewes AG, Phaff HJ & Starr MP (1976). Carotenoids of Phaffia rhodozyma, a red pigmented fermenting yeast. Phytochemistry. **15**: 1003.

Association of Official Analytical Chemists. (AOAC) (1970). Official methods of analysis, 11th ed, Washington, D.C. pp.1015.

Bae M, Lee TH, Yokoyama H, Boetzer HG & Chichester CO (1971). The occurrence of plectaniaxanthin in Cryptococcus laurentii. Phytochem. **10**: 625.

Banno I (1967). Studies on the sexuality of Rhodotorula. J. Gen. Appl. Microbiol. Tokyo **13**: 167.

Bellamy LJ (1975). In: The infra-red spectra of complex molecules. III edition. Chapman & Hall: London.

Bendich A (1989a). Carotenoids and the immune response. J. Nutr. **119**, 112.

Bendich A (1989b). Symposium conclusions: biological actions of carotenoids. J. Nutr. **119**, 135.

Blanch HW and Einsele A (1973). The kinetics of yeast growth on pure hydrocarbons. Biotechnol. BioEngg. **15**: 861.

Bligh EG and Dyer W (1959). A rapid method of total lipid extraction and purification. Can J. Biochem. Physiol. **37 (8)**: 911.

Bobkova TS (1965a). The effect of light, aeration and temperature on the synthesis of carotenoids in some yeasts and Mycobacteria. Prikl. Biokhim. Microbiol. **1**: 234.

Bobkova TS (1965b). Carotenoid pigments of Mycobacteria and yeasts. Mikrobiologiya (English translation). **34**: 229.

Bolton PJ, Rodgers PJ and Dean ACR (1972). Molar growth yields and acid phosphatase synthesis in Klebsiella (Aerobacter) aerogenes growing in carbon-limited chemostat culture. J. Appl. Chem. & Biotechnol. **22**: 941.

Bonelo G, Megias M, Ventosa A, Nieto JJ and Ruiz-Berraquero F (1984). Lethality and mutagenicity in Halobacterium mediterranei caused by N-Methyl-N^1-nitro-N-nitrosoguanidine. Current Microbiol. **11**: 165.

Bonner J, Sandoval A, Tang YW and Zechmeister L (1946). Changes in polyene synthesis induced by mutation in a red yeast (Rhodotorula rubra). Arch. Biochem. Biophys. **10**: 113.

Boze H, Moulin G and Galzy P (1987). Influence of culture condition on the cell yield and amylases biosynthesis in continuous culture by Schwanniomyces castellii. Arch. Microbiol. **1548**: 162.

Braun R, Meyrath J, Stuparek W and Zerlauth G (1979). Feed yeast production from citric acid waste. Process Biochem, **14(1)**: 16.

Britton G (1983). In: The biochemistry of natural pigments, Cambridge University Press, Cambridge: London and New York.

Britton G (1979). Carotenoids and polyterpenoids. In: Specialist Periodical Reports: Terpenoids and steroids, (JR Hanson, ed.), The Chemical Society: London, pp. 218.

Burton GW (1989). Antioxidant action of carotenoids. J. Nutr. **119**: 109.

Camara B, Bardat F and Moneger R (1982). Nature du premier precurseur en C_{40} des carotenoids dans les chromoplasts de fruit de Poivron (Capsicum annuum L.). C.R. Acad. Sci. **294**, Serie III, 549.

Camara B, Payan C, Escoffier A and Moneger R (1980). Les carotenes du fruit de Poivron (Capsicum annuum): mise en evidence du Phytoene trans et du phytofluene trans: comparaison de la biosynthese des differents carotenes. C.R. Acad. **291**, serie D, 303.

Chang KW (1990). Stimulation of carotenoid synthesis in Phaffia rhodozyma by metal ions and superoxide radicals. M.S. thesis, University of Wisconsin, Madison.

Cho CY (1992). Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. Aquaculture **100**: 107.

Choubert G (1979). Tentative utilization of Spirulina algae as a source of carotenoid pigments for rainbow trout. Aquaculture. **18**: 135.

Chu FS and Lilly VG (1960). Factors affecting the production of carotene by Choanephora cucurbitarum. Mycologia **52**: 80.

Ciegler A (1965). Microbial carotenogenesis. Adv. Appl. Microbiol. **7**: 1.

Crueger W & Crueger A (eds) (1989). Strain development. In: Biotechnology, a textbook of industrial microbiology, Sinauer Associates Inc: Sunderland, MA, pp. 9.

Curto R, Tripodo M, Leuzzi U, Giuffre D and Vaccarino C (1992). Flavonoids recovery and SCP production from orange peel. Bioresource Technology. **42**: 83.

Davenport RR (1981). In: Smith's introduction to industrial mycology, VII edition. (AHS Onions, D Allsopp & HOW Eggins, eds), John Wiley & Sons: New York, pp. 65.

Davies BH (1976). Carotenoids. In: Chemistry and biochemistry of plant pigments (Goodwin, TW, ed.), Academic Press Inc: New York, pp.38.

Dawes CJ (ed) (1971). Fixation. In: Biological techniques in electron microscopy. Barnes and Noble: New York, pp. 17.

Dubois M, Gilles K, Hamilton J, Rebers P and Smith F (1956). Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28**: 350.

Eijk GW Van and Roeymans HJ (1982). Distribution of carotenoids and sterols in relation to the taxonomy of Taphrina and Protomyces. Antonie van Leeuwenhoek. J. Microbiol. **48 (82)**: 257.

El-Abyad M, Hamissa F and Gad A (1992). Treatment of beet molasses for citric acid production by a potent strain of Aspergillus niger van Tieghem. Bioresource Technology. **39**: 191.

Elinov NP, Anan'eva EP and Pronina MI (1988). Polysaccharides formed by Rhodotorula rubra mutants induced by N-nitroso-N-methylurea. Mikrobiologiya **57(5)**: 751.

Elinov NP, Vitovskaya GA, Kovalenko AG, Botselko SK, Maryukhta Yu B and Marikhin VA (1980). A study of mannan and its fractions from Rhodotorula rubra. Prikladnaya Biokhimiya i Mikrobiologiya **16(2)**: 249.

Englert G (1991). NMR of carotenoids: Novel experimental techniques. Pure & Appl. Chem. **63(1)**: 59.

Epifanio CE (1979). Composition of yeast and algal diets for bivalve molluscs. Aquaculture. **16**: 187.

Ercoli E and Ertola R (1983). SCP from olive black water. Biotechnol. Lett. **5**: 457.

Estevez R and Almazan O (1973). Influence of concentration of sugars on production of Torula yeasts. Rev. Sobre los Derivados de la Cana de Azucar (Cuba), **7(3)**: 60.

Evans CT & Conrad D (1987). An improved method for protoplast formation and its application in the fusion of Rhodotorula rubra with Saccharomyces cerevisiae. Arch. Microbiol. **148**: 77.

Faust U and Prave P (1983). Biomass from methane and methanol. In: Biotechnology, Vol.3, (HJ Reum and G Reed, eds), Verlag Chemie: Weinheim, pp. 84.

Federici F (1982). A note on milk clotting ability in the yeast genera Cryptococcus and Rhodotorula. J. Appl. Bacteriol. **52**: 293.

Fell JW (1970). Yeasts with heterobasidiomycetous life cycles. In: Recent trends in yeast research, (DG Ahearn, ed), School of Arts and Sciences, Georgia State University: Atlanta, Georgia, pp. 49.

Fell JW, Statzell AC, Hunter IL and Phaff HJ (1969). Leucosporidium gen. nov., the heterobasidiomycetous stage of several yeasts of the genus Candida. Antonie Van Leeuwenhoek **35**: 433.

Ferenczy, LKF and Zsolt J (1974). Fusion of fungal protoplasts. Nature (London), **248**: 793.

Fitt PS, Sharma N and Barua N (1989). Studies on the effects of liquid holding on viability and mutation frequency in N-Methyl-N^1-nitro-N-nitrosoguanidine-treated halophiles. Current Microbiol. **18**: 87.

Flavell RB and Fincham JRS (1968). Acetate-nonutilizing mutants of Neurospora crassa. J. Bacteriol. **95(3)**: 1056.

Forage AJ and Righelato RC (1979). Biomass from carbohydrate. In: Economic Microbiology (AH Rose, editor), Vol. 4, Academic Press: London, pp. 289.

Foss P, Storebakken T, Schiedt K, Liaaen-Jensen S, Austreng E and Streift K (1984). Carotenoids in diets for salmonids. I. Pigmentation of rainbow trout with the individual optical isomers of astaxanthin in comparison with canthaxanthin. Aquaculture. **41**: 213.

Fowell RR (1952). Sodium acetate agar as a sporulation medium for yeasts. Nature, **170**: 578.

Fowell RR (1969). Sporulation and hybridization of yeasts. In: The Yeasts, Vol. 1, Biology of yeasts, (AH Rose & JS Harrison, eds), Academic Press: London & New York, pp.303.

Fracis FJ (1989). Food colorants: Anthocyanins. Crit. Reviews Food Sci. & Nutr. **28**, 273.

Fromageot C and Tchang JL (1938). Sur les pigments de Rhodotorula sanniei. Arch Mikrobiol. **9**: 424.

Fuchsman C (1980). Composition of peat hydrolysates intended for yeast production. In: Industrial Chemistry and Technology, Academic Press, New York, London, Toronto, Sydney & San Francisco, pp. 104.

Fujii T, Ogawa T and Fukuda H (1987). Isobutene production by Rhodotorula minuta. Appl. Microbiol. Biotechnol. **25**: 430.

Gentles AS and Haard NF (1991). Pigmentation of rainbow trout with enzyme treated Phaffia rhodozyma. Progressive Fish Culturist. **53**: 1.

Golubev VI, Manukyan AR, Tsiomenko AB and Ushakov VM (1980). Production of yeast capsular material. Prikladnaya Biokhimiya Mikrobiologiya. **16(4)**: 591.

Gomes EF, Corraze G and Kaushik S (1993). Effects of dietary incorporation of a co-extruded plant protein (rapeseed and peas) on growth, nutrient utilization and muscle fatty acid composition of rainbow trout (Oncorhynchus mykiss). Aquaculture **113**: 339.

Goodwin TW and Britton G (1988). Distribution and analysis of carotenoids. In: Plant pigments (TW Goodwin, ed), Academic Press: London, New York and San Diego, pp. 61.

Goodwin TW (1972). Carotenoids in fungi and non-photosynthetic bacteria. Prog. Ind. Microbiol. Vol. 11, (DJD Hockenhull, ed.), Churchill Livingstone: Edinburgh, pp. 29.

Goodwin TW (1959). The biosynthesis and functions of the carotenoid pigments. Adv. Enzymol. **21**: 295.

Goodwin TW (ed.) (1952). In: Comparative bio-chemistry of the carotenoids. Chapman & Hall, London, pp. 356.

Goodwin TW (1972). Prog. In: Ind. Microbiol (DJD Hockenhull, ed), Vol. 2, Churchill Livingstone, Edinburgh, pp. 29.

Gordon HT (1982). Carotenoids as food colorants. CRC Critical Rev. Food Sci. **18(1)**: 59.

Guiraud JP and Galzy P (1990). Inulin conversion by yeast. In: Yeast biotechnology and biocatalysis, (H Verarchtert and R de Mot, eds). Marcel Dekker: New York, pp. 255.

Guiraud JP (1981). Utilisation des Levures pour la valorisation Industrielle des Polyfructosanes de Type Inuline. These USTL, Montpellier.

Guiraud JP, Devouge C and Galzy P (1979). Selection of yeast with a view to production of proteins on inulin. Biotechnol. Lett. **1**: 461.

Haard NF (1988). Astaxanthin formation by the yeast Phaffia rhodozyma on molasses. Biotech Lett. **10(9)**: 609.

Hagler AN and Ahearn DG (1981). Rapid diazonium blue B test to detect basidiomycetous yeasts. Int. J. Syst. Bacteriol. **31**: 204.

Hamamoto M, Sugiyama J and Komagata K (1987). DNA-DNA reassociation studies of strains in the genera Rhodosporidium and Rhodotorula. J. Gen. appl. Microbiol. **33**: 57.

Hamamoto M, Sugiyama J and Komagata K (1986). DNA base composition of strains in the genera Rhodosporidium, Cystofilobasidium and Rhodotorula determined by reversed-phase high-performance liquid chromatography. J. Gen Appl. Microbiol. **32**: 215.

Hang YD. (1980). Assimilation of lemonade-processing wastewater by yeasts. Appl. Environ. Microbiol. **39**: 470.

Hari RPK, Patel T and Martin AM (1993). Growth kinetics of Rhodotorula rubra on different substrates. J. Basic Microbiol. **33(6)**: 379.

Hari R, Patel T and Martin A (1992). A new strain of Rhodotorula rubra isolated from yogurt. J. Ind. Microbiol. **11**: 43.

Harrison DEF (1976). The requirement of respiration rate in growing bacteria. Adv. Microbiol. Physiol. **14**: 243.

Harrison FC (1928). A systematic study of some Torulas. Trans. R. Soc. Can. Sect. V, **22**: 187.

Hayashi S, Matsuzaki K, Kawahara T, Takasaki Y and Imada K (1992). Utilisation of soybean residue for the production of ß-fructofuranosidase. Bioresource Technology. **41**: 231.

Hayman EP, Chichester CO & Simpson KL (1974). Effects of CPTA upon carotenogenesis and lipoidal constituents in Rhodotorula species. Phytochem. **13**: 1123.

Hesseltine CW and Anderson RF (1957). Microbiological production of carotenoids. I. Zygospores and carotene produced by intraspecific and interspecific crosses of Choanephoraceae in liquid media. Mycologia **49**: 449.

Hopwood DA, Wright HM, Bibb MJ & Cohen SN (1977). Genetic recombination through protoplast fusion in Streptomycetes. Nature (London), **268**: 171.

Hours R and Garzon C (1992). Citrus waste: An alternative substrate for pectinase production in solid-state culture. Bioresource Technology. **39**: 93.

Hunter RS (ed.) (1975). Uniform color scales. In: The measurement of appearance. John Wiley & Sons: New York, London, Sydney & Toronto, pp. 102.

Hynes MJ, and Kelly JM (1977). Pleiotropic mutants of Aspergillus nidulans altered in carbon metabolism. Mol. Gen. Genet. **150**: 193.

Imrie F (1969). Fermentation media: sugar and molasses. In: Proc. Biochem. January, 34.

Ingram M (1955). In: An introduction to the biology of yeasts. Pitman: London, pp.273.

Jarl K (1969). Symba Yeast Process. Food Technol. **23**: 23.

Jeffrey SW (1974). Profiles of photosynthetic pigments in the ocean using thin-layer chromatography. Marine Biology. **26**: 101.

Johnson EA and An G.-H (1991). Astaxanthin from microbial sources. CRC Crit. Rev. Biotech. **11(4)**: 297.

Johnson EA, An G.-H and Schuman DB (1989). Isolation of Phaffia rhodozyma mutants with increased astaxanthin content. Appl. Environ. Microbiol. **55**: 116.

Johnson E, Villa T and Lewis M (1980). Phaffia rhodozyma as an astaxanthin source in salmonid diets. Aquaculture. **20**: 123.

Johnson EA and Lewis MJ (1979). Astaxanthin formation by the yeast Phaffia rhodozyma. J. Gen. Microbiol. **115**: 173.

Johnson EA, Villa TG, Lewis MJ and Phaff HJ (1978). A simple method for the isolation of astaxanthin from the basidiomycetous yeast Phaffia rhodozyma. Applied and environmental Microbiol. **35(6)**: 1155.

Johnson EA, Conklin DE and Lewis MJ (1977). The yeast Phaffia rhodozyma as a dietary pigment source for salmonids and crustaceans. J. Fish. Res. Board Can. **34**: 2417.

Karlstrom O (1968). Mutants of Escherichia coli defective in ribonucleoside and deoxyribonucleoside catabolism. J. Bacteriol. **95(3)**: 1069.

Karnovsky MJ (1965). A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. cell biol. **27**: 137.

Kawabata Y, Uchida H, Kusakabe I, Ono T and Murakami K (1992). Production of xylose from xylan with intracellular enzyme system of Aspergillus niger 5-16. J. Ferment. & BioEngg. **74**: 153.

Komagata K, Goto S, Sugiyama J and Hamamoto M (1987). Saitoella, a new anamorph genus in the Cryptococcaceae to accomodate two Himalayan yeast isolates formerly identified as Rhodotorula glutinis. J. Gen. Appl. Microbiol. **33**, 75.

Kosonogova L and Evdokimova G (1977). In: The Production of Feed Yeast from Peat (V Rakovskii, ed.), Nauka i Tekhnika Minsk, pp. 173.

Kotik LV, Tolokonnikov GY and Dubrovin N (1979). The effect of krill meal additions to feeds on muscle pigmentation in the rainbow trout (Salmo gairdneri). J. Ichthyol. **19**: 119.

Kreger-Van Rij NJW (ed) (1984). General classification of the yeasts. In: The yeasts, a taxonomic study, III edition, Elsevier Science Publishers BV: Amsterdam, pp.1.

Kreger-van Rij NJW and Veenhuis M (1971). A comparative study of the cell wall structure of basidiomycetous and related yeasts. J. Gen. Microbiol. **68**: 87.

Kreger-van Rij NJW (1969). The taxonomy and systematics of yeasts. In: The yeasts. Vol.1, Biology of yeasts (AH Rose and JJ Harrison, eds). Academic Press: London and New York.

Krinsky NI and Deneke SM (1982). Interaction of oxygen and oxy-radicals with carotenoids. J. Natl. Cancer Inst. **69**: 205.

Krinsky, NI (1979). Carotenoid protection against oxidation. Pure & Appl. Chem. **51**: 649.

Kuhad RC and Singh A (1993). Lignocellulosic biotechnology: current and future prospects. CRC Crit. Rev. Biotech. **13**: 151.

Kuo HC, Lee TC, Kamata T and Simpson KL (1976). Red crab processing waste as a carotenoid source for rainbow trout. Alimenta. **15**: 47.

Kurth EF (1946). Yeasts from wood sugar stillage. Ind. Eng. Chem. **38(2)**: 204.

Kurth EF and Cheldelin VH (1946). Feeding yeasts from wood sugar stillage. Ind. Eng. Chem. **38**: 617.

Laine JJ and Gyllenberg HG (1969). Feeding of rainbow trouts with Rhodotorula sanneii preparations. I. General outline and principal results. Lebensmittel-Weissenschaft Und technologie. **2(2)**: 48.

Lambertsen G and Braekken OR (1971). Method of analysis of astaxanthin and its occurrence in some marine products. J. Sci. Food Agric. **22**: 99.

Lee HC, Chin C and Hwang W (1991). Isolation and characterization of mutants of Clostridium saccharoperbutylacetonicum fermenting bagasse hydrolysate. J. Ferment. & BioEngg. **72(4)**: 249.

Lee RG, Neamtu G.Gh, Lee TC and Simpson KL (1978). Pigmentation of rainbow trout with extracts of floral parts from Tagetes erecta and Curcubita maxima marcia. Rev. Roum. Biochem. **15 (4)**: 287.

Leenheer AP De and Nelis HJ (1991). Microbial sources of carotenoid pigments used in foods and feeds (A review). J. Appl. Bacteriol. **70**: 181.

Levine SA (1977). Isolation and characterization of catalase deficient mutants of Salmonella typhimurium. Mol. Gen. Genet. **150**: 205.

Lewis MJ, Ragot N, Berlant MC and Miranda M (1990). Selection of astaxanthin overproducing mutants of Phaffia rhodozyma with ß-ionone. Appl. Environ. Microbiol. **56(9)**: 2944.

Liaaen-Jensen S and Andrewes AG (1985). Analysis of carotenoids and related polyene pigments. Methods in Microbiol. **18**: 235.

Litchfield JH (1977). Comparative technical and economic aspects of single cell protein processes. Adv. Appl. Microbiol. **22**: 267.

Liu IS, Lee TH, Yokoyama H, Simpson, KL & Chichester CO (1973). Isolation and identification of 2-hydroxy plectaniaxanthin from Rhodotorula aurantiaca. Phytochem. **12**: 2953.

Lodder J and Kreger-van Rij NJW (1954). Classification and identification of yeasts. Lab. Prac. **3(12)**: 483.

Lodder J (1934). Die anaskosporogenen Hefen, I. Half te, Verh. Kon. Ned, Acad. Wet, Afd. Natuurk, Sect 11 **32**: 1.

Lowry O, Rosebrough N, Farr A and Randall R (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265.

Lu C (1993). Study of the growth parameters of the yeast Rhodotorula rubra and its carotenoid production. M.Sc. Thesis, Memorial University of Newfoundland, St. John's, Canada.

Mancilha I, Roberto C, Felipe G, Lacis S and Silva S (1991). Utilization of sugarcane bagasse hemicellulosic hydrolysate by Candida guilliermondii for xylitol production. Bioresource Technology. **36**: 271.

Marchant R and Smith DG (1968). Bud formation in Saccharomyces cereviseae and a comparison with the mechanism of cell division in other yeasts. J. Gen. Microbiol. **53**: 163.

Martin AM, Goddard S and Bemister P (1993a). Production of Candida utilis biomass as aquaculture feed. J. Sci. Food Agric. **61**: 363.

Martin AM, Acheampong E and Patel TR (1993b). Production of astaxanthin by Phaffia rhodozyma using peat hydrolysates as substrate. J. Chem. Tech. Biotechnol. **58**: 223.

Martin AM, Acheampong E, Patel TR and Chornet E (1992). Study of growth parameters for Phaffia rhodozyma cultivated in peat hydrolysates. Appl. Biochem. & Biotechnol. **37**: 235.

Martin AM, Chintalapati SP and Hoover R (1990). Assessment of acid hydrolysates of peat as substrate for Scytalidium acidophilum ATTC 26774 fungus. J. Chem. Tech. Biotechnol. **49**: 3.

Martin M and Manu-Tawiah W (1989a). Study on the acid hydrolysis of peat: composition of the extracts from Sphagnum peat. J. Chem. Tech. Biotechnol. **45**: 171.

Martin AM and Manu-Tawiah W (1989b). Peat extract as a carbon source for the growth of Pleurotus ostreatus mycelium. J. Sci. Food. Agric. **47**: 243.

Martin AM and White MD (1986). Studies on the production of Scytalidium acidophilum biomass. Appl. Microbiol. Biotechnol. **24**: 84.

Marzolf JJ (1977). Point sur la situation actuelle de la production de levures cultivees sur lactoserum. In: Colloque: Les Lactoserums, une richesse alimentaire, APRIA, Paris, 73.

Mathur S, Mathur A, Barnie S and Kulshreshtha R (1993). Fungal biomass production on waste leather chharri and possible utilisation for feed supplement. Bioresource Technol. **43**: 95.

Matty AJ and Smith P (1978). Evaluation of a yeast, a bacterium and an alga as a protein source for rainbow trout. I. Effect of protein level on growth, gross conversion efficiency and protein conversion efficiency. Aquaculture. **14**: 235.

Maxwell WA, Mcmillan JD and Chichester CO (1966). Function of carotenoids in protection of Rhodotorula glutinis against irradiation from a gas laser. Photochem. Photobiol. **5**: 567.

Mazia D, Schatten G & Sale W (1975). Adhesion of cells to surfaces coated with poly-lysine. J. Cell Biol **66**, 198.

Merlin JC. (1985). Resonance Raman spectroscopy of carotenoids and carotenoid containing systems. Pure Appl. Chem. **57(5)**: 785.

Meyers S (1977). Using crustacean meals and carotenoid fortified diets. Feedstuffs. May 9: 26.

Miller MW, Yoneyama M and Soneda M (1976). Phaffia, a new yeast in the Deuteromycotena (Blastomycetes). Int. J. Systematic bacteriol. **26** (2): 286.

Moon R (1989). Comparative aspects of carotenoids and retinoids as chemopreventive agents for cancer. J. Nutr. **119**, 127.

Moreira A, de Menezes TJB and Avakaki T (1976). Col. Inst. Technol. Alim. (Campinas) **7(1)**: 97.

Morre MM, Breedveld MW and Autor AP (1989). The role of carotenoids in preventing oxidative damage in the pigmented yeast, Rhodotorula mucilaginosa. Arch. Biochem. Biophys. **270**: 419.

Mortimer RK & Hawthorne DC (1969). Yeast genetics. In: The yeasts, Vol. 1, Biology of yeasts, (AH Rose & JS Harrison, eds), Academic Press: London & New York, pp. 386.

Moss GP and Weedon BCL (1976), Chemistry of the carotenoids. In: Chemistry and biochemistry of plant pigments, (TW Goodwin, ed), Academic Press: London, New York and San Francisco, pp.149.

Murad H and Foda M (1992). Production of yeast polygalacturonase on dairy wastes. Bioresource Technology. **41**: 247.

Murado M, Siso I, Gonzalez P, Montemayor I, Pastrana L and Pintado J (1993). Characterization of microbial biomass and amylolytic preparations obtained from mussel processing waste treatment. Bioresource Technology. **43**: 117.

Nakase T and Komagata K (1971). Significance of DNA base composition in the classification of yeast genera Cryptococcus and Rhodotorula. J. Gen. Appl. Microbiol. **17**: 121.

Nakayama T, Mackinney G and Phaff HJ (1954). Carotenoids in asporogenous yeasts. Antonie van Leeuwenhoek **20**: 217.

Nand K, Joseph R and Ramachandra Rao TN (1973). Use of griseofulvin for the isolation of auxotrophic mutants of Rhodotorula sp. Experientia **29/2**: 237.

Neale S (1976). Mutagenicity of nitrosoamides and nitrosamines in micro-organisms and plants. Mutat. Res. **32**: 229.

Needham AE (1974a). An Introduction. In: The significance of zoochromes: zoophysiology and ecology, Springer-Verlag: New York and San Francisco, pp. 3.

Needham AE (1974b). The basis and significance of color in molecules. In: The significance of zoochromes: zoophysiology and ecology. Springer-Verlag: New York and San Francisco, pp. 5.

Newell SY and Fell JW (1970). The perfect form of marine occurring yeast of the genus Rhodotorula. Mycology **62**: 272.

Newell SY and Hunter IL (1970). Rhodosporidium diobovatum, sp.H., the perfect form of an asporogenous yeast (Rhodotorula sp.) J. Bacteriol. Oct. 503.

Nieto JJ, Fernandez-Castillo R, Megias M and Ruiz-Berraquero F (1992). Ethyl methanesulfonate mutagenesis in extremely halophilic Archaebacteria: Isolation of auxotrophic mutants of Haloferax mediterranei and Haloferax gibbonsii. Current Microbiol. **24**: 41.

Nikolaev PI, Paleeva MA, Pomortseva NV, Samokhvalov GI, Vakulova LA and Lopatik MD (1966). Izobret. Prom. Obraztsy Tovarnye Znaki **43**: 47.

Nishio N and Nagai S (1981). Single cell protein production from mandarin orange peel. Eur. J. Appl. Microbiol. Biotechnol. **11**: 156.

Ogrydziak DM (1993). Yeast extracellular proteases. Crit. Rev. Biotech. **13(1)**: 1.

Oguntimein G, Vlach D and Moo-Young M (1992). Production of cellulolytic enzymes by Neurospora sitophila grown on cellulosic materials. Bioresource Technology. **39**: 277.

Okagbue RN and Lewis MJ (1985). Influence of mixed culture conditions on yeast-wall hydrolytic activity of Bacillus circulans WL-12 and on the extractability of astaxanthin from the yeast Phaffia rhodozyma. J. Appl. Bacteriol. **59**: 243.

Okagbue R and Lewis M (1984a). Use of alfalfa residual juice as a substrate for propagation of the red yeast Phaffia rhodozyma. Appl. Microbiol. & Biotechnol. **20**: 33.

Okagbue RN and Lewis MJ (1984b). Autolysis of the red yeast Phaffia rhodozyma: a potential tool to facilitate extraction of astaxanthin. Biotech Lett. **6(4)**: 247.

Ostrander J, Marhssen C, Laston J & McCurrough J (1976). Sensory testing of pen-reared salmon and trout. J. Food Sci. **41**: 386.

Owen NE, Chaure PT and Connerton IF (1992). Isolation and characterization of new fluoroacetate resistant/acetate non- utilizing mutants of Neurospora crassa. J. Gen. Microbiol. **138**: 2599.

Parades-Lopez O, Camargo-Rubio E and Ornelas-Vale A (1976). Influence of specific growth rate on biomass yield, productivity and composition of Candida utilis in batch and continuous culture. Appl. Environ. Microbiol. **31(4)**: 487.

Peppler HJ (1979). Production of yeasts and yeast products. In: Microbial Technology, Vol. 1, 2nd. edition, (HJ Peppler & D Perlman, eds.), Academic Press, New York, Chap.5.

Phaff HJ, Miller MW and Mrak EM (1978). In: The life of yeasts, II edition. Harvard Univ. Press: Cambridge, pp. 19.

Phaff HJ (1977). Enzymatic cell wall degradation. In: Food Proteins, Advances in chemistry series (RE Feeney & JR Whitaker, eds.), American chemical society: Washington DC, pp. 244.

Phaff HJ, Mrak EM and Williams OB (1952). Yeasts isolated from shrimp. Mycologia **44**: 431.

Pirt SJ (1975). Energy and carbon source requirements. In: Principles of microbe and cell cultivation. Blackwell Scientific Publications: Oxford, pp. 63.

Prescott S and Dunn C (eds) (1959). The propagation of yeast. In: Industrial Microbiology (III edition), McGraw-Hill Book Company Inc., New York, Toronto & London, pp.60.

Qureshi AA, Barnes FJ and Porter JW (1972). Lycopersene and prelycopersene pyrophosphate intermediates in carotene biosynthesis. J. Biol. Chem. **247**: 6730.

Raitsina G and Evdokimova G (1977). In: The Production of Feed Yeast from Peat (V. Rakovskii, Editor), Nauka i Tekhnika Minsk, pp.113.

Ratledge C and Tan K-H (1990). oils and fats: production, degradation and utilization by yeast. In: Yeast biotechnology and biocatalysis, (H Verachtert and R de Mot, eds), Marcel Dekker Inc.: New York, pp.223.

Ratledge C & Evans CT (1987). Lipids and their metabolism. In: The yeasts, Vol. III, II ed., Metabolism and physiology of yeasts, (AH Rose & JS Harrison, eds), Academic Press: London, San Diego & Toronto, pp. 367.

Ratledge C (1981). Yeasts and moulds as sources of oils and fats. In: New sources of fats and oils, (EH Pryde, LH Princen and KD Mukherjee, eds.), Amer. Oil. Chem. Soc. pp. 159.

Revah-Moiseev S and Carroad A (1981). Conversion of the enzymatic hydrolysate of shellfish waste chitin to single-cell protein. Biotechnol. BioEngg. **23**: 1067.

Reynolds ES (1963). The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208.

Rhodes A and Fletcher D (1966). Nutrition of microorganisms. In: Principles of industrial microbiology. Pergaman Press: Oxford, pp. 58.

Riviere J (1977). Culture media. In: Industrial applications of Microbiology. John Wiley & Sons: New York, pp. 27.

Rodriguez-Vazquez, Villanueva-Ventura and Rios-Leal E (1992). Sugarcane bagasse pith dry pretreatment for single cell protein production. Bioresource Technology. **39**: 17.

Rogers PJ and Stewart PR (1974). Energetic efficiency and maintenance energy characteristics of Saccharomyces cerevisiae (wild type and petite) and Candida parapsilosis grown aerobically and micro-aerobically in continuous culture. Arch. Microbiol. **99**: 25.

Rolz C (1984). Microbial biomass from renewables: a second review of alternatives. In: Annual reports on fermentation processes, Vol. 7, (GT Tsao, ed.), Academic Press Inc., San Diego, Orlando, pp. 213.

Rose AH (1968) (ed.). Growth, reproduction and survival. In: Chemical Microbiology, II edition. Plenum press: New York, pp. 238.

Sadowski R & Wojcik W (1983). Chromatography of chloroplast carotenoids on magnesium oxide thin layers. J. Chromatogr. **262**: 455.

Saeman JF, Locke EG and Dicksman GK (1945). The production of wood sugar in Germany and its conversion to yeast and alcohol. FIAT Final Report 499, Nov. 14.

Salares VR, Young NM, Bernstein HJ & Carey PR (1977). Resonance Raman spectra of lobster shell carotenoproteins and a model astaxanthin aggregate. A possible photobiological function for the yellow protein. Analyt. Chem. **16**: 4751.

Sassi G, Ruggeri B, Specchia V and Gianetto A (1991). Citric acid production by A. niger with banana extract. Bioresource Technology **37**: 259.

Savolainen JET and Gyllenberg HG (1970). Feeding of rainbow trout with Rhodotorula sanneii preparations. III. Amounts and qualities of carotenoids. Lebensm. Wiss. Technol. **3(1)**: 18.

Schiedt K, Leuenberger FJ, Vecchi M and Glinz E (1985). Absorption, retention and metaboloic transformation of carotenoids in rainbow trout, salmon and chicken. Pure & Appl. Chem. **57(5)**: 685.

Schmidt PJ and Cuthbert RM (1969). Color sorting of raw salmon. Food Technol. **23**: 232.

Seabright Corporation (1994). Personal communication, Dave King, Memorial University of Newfoundland.

Selim M, Elshafei A and El-Diwany A (1991). Production of single cell protein from yeast strains grown in Egyptian vinasse. Bioresource Technology. **36**: 157.

Shailubhai K, Rao NN and Modi VV (1984). Treatment of petroleum industry oil sludge by Rhodotorula spp. Appl. Microbiol. Biotechnol. **19**: 437.

Sheehan BT and Johnson MJ (1971). Production of bacterial cells from methane. Appl. Microbiol. **21(3)**: 511.

Sikyta R (ed.) (1983a). Substrates for microbial processes. In: Methods in Industrial Microbiology. John Wiley & Sons Chichester, New York, Brisbane & Toronto.

Sikyta RB (ed.) (1983b). Kinetics of microbial processes. In: Methods in Industrial Microbiology, John Wiley & Sons : Chichester, New York, Brisbane & Toronto, pp. 176.

Simard RE and Cameron A (1974). Fermentation of spent sulfite liquor by Candida utilis. Pulp Paper Mag. Can. **75**: 107.

Simpson KL, Chichester CO and Phaff HJ (1971). Carotenoid pigments of yeasts. In: The yeasts, vol.2, Physiology and biochemistry of yeasts (AH Rose & JS Harrison, eds.), Academic Press: New York, pp. 493.

Singh H, John J and Cama HR (1973). Separation of ß-apocarotenals and related compounds by reversed phase paper and thin layer chromatography. J. Chromatogr. **75**: 146.

Sipiczki M & Ferenczy L (1977). Protoplast fusion of Schizosaccharomyces pombe auxotrophic mutants of identical mating type. Molecul. Gen. Genet. **157**: 77.

Slater ML (1976). Rapid nuclear staining method for Saccharomyces cerevisiae. J. Bacteriol. **126(3)**: 1339.

Smith KC (1978). Multiple pathways of DNA repair in bacteria and their roles in mutagenesis. Photochem & Photobiol. **28**: 121.

Spencer JFT, Spencer DM & Reynolds N (1988). Genetic manipulation of non-conventional yeasts by conventional and non-conventional methods. J. Basic. Microbiol. **28(5)**: 321.

Spinelli J and Mahnken C (1978). Carotenoid deposition in pen-reared salmonids fed diets containing oil extracts of red crab (Pleuroncodes planipes). Aquaculture. **13**: 213.

Spurr AR (1969). A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastrut. Res. **26**, 31.

Stanbury PF and Whitaker A (Eds) (1984). Media for industrial fermentations. In: Principles of fermentation technology, Pergamon Press: Oxford, New York & Toronto, pp. 74.

Stewart I and Wheaton TA (1971). Continuous flow separation of carotenoids by liquid chromatography. J. Chromatogr. **55**: 325.

Stewart WDP (1974). Carotenoids and biliproteins. In: Algal physiology and biochemistry, University of California Press: Berkeley, pp. 176.

Storck R, Alexopoulos CJ and Phaff HJ (1969). Nucleotide composition of deoxyribonucleic acid of some species of Cryptococcus, Rhodotorula and Sporobolomyces. J. Bacteriol. **98**: 1069.

Storebakken T, Foss P, Austreng E and Liaaen-Jensen S (1985). Carotenoids in diets for salmonids. II. epimerization studies with astaxanthin in Atlantic salmon. Aquaculture. **44**: 259.

Suhasukan S and Yasin B (1986). SCP production from citrus wastes by using constituted mixed fungal mycelium. Process Biochem. **21**: 50.

Tada M and Shiroishi M (1982a). Mechanism of photoregulated carotenogenesis in Rhodotorula minuta. I. Photocontrol of carotenoid production. Plant & Cell Physiol. **23(3)**: 541.

Tada M and Shiroishi M (1982b). Mechanism of photoregulated carotenogenesis in Rhodotorula minuta.II. Aspects of photoregulative reaction. Plant & Cell Physiol. **23(3)**: 549.

Tada M and Shiroishi M (1982c). Mechanism of photoregulated carotenogenesis in Rhodotorula minuta. V. Photoinduction of 3-OH-3-methyl-glutaryl coenzyme A reductase. Plant & Cell Physiol. **23(4)**: 615.

Taylor RF (1992). Personal Communication, Arthur D. Little Inc, Massachussetts.

Taylor RF, Farrow PE, Yelle LM, Harris JC & Marenchic TG (1990). Advances in HPLC and HPLC-MS of carotenoids and retinoids. In: Carotenoids: Chemistry and biology, (NI Krinsky, ed), Plenum Press: New York, pp. 105.

Thomson RH (1976). In: Chemistry and biochemistry of plant pigments, Academic Press: London, New York and San Francisco.

Thomson RH (1971). In: Naturally occurring quinones, Academic Press: London and New York.

Tidemann E, Raa J, Stormo B and Torrissen O (1984). Processing and utilization of shrimp waste. In: Engineering and Food, (BM Mckenna, ed.), Vol. 2, Elsevier, Applied Science Publishers: Amsterdam, pp. 583.

Torrissen OJ, Hardy RW and Shearer KD (1989). Pigmentation of salmonids-carotenoid deposition and metabolism. CRC Crit. Rev. Aquat. Sci. **1**: 209.

Torrissen OJ (1986). Pigmentation of salmonids - a comparison of astaxanthin and canthaxanthin as pigment sources for rainbow trout. Aquaculture. **53**: 271.

Torrissen OJ (1985). Pigmentation of salmonids: factors affecting carotenoid deposition in rainbow trout (Salomo gairdneri). Aquaculture. **46**: 133.

Tswett M (1906). Adsorptionsanalyse und chromatographische Methode. Ber. dtsch. bot. Ges. **24**: 384.

Ushio K, Ohtsuka H and Nakata Y (1991). Lipid composition of an obligate osmophilic mutant in Zygosaccharomyces rouxii. J. Ferment. & BioEngg. **72(3)**: 210.

Van der Walt JP and Yarrow D (1984). Methods for the isolation, maintenance, classification and identification of yeasts. In: The yeasts, a taxonomic study, III ed. (NJW Kreger-Van Rij, ed.). Elsevir Science Publishers B.V., Amsterdam, pp. 45.

Varesmaa E, Laine JJ and Gyllenberg HG (1969). Feeding of rainbow trouts with Rhodotorula sanneii preparations. II. Fatty acid composition of lipids and amino-acid composition of proteins. Lebensmittel wissenschaft und technologie. **2(2)**: 51.

Vaskivniuk VT and Kvasnykov EI (1968). Growth of Rhodotorula Harrison in media containing n-alkanes. Mikrobiologiya **37**: 876.

Vecher AS and Kulikova AN (1968). Changes in polyene compounds at various stages of carotenoid development of Rhodotorula gracilis. Mikrobiologiya (English translation). **37(4)**: 676.

Vecher AS, Karabava G Ya and Kulikova AN (1967). Vesti. Acad. Nauk Brlarus. SSR. Biol. Nauk **3**: 87.

Vecher AS, Gurinovich ES, Prokazov GF, Lukashik AN and Astapovich NI (1965). Izv. vyssh. Uchebn. Zavedenii, Pishch. Technol. **2**: 45.

Vetter W, Englert G, Rigassi N & Schwieter U (1971). Spectroscopic methods. In: Carotenoids, (O Isler, H Gutmann & V Solms, eds), Birkhauser: Basle, pp.189.

Villoutreix J (1960). Influence de dirers agents Chimiques sur la carotenogenese de Rhodotorula mucilaginosa. Biochim. biophys. Acta **40**: 434.

Watson ML (1958). Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. cytol **4**, 475.

Weijman ACM and Rodrigues de Miranda L (1988). Carbohydrate patterns of Candida, Crytococcus and Rhodotorula species. Antonie Van Leeuwenhoek **54**: 535.

Wickerham LJ and Burton KA (1954). A simple technique for obtaining mating types in heterothallic diploid yeasts, with special reference to their uses in the genus Hansenula. J. Bacteriol. **67**: 303.

Widdowson D and White PJ (1976). The nutritional requirements of methicillin- dependent and -resistant strains of Pediococcus cerevisiae. J. Gen. Microbiol. **92(263)**: 138.

Winge O (1935). On haplophase and diplophase in some Saccharomycetes, C. r. Trav. Lab. Carlsberg. Ser. Physiol. **21**: 77.

Wittmann H (1957). Untersuchungen uber die Vevanderung der carotinoidkomponenten von Rhodotorula rubra in Abhangigkeit von Ernahrungsbedingungen. Arch. Mikrobiol. **25**: 373.

Wright JD and Ratledge C (1991). Isolation of two Rhodotorula rubra strains showing differences in the degradation of aromatic compounds. Appl. Microbiol. Biotechnol. **35**: 94.

Wyszecki G and Stiles WS (1967). Concepts and methods, quantitative data and formulas. Color Science. John Wiley & Sons, New York, pp.628.

Yamada Y and Kondo K (1973). Coenzyme Q system in the classification of the yeast genera Rhodotorula and Cryptococcus, and the yeast like genera, Sporobolomyces and Rhodosporidium. J. Gen. Appl. Microbiol. **19**: 59.

Zabriskie DW, Armiger WB, Phillips DH and Albano PA (eds.) (1988). Fermentation media formulation. In Traders' guide to fermentation media formulation. Traders Protein, Tennessee, pp.1.

Zagalsky PF, Gilchrist BM, Clark RJH & Fairclough DP (1983). The Canthaxanthin-lipovitellin of Branchipus stagnalis (L) (Crustacea: Anostraca): A resonance Raman and circular dichroism study. Comp. Biochem. Physiol. **75B(1)**: 163.

Zamenhof S and Eichhorn HH (1967). Study of microbial evolution through lots of biosynthetic functions: establishment of 'defective' mutants. Nature (Lond.) **216**: 456.

Zhukova GM, Chikileva EA, Nikolaeva ND and Ashikmina GF (1976). Tr. Vses. Nauchno-Pruizvod Ob' edin. Bum. Prom.

Sti. Persk. Fil. 2I 83, CA 89, 4571X.

Ziegler RG (1989). A review of epidemiologic evidence that carotenoids reduce the risk of cancer. J. Nutr. **119**, 116.

Zvyagintseva IS and Pitryuk IA (1975). Lecithinase activity of several yeast species. Mikrobiologiya USSR **44**:1119.

Zvyagintseva IS (1972). Lipase activity of some yeasts. Mikrobiologiya USSR **41**: 24.