SOME ASPECTS OF THE TAXONOMY, GENETICS, CAROTENOGENESIS AND CHEMICAL COMPOSITION OF A RED YEAST *Rhodotorula rubra* TP1

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By

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ABSTRACT

A new strain of yeast was isolated from yogurt and tentatively identified as *Rhodotorula rubra* TP1. It was reported that the yeast produced structures resembling ascospores and, hence, was the first report of a sexual stage in *Rhodotorula rubra*. Preliminary studies in fish feeding trials indicate that the yeast may be an attractive candidate for use in the aquaculture industry for coloring fish muscle. To exploit the potentials of the new yeast isolate, a comprehensive study was undertaken to: 1) confirm the phylogenetic affinity of the new isolate using molecular, biochemical and physiological techniques; 2) identify and quantify the pigments produced by the yeast; 3) isolate mutants with increased pigment production; 4) examine the cell wall for industrially useful polysaccharides; 5) isolate and characterize the carotenogenic enzymes.

Studies on the sexuality of the new isolate could not confirm the production of ascospores or any structures resembling spores. To determine the exact phylogenetic relationship of the new isolate, various biochemical and physiological tests were carried out. These tests included assimilation of various carbon and nitrogen sources, isozyme electrophoresis and analysis of cellular long-chain fatty acids.

Based on the comparison of the electrophoretic mobilities (μ) of 8 isozymes in the new isolate and 8 other yeast isolates (*Rhodotorula rubra* ATCC 9449, *Saccharomyces cerevisiae*, *Phaffia rhodozyma*, *Rhodotorula glutinis*, *Rhodotorula graminis*, *Rhodotorula minuta*, *Rhodosporidium toruloides* and *Cryptoccocus*

macerans), it was concluded that the new isolate could not be distinguished from *R. rubra* and should therefore be considered a variant strain. The study also suggested that cellulose acetate electrophoresis could be an invaluable taxonomic tool for the identification of isolates of yeast.

Similarly, on the basis of the numerical analysis of the cellular long-chain fatty acid composition 9 isolates, assimilation patterns of various carbon sources, Diazonium blue (DBB), urease and nitrate tests, the new isolate should be confirmed as a variant strain of *R. rubra*.

To determine whether the phylogeny of the new isolate could be determined on the basis of other identification protocols aside from the biochemical and physiological tests, a portion of the ribosomal DNA (rDNA) and internal transcribed spacer was amplified by the polymerase chain reaction and then sequenced. Comparison of the DNA sequences of the small subunit ribosomal RNA (18S rRNA) coding regions and the internal transcribed spacer (ITS) of the new isolate with those of other yeast isolates revealed that the new yeast isolate may be a variant strain of *R. rubra*. The new isolate had 93 and 100% sequence similarity with *R. rubra* ATCC 9449 for the 18S rRNA and ITS sequences, respectively. Furthermore, the evolutionary distances estimated from the 18S rRNA genes and the ITS sequences of the two organisms were 0.015 and 0.000, respectively.

Using gas liquid chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy, it was determined that the cell wall polysaccharides of the new isolate consisted of mainly mannans with β-(1→3) and

β-(1→4) mannopyranosyl units. The major monosaccharides of the cell wall were determined to be mannose (50.53%), glucose (25.53%), galactose (12.27%), fucose (8.6%) and rhamnose (3.2%).

Characterization of pigments produced by the new isolate involved the use of column and thin layer chromatography, high performance liquid chromatography and light spectroscopy. The new isolate was found to produce β-carotene, torulene, torularhodin, phytoene and phytofluene with percentage compositions of 39.85, 30.65, 24.5, 2.2 and 3.1%, respectively.

Studies on the genetics of the new isolate included mutagenesis with nitrosoguanadine. Several mutants with increased production of pigments were isolated. Some of these mutants produced the same types of pigments produced by the parental strain while others were found to be blocked at the hydrogenation step and, therefore, produced only β- carotene.

The preliminary studies on the isolation and characterization of the carotenogenic enzymes involved the solubilization and polyethylene glycol precipitation of a cell-free 40,000 x g supernatant fraction that converted [^{14}C]MVA to phytoene, β-carotene, torulene and torularhodin. The effects of three non-ionic detergents, Tweens 40, 60 and 80 over a 0.5 to 3 % (w/v) concentration range on enzyme activity and protein release were investigated. Enzymatic activity was retained with all three detergents, however, 1% Tween 60 was found to be the least inhibitory.

The efficacy of the new isolate to color the flesh of rainbow trout was demonstrated in a 16 week feeding trial. Even though a commercial canthaxanthin containing diet induced better pigmentation than the test yeast supplemented diet, the level of pigmentation obtained with the test yeast was comparable to the level reported for rainbow trout as sufficient for adequate visual color impression. The highest growth rate was obtained with fish fed a diet containing no pigment (negative control group) and the lowest growth rate was observed in fish fed a diet supplemented with test yeast. While the proximate analysis of the flesh showed significant increases in the total protein content of fish in all groups at the end of the feeding trial, the levels were found to be lower than those reported for rainbow trout by other workers and were found to be associated with increased moisture content. Finally, it was observed that the test yeast fed fish had increased polyunsaturated fatty acids content whereas the fatty acid profile remaining relatively unchanged in all other groups.

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CHAPTER 1

GENERAL INTRODUCTION

Salmonid farming increased substantially in the 1980s creating a large market for carotenoids, pigments found in salmonids and crustaceans. As fish are unable to synthesise carotenoids *de novo*, these have to be added to their diets when reared in aquaculture farms to impart the attractive pigmentation to the animals and also to contribute to consumer appeal in the market place. This is usually done at considerable expense to the farmer (Johnson and An, 1991).

Different sources of carotenoids have been used to pigment the flesh of farmed-salmonid fish, for example crustacean and crustacean processing waste (Storbakken *et al.*, 1985), plant and plant products, e.g. marigold and squash flowers (Lee *et al.*, 1978), the green algae *Spirulina* (Choubert, 1979), the yeast *Phaffia rhodozyma* (Johnson *et al.*, 1980, gentles and Haard, 1990; 1991) and synthetic sources of astaxanthin and canthaxanthin (Torrissen, 1986).

The high cost of the synthetic carotenoids, coupled with consumers and regulatory agencies' concern about the safety of synthetics as food additives, and the general instability of the artificially added carotenoids in the food mixture (Johnson *et al.*, 1980), has resulted in a renewed interest in biological sources of pigments.

A new strain of yeast tentatively identified, as *Rhodotorula rubra* TP1 was isolated from yogurt (Hari *et al.*, 1992). In comparison with *Phaffia rhodozyma*, this new strain is unique in that it has a faster growth rate and grows very well on cheap

industrial by-products. In a preliminary study, *R. rubra* TP1 was fed to rainbow trout as source of carotenoids and the preliminary results indicated that yeast promises to be a good source of pigment and nutrients for the salmonid industry (Sangha, 1994). It was however reported that the total color difference between the flesh of fish fed commercial canthaxanthin and those fed the test yeast was greater in the first six weeks and then decreased to one third at the end of the feeding trial (Sangha, 1994). This has led to the speculation that uptake of pigments from whole cells is slower. To confirm this, a feeding trial longer than that conducted by Sangha (1994) and employing whole yeast cells as well as pigment extracts from the yeast as sources of carotenoid is needed.

Studies done to determine the taxonomic relationship of the new yeast isolate were inconclusive and need to be completed if the yeast is to have any industrial application in the future. For example, the new isolate was found to produce ascospores which is very uncharacteristic of the Cryptococacceae (imperfect yeast) to which most of the pigment producing yeast belong. The determination of the taxonomy of the new isolate was therefore necessary. Furthermore, there was a lack of information on the chemistry, quantity and nature of the pigment produced by the new isolate. Determination of the exact nature and concentration of the pigment produced by the yeast was also essential if any commercialization of the yeast is to be carried out in the future.

Yeast cell walls are known to contain large quantities of glucans (Gorin and Spencer, 1970). Glucans have become important commercially as therapeutic agents

for protecting fish stocks against bacterial infections (Azuma, 1987). The presence of glucans in the cell wall of this isolate may increase the commercialisation potential. Last but not the least, the problem with *Phaffia rhodozyma*, a pigment containing yeast that is on the threshold of being commercialised, is the relatively low concentration of the pigment astaxanthin (An *et al*, 1989). Research to produce mutants with high yields of astaxanthin is well underway (An *et al.*, 1989; 1991; Lewis *et al.*, 1990). If *R. rubra* TP1 is to compete with *P. rhodozyma*, then mutants that produce pigment concentrations comparable to that produced in *P. rhodozyma* mutants will be required. Furthermore, isolation and characterization of the carotenogenic enzymes in this yeast will aid in the cloning of the carotenogenic genes which may eventually boost the commercialization of this new isolate.

The objectives of the present study were, therefore, to:

1. Identify the unknown yeast strain using biochemical and molecular biology techniques.
2. Conduct genetic studies involving mutagenesis to isolate pigment hyper-producing mutants.
3. Identify and characterize the pigments produced by the new isolate.
4. Examine the cell wall of the new isolate for the presence of glucans and other immunologically important polysaccharides.
5. Isolate and characterize the carotenogenic enzymes of the new isolate.
6. Determine the efficacy of the new isolate to color the flesh of fish.

CHAPTER 2

LITERATURE REVIEW

2.1 Carotenoids

Carotenoids represent one of the most important and widespread groups of naturally occurring pigments. They are found in animals, algae, plants, fungi and bacteria and impart the yellow, orange and red colors to leaves, fruits, vegetables, flowers, dairy products, shrimp, lobster and the plumage of exotic birds (Bramley, 1985). They have also been implicated in other functions, for instance, photoprotection, membrane stabilization, phototropism and phototaxis, vitamin A/retinoid metabolism, reproduction and electron transport (Goodwin 1972; Mathis and Scheck, 1982).

2.1.1 Chemistry of carotenoids

Carotenoids belong to the class of polyenes. Their structure is based on a 5-carbon isoprene unit ($CH_2=C(CH_3)CH-CH_2$) and eight of such units are usually linked to form a C_{40} compound. Almost all carotenoids either are, or are derived from this C_{40} compound, e.g. xanthophylls, carotenes, retro-carotenoids, seco- and apo-carotenoids (Britton, 1983). The polyene chain is the most prominent feature of the carotenoids and may be made up of three to fifteen conjugated double bonds. The

conjugated double bonds form a chromophore that is responsible for the characteristic absorption spectrum and color of the given carotenoid molecule.

2.1.2 Biosynthesis of carotenoid.

The pathway for the production of carotenoid is given in Figs. 2.0 to 2.2. Mevalonic acid, the first important compound formed in this pathway, is formed from the condensation of three molecules of acetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HGM-CoA). HGM-CoA then undergoes reduction to form mevalonic acid which, in the presence of adenosine triphosphate (ATP), is converted to mevalonic pyrophosphate (MVAPP). In the presence of ATP, MVAPP is converted to the first 5-carbon isoprene unit, isopentenyl pyrophosphate (IPP) which in turn undergoes a series of isomerization, condensation and dimerization reactions to form phytoene, the basic C_{40} acyclic structure (Fig. 2.0). The phytoene undergoes a series of sequential desaturation reactions (Fig. 2.1 and 2.2) to form phytofluene, neurosporene and finally to either acyclic lycopene or cyclic β-zeacarotene both yielding γ-carotene which is eventually converted to more unsaturated carotenoids. Both pathways may operate simultaneously in many fungi (Bramley and Mackenzie, 1988). At each stage, two hydrogen atoms are removed by trans-elimination from adjacent positions (McDermott *et al.*, 1973) to introduce a new double bond and extend the conjugated polyene chromophore by two double bonds. In certain fungi, cyclization at one end of the molecule takes place after desaturation of the 7,8-double bond although the second cyclization at the opposite end of the carotene molecule

must await corresponding desaturation. Even though most naturally occurring carotenoids are reported to be xanthophylls rather than carotenes (Bramley and Mackenzie, 1988), little is known about the biosynthesis of oxygenated carotenoids. Hydroxylation at the C-3 and C-3 ' has been reported to occur late in the pathway and involved mixed function oxidase reactions (Britton, 1982). In higher plants and algae, phytoene is desaturated to lycopene (ψ,ψ-carotene) by a series of four didehydrogenations, the intermediates being phytoene and phytofluene (7,8,11,12,7',8'–hexahydro-ψ,ψ-carotene) (Bramley and Mackenzie, 1988). In some fungi the desaturation proceeds a step further to yield, 3,4 dehydrolycopene (3,4 didehydro-ψ,ψ-carotene) (Liaaen-Jensen, 1985). Variations in this desaturation sequence has been reported in some microorganisms, e.g. *Rhodosporidium rubrum* (Davies, 1970).

The direct enzymatic conversion of phytoene into colored carotenoids in the laboratory has proven to be difficult to achieve even though some reactions have been demonstrated in cell free extracts of some organisms (Beyer *et al.*, 1985; Kushwaha *et al.*, 1970; Camara and Moneger, 1982). Simpson *et al.* (1971) and Johnson and An (1991) have proposed that torularhodin and torulene, which are produced by *R. rubra* are derived directly from β-zeacarotene and γ-carotene (Fig. 2.1).

2.1.3 Enzymes involved in carotenoid biosynthesis.

Numerous enzymes are involved in the production of carotenoids including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and reductase, mevalonate (MVA) kinase, mevalonate 5-phosphate (MVAP) kinase, pyrophosphomevalonate decarboxylase, isopentenyl pyrophosphate isomerase, farenesyl pyrophosphate synthetase, geranylgeranyl pyrophosphate synthetase, phytoene synthetase and phytoene desaturase. Only phytoene synthetase and phytoene desaturase, the enzymes involved in the formation of colored carotenoids will be discussed in detail.

2.1.3.1 Phytoene synthetase.

Phytoene synthetase is the enzyme that catalyses the tail to tail condensation of GGPP to form the first C_{40} carotenoid, phytoene (7,8,11,12,7',8',11',12'-octadehydro-ψ, ψ-carotene) via the intermediate prephytoene pyrophosphate. Dogbo *et al.* (1988) isolated and characterized the enzyme from *Capsicum* chromoplasts, and reported that a monomeric protein with molecular weight 47,500 forms prephytoene and phytoene. The specific activity of the enzyme was 4000 nmol. of GGPP incorporated into phytoene per mg protein per hour. The enzyme was strictly dependent on Mn^{2+} and no other divalent cation stimulated activity. Dogbo *et al.* (1988) concluded that this selectivity may be one of the factors regulating the competition with potentially rival enzymes converting GGPP into other plastid terpenoids.

Studies on the partially purified phytoene synthetase from tomato fruits plastids, on the other hand, gave an unstable complex enzyme with a molecular weight of 200,000 which also has an absolute requirement for Mn^{2+} (Maudinas *et al.*, 1975, 1977).

2.1.3.2 Phytoene desaturase (PDS).

Phytoene desaturase (PDS) or phytoene dehydrogenase is the enzyme that catalyses the sequence of four didehydrogenations which convert phytoene into the first C_{40} carotene of the pathway. The number of enzymes required for this sequence is unknown and probably varies among organisms (Fraser and Bramley, 1993) and little is known of its structural and regulatory properties (Johnson and An, 1991). Isolation and genetic characterization of mutants in fungi and bacteria have indicated that one enzyme is responsible for the dehydrogenation in some organisms, whereas two are needed in others (Dogbo and Camara, 1987; Schmidhauser *et al.*, 1990). Molecular biology techniques have been employed to isolate and purify the Synechoccus PDS from an overexpressing strain of *Escherichia coli* (Fraser *et al.*, 1993). The recombinant phytoene desaturase has an apparent molecular weight of 53 Kda on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fraser *et al.*, 1993). Similarly, Schmidt *et al.* (1989) used molecular biology techniques to isolate and characterize PDS from photosynthetic organisms and higher plant chloroplasts. The molecular weights of the immunoreactive proteins from

Rhodobacter, *Aphanocapsa*, rape and spinach were estimated to be around 64Kda, whereas that of *Bumilleriopsis* was around 55Kda. Linden *et al.* (1993) also reported the molecular weights of the immunoreactive proteins from tobacco and spinach to be around 62 and 64 Kda, respectively.

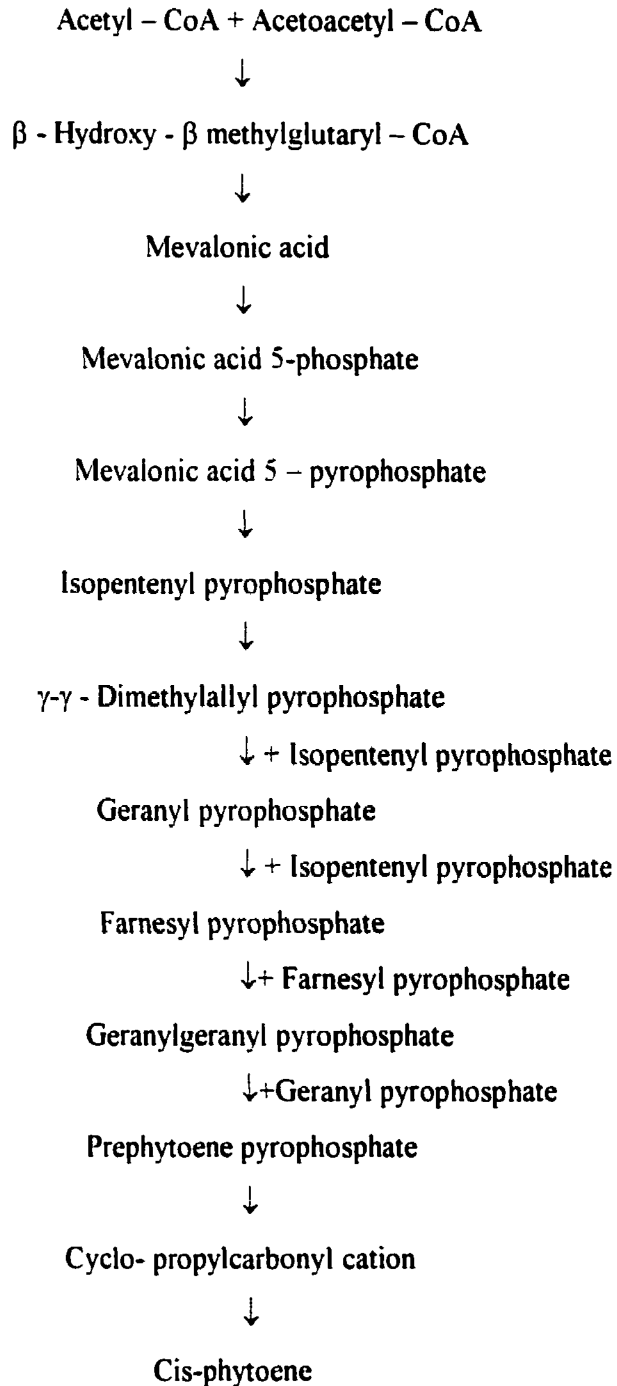


Fig. 2.0 Conversion of acetyl-CoA to cis-phytoene (Modified from Simpson *et al.*, 1971; Britton 1983).

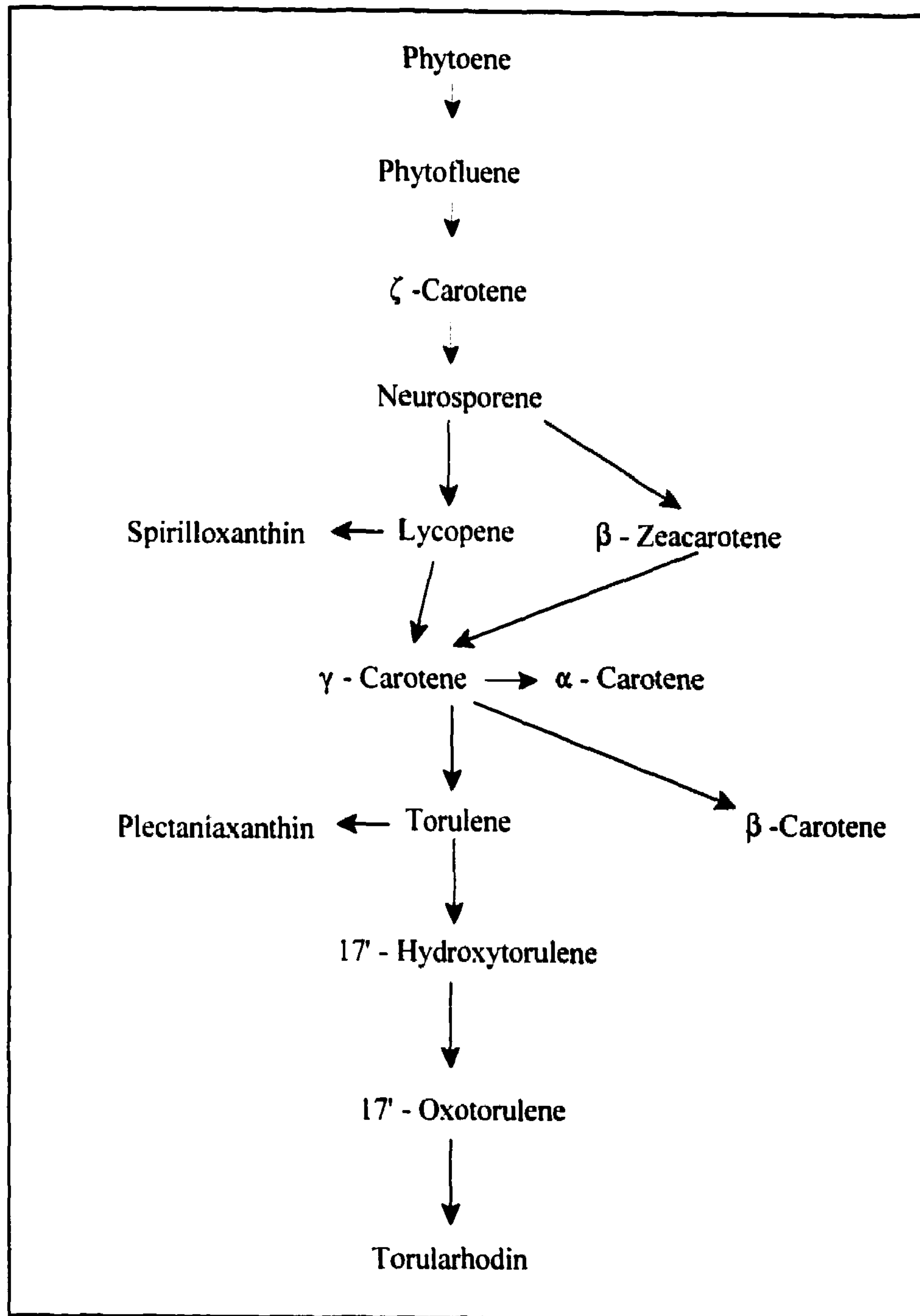


Fig. 2.1. Conversion of cis-phytoene to torulene and torularhodin (Simpson *et al.*, 1991).

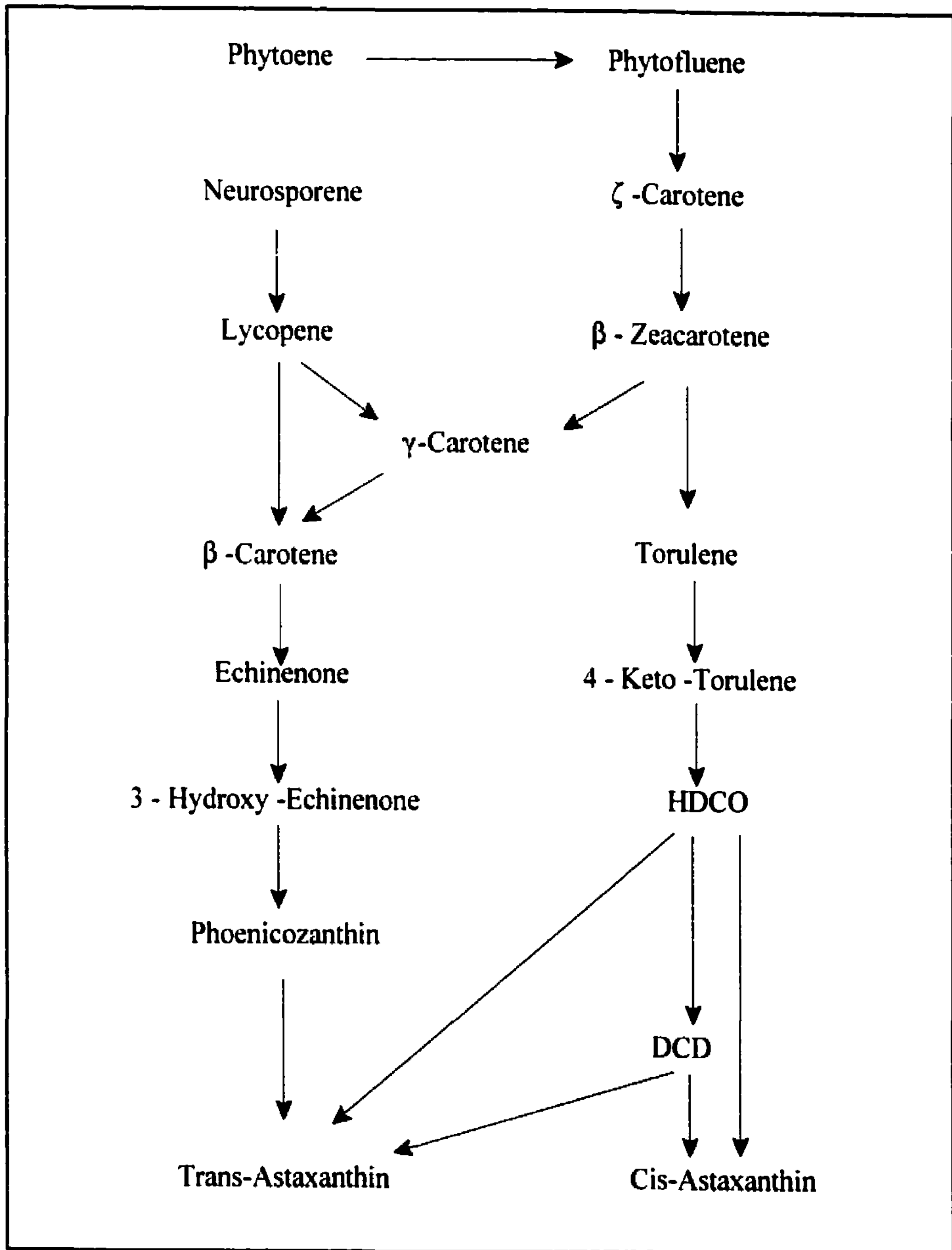


Fig. 2.2. The astaxanthin biosynthetic pathway. (Modified from Andrewes et al., 1976; Johnson and An, 1991).

2.2 Commercial applications of carotenoids.

Carotenoids play crucial roles in photosynthesis and photooxidative protection, nutrition, vision and cellular differentiation. The ability to perform these functions coupled with the wide range of applications and uses of carotenoids are a consequence of the light absorbing properties of the polyene chromophore. In humans and animals, however, the importance of carotenoids is not based on the light absorbing properties of the pigments.

I. Aquaculture and the food industry.

Because consumer acceptance of salmonid depends on the pink coloration of the fish flesh, it is important that these animals, whether they originate in the wild or are farmed, are pigmented. Carotenoids are used in the aquaculture industry to pigment the flesh of fish, and as a result, there has been a dramatic increase is the use of carotenoids in the past few years. Carotenoids are also used in the food industry to pigment products such as chicken egg yolk and bakery products (Anderson *et al.*, 1991). Similarly, carotenoids are used in the pharmaceutical and cosmetic industries to color tablets coatings, suppositories, lipsticks, vitamin emulsions, fat-based ointments and a host of other products. (Johnson and Schroeder, 1995).

2. Nutrition and health.

A. Vitamin A and retinoids: Carotenoids have been implicated in important metabolic functions in humans and animals. Vitamin A, acting as the chromophore of the visual pigments in the eye is central to the process of vision, and vitamin A deficiency, therefore, results in various eye diseases such axerophthalmia, blindness and premature death, particularly in children (Britton *et al.*, 1995). Vitamin A also plays a role in the maintenance and growth of the reproductive efficiency of the systemic functions, maintenance of epithelial tissues and prevention of their keratinization (Britton *et al.*, 1995). β-carotene and structurally related compounds serve as precursors for vitamin A, retinal and retinoic acid in animals and thus play essential roles in nutrition, vision and cellular differentiation.

B. Antioxidant potentials and prevention of diseases: Carotenoids play a role in enhancing the immune response system, and in protecting against such diseases as cancer by quenching oxygen radicals (Goodwin 1986, Bendich and Olson, 1989). Recent studies utilizing both statistical correlation and model experiments with animal systems have suggested that carotenoid can provide protection against cancer, heart diseases and AIDS (Britton *et al.*, 1995). Epidemiological studies have also shown that vitamin A, carotenoids and provitamin A carotenoids can be effective antioxidants for inhibiting the development of heart disease (Manson *et al.*, 1991; Gaziano *et al.*, 1992; Street *et al.*, 1994; Greenberg *et al.*, 1996; Riuiz Rejon *et al.*, 1997; Evans *et al.*, 1998). Similarly, extensive *in vitro* and limited *in vivo* studies

have shown that individual antioxidants such as vitamins A, E, C and carotenoids induce cell differentiation and growth inhibition to various degrees in rodents and human cancer cells (Prasad *et al.*, 1999). Retinoids have also been demonstrated to act against the development of cancer of the mammary glands, urinary bladder, lungs, skin, pancreas, colon and esophagus (Moon, 1989). However, humans and animals are incapable of *de novo* synthesis of carotenoids, and therefore the pigments should be provided in the diet as a source of vitamin A.

2.3 Value of the carotenoid and implications of future business.

Farm rearing of salmonids has grown dramatically in recent years. In 1989 alone over 200,000 tons of salmon were produced worldwide (Bjørndahl, 1990). It is expected that farmed salmon will dominate the salmonid market by the year 2000 with the output expected to exceed 460,000 tons annually, and that Norway, Canada, Chile and Japan will emerge as the world leading producers of cultured salmonids (Johnson and Schroeder, 1995). These developments, in conjunction with the dramatic increase in the amount of trout being farmed around the world, have resulted in a dramatic increase in the amount of carotenoids being used in the fish farming industry (Johnson and Schroeder, 1995) and this trend is expected to continue. Furthermore, the interest that has been generated in the role carotenoids play in the prevention of various human ailments is bound to ensure a steady increase in the demand and market for carotenoids.

2.4 Sources of carotenoid.

Carotenoids can be obtained from various sources among, which are crustacean and crustacean processing waste, chemical synthesis, plants, algae and microorganisms such as fungi and yeast.

2.4.1 Crustacean and crustacean processing waste.

The major pigment that occurs in crustaceans is astaxanthin and several studies have been conducted on the use of crustacean and crustacean processing waste as a pigment source for aquaculture of fish (Torrissen *et al.*, 1989). Since the level of carotenoid reported in most crustaceans is very low (Torrissen *et al.*, 1989; Lambersten and Braekkan, 1971), large quantities of the chitinous products must be incorporated into the diets of the fish to achieve the desired level of coloration. Since the crustacean wastes have low protein levels and high levels of ash, moisture and chitin (Torrissen *et al.*, 1989), incorporating large quantities of these materials in fish feed results in a moist diet that is nutritionally imbalanced and may induce mineral imbalances in the fish (Spinelli and Mahnken, 1978). Crustacean waste therefore has limited potential as a source of carotenoid for cultured fish (Torrissen *et al.*, 1989).

2.4.2 Synthetic carotenoids.

The principal sources of carotenoid used in the aquaculture industry are synthetic canthaxanthin and astaxanthin called "Carophyll Pink" which is produced and marketed by Hoffman-LaRoche (Basel, Switzerland). This pigment is reported to be absorbed and deposited better than its predecessor "Roxanthin" or "Carophyll Red" produced and marketed by the same company in the 60s (Foss *et al.*, 1984, 1987; Storebakken *et al.*, 1987; Torrissen, 1986). Megadoses of canthaxanthin gained by eating the so-called artificial 'suntan' pills are reported occasionally to be toxic, causing reduced vision and anemia (Herbert, 1991; Mathews-Roth, 1991). However, the toxicity of megadoses of canthaxanthin is not well-documented (Herbert, 1991, Mathews-Roth, 1991) and the U.S. Food and Drug Administration (FDA) has classified canthaxanthin as a 'generally recognized as safe' (GRAS) substance. However, astaxanthin does not have a GRAS classification in the U.S. (Tangerås and Slinde, 1994). For these reasons, the difficulties encountered in the synthesis of these compounds, and the increasing wariness by farmers and consumers of the incorporation of synthetic chemicals in fish diets, have contributed to the search for natural sources of carotenoids (An *et al.*, 1991).

2.4.3 Plants and algae.

Some green algae belonging to the subphylum Chlorophycaea possess the ability to synthesize ketonic xanthophylls, echinenone, canthaxanthin and astaxanthin (Wettern and Weber, 1979; Nakayama, 1962). The best-known astaxanthin-producing alga is *Chlamydomonas nivalis* (Torrissen *et al.*, 1989). The green alga *Duanelíella salina* produces high quantities of β-carotene when placed under stressful conditions. *Haematococcus pluvialis* also produces high levels of astaxanthin depending on the culture conditions and method of cultivation (Droop, 1955; Goodwin and Jamikron, 1954). However about 87% of the astaxanthin found in these organisms are esterified, which may affect deposition and metabolism in some animals (Johnson and An, 1991). It has also been reported that salmon fed algae-based diet deposited only a minimal amount of astaxanthin.

There are numerous reports documenting the use of various plants as possible pigment sources for fish and lobster. For example, Peterson *et al.* (1966) and Isler (1971) reported the pigmentation of salmonid flesh with capsxanthin–containing paprika. Lee *et al.* (1978) observed an increased deposition of lutein and canthaxanthin in the flesh of rainbow trout fed diets containing marigold (*Tagetes erecta*) and squash flower (*Cucurbita maxima Marcus*) extracts. Based on the overall results of these studies, however, Torrissen *et al.* (1989) concluded that products from higher plants have little potential for use as pigment sources in practical fish diets.

2.4.4 Fungi

Most carotenogenic fungi produce carotenes such as β-carotene and γ-carotene. The yeasts which produce carotenoids belong to the taxa Deuteromycetes and Basidiomycetes (Britton, 1983). Phaff *et al.* (1978) classified carotenogenic yeasts into six genera, *Sporidiobolus*, *Rhodosporidium*, *Sporobolomyces*, *Rhodotorula*, *Cryptococcus*, and *Phaffia* and in 1987, Komagata added a seventh, *Saitoella*. Of the seven genera, *Phaffia* is different in that it produces astaxanthin (Andrewes *et al.*, 1976a) while yeasts belonging to the genera *Cryptococcus*, *Sporobolomyces* and *Rhodotorula* produce mainly γ-carotene, β-carotene, torulene and torularhodin (Simpson *et al.*, 1971; Goodwin, 1972). Other pigments such as ζ-carotene, phytofluene β-zeacarotene and phytoene may also be found in *Rhodotorula* and *Rhodosporidium* (Haymen *et al.*, 1971). Some *Rhodotorula* and *Cryptococcus* species have also been reported to produce plectaniaxanthin (Ratledge and Evans, 1987) whereas 2-hydroxy plectaniaxanthin has been reported in *Rhodotorula aurantiaca* (Liu *et al.*, 1973).

Although astaxanthin is rarely found in fungi, it has occasionally been isolated from the Basidiomycete *Peniophora* and *Perquercina* of the Aphyllophorales (Goodwin, 1972). In yeast, *Phaffia rhodozyma* produces astaxanthin and has been tested as the coloring component of the diets of farmed salmonids with very promising results (Johnson *et al.*, 1980).

Currently there is economic interest in *P. rhodozyma* as a biological source of astaxanthin. However, because the absolute configuration of the yeast's astaxanthin differs significantly from that normally occurring in salmonids, the commercial use of *P. rhodozyma* could possibly be slowed down (or even stopped) by regulatory agencies. Other obstacles such as the rigid cell wall which limits pigment extractability, a slow growth rate and poor digestibility of whole cells by the fish have prevented the commercial use of *P. rhodozyma* (Johnson *et al.*, 1980).

2.5 Yeast taxonomy.

Yeasts are classified among Eumycota and are found in the Ascomycetes, Basidiomycetes and Deuteromycetes (Imperfect fungi). (Table 2.0) (Kreger-van Rij, 1984).

Table 2.0. Taxonomy of yeast

Subdivision	Class	Order	Family
Ascomycotina	Hemiascomycetes	Endomycetales	Spermophthoraceae Saccharomycetaceae
Basidiomycotina		Ustilaginales	Filobasidiaceae Teliospres forming yeast
		Tremellales	Sirobasidiaceae Tremellaceae
Deutermycotina (Form sub-division)	Blastomycetes		Cryptococcaceae Sporobolomycetaceae

Adapted from Kreger-van Rij, (1984).

2.5.1 Methods used in yeast taxonomy.

Traditionally, morphological and physiological characteristics and, to a lesser extent, genetic characteristics have been used for the classification of yeasts. The main characteristics that are presently used to classify yeasts are morphological characteristics, sexual reproduction, biochemical features and physiological characteristics which primarily serve to describe, differentiate and identify yeast strains. They also serve to describe, characterize, and differentiate species and to a lesser extent, genera (Kreger-van Rij, 1984; Barnett *et al.*, 1983). Although these characteristics may provide valuable information for the identification of the yeast, it is often time consuming, inadequate and sometimes not completely reliable. The search for new methods of identification therefore continues to this day.

2.5.2 Molecular taxonomy.

The principles and methods of molecular biology are becoming increasingly important in yeast taxonomy as it has in clarifying the relationship between various organisms ranging from bacteria to man and other forms of life. Molecular taxonomy based on the determination of G + C content of molecular genomes and re-association of RNA and DNA in different yeast species were the first methods used (Martini *et al.* 1972, Kurtzman *et al.*, 1983; Nakase and Komagata, 1970 a, b; Meyer and Phaff, 1970). The DNA-RNA re-association is used by most yeast taxonomists when the

equipment is available to determine the relationships between yeast species (Kurtzman and Phaff, 1987; Kurtzman, 1990; Kurtzman *et al.*; 1983). The highly conserved sequences of the ribosomal RNAs (rRNA) and the ribosomal DNAs (rDNA) allow the determination of the relationship among different types of yeast. Numerous investigators (Kurtzman and Robnett, 1991; Gueho *et al.* 1990; Mendonça-Hagler *et al.* 1993; Molina *et al.*, 1992, Fell *et al.*, 1999, 1998) have successfully used this as a taxonomic criterion in an effort to determine the relationship between various yeast isolates. With the advent of PCR (Polymerase chain reaction), sequence analysis of rRNAs and rDNAs has become more powerful.

2.5.3 Polymerase chain reaction (PCR) and DNA sequencing.

DNA sequencing provides the most exhaustive approach to recover information from macromolecules. With the invention of PCR by Mullis and Faloona in 1987, sequencing of nucleic acids has become easy at a reasonable cost (Minelli, 1993). The technique generates hundreds of billions of identical molecules of DNA from a single molecule in just a few hours. It has since become the standard bearer of the molecular systematists' tools, unrivaled in its power and complexity by any other procedure available to the molecular systematist (Palumbi, 1996).

PCR is a simple cyclic process consisting of three major steps: (1) a denaturation step during which the DNA strands are separated by heating; (2) an

annealing step during which the separated strands are cooled so that oligonucleotide primers can bind to appropriate sites in the template DNA; and, (3) an extension period during which the enzyme DNA Taq polymerase synthesizes the target DNA segment by adding nucleotides to them to replicate the complementary strands of the DNA molecule (Mullis, 1990, Palumbi, 1996).

The products of PCR are usually fragments of DNA of defined length that can be sequenced directly by manual sequencing using the dideoxy sequencing method (Sanger *et al.* 1977) or by automated sequencing using an automated sequencing machine (Hills and Moritz, 1990). The DNA sequences obtained this way provide a wealth of information that can be used to discriminate between morphologically indistinguishable taxa.

2.5.4 Ribosomal DNA

Ribosomes are small, complex spherical bodies (about 20 to 30nm in diameter) which act as sites for protein synthesis. Because protein synthesis is a prerequisite for life as we know it, ribosomes are universally present in all living systems.

The ribosomal DNA (rDNA) of an eukaryotic genome is composed of three gene coding regions: (1) a large subunit (LSU) rRNA also called the 28S subunit; (2) a 5.8S rRNA; and (3) the small subunit (SSU), also called the 18S subunit. Flanking

the 5.8S subunit on either side are two non-coding DNA spacer segments referred to as the internal transcribe spacers (ITS). Between each coding region are largely non-transcribed regions called the intergenic spacer (IGS) (Hillis and Dixon, 1991).

The nuclear rDNA of the eukaryote consists of several hundred tandemly repeated copies of the transcription unit and non-transcribed spacer region. The number of copies of this transcription unit may be as few as one as in *Tetrahymena* or as many as several thousand in other organisms (Appels and Honeycutt, 1987). As a result there can be a high concentration of rDNA, which makes amplification easier. Another reason why rDNA is useful in phylogenetic analysis is that the different repeat units of rDNA evolve at different times and there is, therefore, a high degree of variability within each of the repeat units. The coding regions evolve slowly and are, therefore, the most highly conserved regions. The ITS and the IGS of the rRNA repeat unit evolve fastest and may vary among species within a genus or among populations (White *et al.* 1990).

The 18S rRNA is the most studied nuclear gene in eukaryotes. In addition, the presence of highly conserved sequences within this region makes it useful for constructing 'universal' primers that are useful for sequencing rRNA or rDNA for many species and for amplification of the region of interest in PCR reactions (White *et al.* 1990, Hillis, 1990). The variation within the large subunit and the internal transcribed spacer region have also been used to identify species or strains, to study hybridization and markers for population genetic studies (Hillis *et al.* 1991; Sites and

Davis, 1989; Rogers, 1986). Partial base sequences of 18S and 26S regions have been used to reclassify a number of yeast species previously put into different genera (Yamada *et al.* 1994a, 1994b, 1994c, 1995).

2.5.5 Protein Electrophoresis

Electrophoretic comparison of enzymes has proven to be very useful in microbial systematics in that it has not only become one of the cost-effective methods of investigating genetic phenomena at the molecular level (Murphy *et al.* 1996), but also in the differentiation of closely related organisms. With the inception of starch gel electrophoresis (Smithies, 1955) and the histochemical visualization of enzymes on gels (Hunter and Markert, 1955), numerous investigations have been launched using both enzymatic and non-enzymatic protein electrophoresis. These investigations have looked into enzyme efficiency, estimation and understanding genetic variability in natural populations, recognition of species boundaries and phylogenetic relationships among various organisms (Murphy *et al.*, 1996). These types of analyses have resulted in the identification of closely related species, groups of populations or reproductively isolated species that were hitherto undistinguishable by conventional morphological characteristics.

Protein electrophoresis can be used to gather two forms of data simultaneously. These are isozyme and allozyme analysis. Isozymes or multiple

molecular forms of enzymes, are enzymes that share a common substrate but differ in electrophoretic mobility (Markert and Muller, 1959). Allozymes, a subunit of isozymes, are variants of polypeptides representing different allelic alternatives of the same gene locus. Both enzymes can be separated on the basis of their net charge and size and are important in molecular systematics (Murphy *et al.*, 1996).

Protein electrophoresis can be accomplished in several types of support media including starch, polyacrylamide, and agarose gels, and cellulose acetate membranes. Among these support media, polyacrylamide gel electrophoresis (PAGE) is the method of choice among biochemists (Wendel and Weedon, 1989) and molecular systematists (Murphy *et al.* 1996).

PAGE has been used to compare the electrophoretic mobility of numerous yeast enzymes. For example Yamazaki and Komagata (1981) used PAGE to compare enzymes in 108 strains of the genera *Rhodotorula* and *Rhodosporidium* and the isozyme patterns obtained allowed them to divide the strains into a number of distinct groups. Similarly, Okunishi *et al.* (1979) and Hamamoto *et al.* (1986) used PAGE to discriminate between various species of *Rhodosporidium, Rhodotorula* and *Cystofilobasidium* genera.

Even though cellulose acetate has been around for decades, its use has been restricted to diagnostic applications in the clinical settings (Herbert and Beaton, 1993). It is however deemed by some authorities as a simpler, more rapid type of electrophoresis, which is more sensitive, and provides a superior resolution to starch

or PAGE (Eastel and Boussy, 1987). There are no reports of cellulose acetate electrophoresis been used in yeast taxonomy.

2.5.6 The value of fatty acid analysis in the taxonomy of yeasts.

According to Ratledge (1982), oleaginous (oil containing) microorganisms are chiefly confined to algae, fungi including yeasts and some cyanobacteria. Some bacteria also accumulate high concentrations of complex lipids which are more diverse than the polar lipids found in most biological cells (Tornabene, 1985). The use of lipid analyses as a tool in bacterial taxonomy is well established. The first attempt to associate lipid composition with the identification of bacteria was made by Abel *et al.* (1963), who illustrated that the fatty acid profile could be used to discriminate between heterogeneous bacteria. Since then, some of the most valuable taxonomic studies on coryneform bacteria and actinomycetes genera have utilized fatty acid analysis and the occurrence of mycolic acid as major taxonomic tools (Collins *et al.*, 1982; Minnikin *et al.*, 1978; Bousfield *et al* 1983; Athalaye *et al.*, 1985)

Cellular fatty acids have also been found in almost all yeasts and analyses of the fatty acid composition of most of the major species of yeast is well documented. In ascomycetous and basidiomycetous yeasts, fatty acids ranging from C_{14} to C_{20} have been detected and used in the classification of these organisms (Cottrell *et al.*,

1985; Kock *et al.*, 1986; Smit *et al.*, 1987, van der Weisthuzen *et al.*, 1987; Viljoen *et al.*, 1986). Ratledge (1994) reviewing fatty acid analyses in various yeasts concluded that the major fatty acids found in yeast are palmitic acid (16:0), palmitoleic acid (16:1), oleic acid (18:0) and linoleic acid (18:2). Oleic acid is the most abundant fatty acid and linoleic acid can be the second most abundant fatty acid in most types of yeast whereas stearic acid is usually a minor constituent and rarely exceeds 10% of the total fatty acids (Ratledge, 1994). Medium chain fatty acids (12:0 to 14:0) occur in trace amounts (less than 1%) (Rattray, 1988) whereas long-chain fatty acids (20:0 to 24:0) occur in few yeasts and are probably present in trace amounts in many species (Ratledge, 1994). Polyunsaturated long chain fatty acids are not common, however, the presence of di-homo-γ-linolenic acid (20:3) and arachidonic acid (20:4) has been reported in the yeast, *Dipodascopsis uninucleata* (Botha *et al.*, 1992).

2.5.7 Cellular carbohydrate composition of yeast cell wall.

The cellular carbohydrate composition of yeast cell wall has been used in the taxonomy of yeast. Sugiyama *et al.* (1985) used this approach together with other biochemical analyses to examine 108 strains of 61 species of the genera *Rhodosporidium*, *Leucosporidium* and *Rhodotorula* and concluded that cellular carbohydrate composition profile is a valuable tool in the chemotaxonomy of yeast. Other workers have used the 'intact whole cell approach' to profile the cellular carbohydrate composition of yeast and yeast-like organisms and have all concluded

that this approach is useful in yeast taxonomy (Von Arx and Weijman, 1979; Weijman, 1977, 1979; Weijman *et al.* 1982; Weijman and Rodrigues de Miranda, 1988a).

2.6. Yeast genetics.

Yeast genetics as a discipline began in 1935, when Winge and Lausten (1937) demonstrated the alternation of haploid and diploid phases in *Saccharomyces cerevisiae*. They isolated tetrads of spores from asci of *S. cerevisiae*, observed differences in the morphology of giant colonies on wort gelatin and demonstrated Mendelian segregation in *Saccharomycodes ludwigii*, a species in which long and short cells segregate according to Mendelian rules. Later, Lindergren (1949) demonstrated Mendelian segregation of melibiose fermentation by tetrad analysis of hybrid of *S. cerevisiae* x *S. carlsbergensis (S. pastorianus)* while the genes for fermentation of rhamnose, maltose, galactose, and sucrose were investigated by Lindergren (1949) and Winge and Roberts (1952).

These classic works laid the foundation for modern yeast genetics, which has progressed rapidly, and have increasingly become molecular in nature. Yeasts have been found to present many advantages for genetics studies, e.g. they are easy to clone, handle and store, have rapid growth rate, are adaptable to replica plating, micro-manipulation and can be subjected to an array of biochemical procedures (Motimer and Hawthorne, 1970).

2.6.1 Methods for genetics manipulation.

1. Alteration of DNA by chemicals and radiation (Mutagenesis): The four-nucleotide bases of DNA molecules are highly reactive groups capable of interacting chemically with a variety of compounds. Some of the compounds are natural products of biochemical reactions that break down metabolites inside cells while cells take the others up from their environment. The energy absorbed from radiation has the effect of increasing the reactivity of DNA or of substances occurring naturally in cells or taken up from surroundings. This reactivity frequently results in chemical modifications of the DNA, resulting in the formation of mutants.

A. Mutagenesis with chemical agents: Although chemical modifications of the DNA bases are potentially almost endlessly varied, the most likely possibilities fall into three categories.

I. Base analogs: These are chemicals that mimic normal DNA bases. They are incorporated into replicating DNA but, because of structural similarity, they have different pairing properties and cause much more frequent mispairing than normal. Two widely used examples are 5 – bromouracil (BU) and 2 – aminopurine (2AP).

II. Mutagens affecting non-replicating DNA: There are a number of chemicals, which cause direct damage to non-replicating DNA. Nitrous acid (HNO_2), ethylmethane sulfonate (EMS), N-methyl-N-nitroso urea, N-methyl-N-nitro-N-nitrosoguanidine (MNNG or NTG), methyl sulfonate (MMS), mustard gas and many others are examples of this category. These

compounds, known as akylating agents, add methyl or ethyl groups to DNA bases, converting them to modified bases that may pair differently from the original A,T,C and G types. Others cause changes that block normal base pairing, leading to non-specific, random entry of nucleotides into the copy chain at positions opposite an alkylated template base. Addition of cyclic groups may be so large that passage of the replicating enzyme is blocked. Furthermore, DNA may be prevented from unwinding for replication or transcription resulting in "skips" in which the copy chain has an open break of greater or shorter length.

III. Frame shift mutagens: These are compounds that intercalate into the DNA molecule causing errors and resulting in an alteration of the reading frame thus leading to the formation of faulty protein or no protein at all. Examples of frame shift mutagens are acridine orange, proflavine and acriflavine. Although acridines are useful in research they are not suitable for routine isolation of mutants in strain development (Crueger and Crueger, 1989).

B. Mutagenesis resulting from radiation: Radiation-induced mutagenesis may result from direct absorption of energy by parts of the DNA molecule itself or by chemical activation of substances absorbing radiation in the vicinity of DNA. Both ultraviolet and ionizing radiation are used in mutagenesis studies.

I. Ultraviolet radiation: Ultraviolet light is one of the radiation sources that directly induce alterations in DNA molecules. Short-wavelength radiation between 200–300 nm is the most effective for mutagenesis. The most

common result of exposure to this form of radiation is the formation of thymine dimers between adjacent pyridines or pyrimidines of complementary strands. Long-wavelength ultraviolet radiation between 300 and 400 nm has less lethal effects on cells, however, in the presence of various dyes, which interact with DNA, greater death rates and increased mutations occur.

II. Ionizing radiation: Ionizing radiation creates reactive substances that interact with atoms of DNA bases in various ways. It is, however, used in mutagenesis only if others mutagens cannot be used (Crueger and Crueger, 1989). Ionization radiation includes X-rays, β-rays, and γ-rays.

2. Protoplast Fusion (spheroplast and nucleus-protoplast fusion): This procedure was first used in making "hybrid" plant and animal cells and has now been applied to yeast. In spheroplast fusion, the cell wall of the yeast is enzymatically removed, the resulting protoplasts are then mixed in stabilizing solutions of sorbitol or potassium chloride with the addition of calcium in a fusogenic agent such as polyethylene glycol (PEG). The resulting mixture is incubated at appropriate temperature for 30 minutes, and plated on osmotically stabilized media. Since there are no constraints on the recombination process, this technique results in the production of various classes of hybrids. Protoplast fusion has been performed in *Streptomyces* sp. (Hapwood *et al.*, 1977). Protoplasts from basidiomycetous yeasts are difficult to obtain, however, protoplasts have been obtained from the genera *Rhodosporidium*, *Cryptococcus* and *Phaffia* (Spencer and Spencer, 1997).

3. Nucleus-protoplast fusion: This involves the isolation of viable nuclei from a donor strain and the fusion of the nuclei with protoplast of the recipient strain in the conventional manner. The method has been applied to protoplasts from strains of *S. cerevisiae* and nuclei from the filamentous fungi, *Fusarium monoliforme* and *Trichoderma reesii* (Spencer and Spencer, 1997); yeast protoplasts and animal cells (Ward, 1984); protoplasts of petite mutants of *S. cerevisiae* and human blood platelets that contain mitochondria but no nuclei (Spencer and Spencer, 1997).

4. Recombinant DNA techniques: This process involves the transformation of *Escherichia coli* by a plasmid from yeast carrying the desired gene to be introduced into the recipient strain. DNA from the bacteria strain is amplified, re-isolated and used to transform the recipient yeast. Yeast transformants are subsequently selected and then tested for the presence of the desired gene (Spencer *et al.* 1988).

5. Electroporation: This technique uses pulses of an electric field to separate intact chromosomes. Yeast cells embedded in agarose gel blocks are enzymatically lysed, subjected to pulses of electrical current of unequal duration. The currents are reversed at intervals of several seconds producing a homogenous electric field, which results in sharper separation of bands. This technique has been used for karyo-typing of a number of yeast species (Spencer *et al.* 1988).

2.7 Economic importance of yeast.

2.7.1 Fermented foods and beverages.

Controlled fermentation of food originated in China, and was initially used for the preservation of cereals and legumes. The practice has since been extended to many other foods. Vegetable products having a meat-like texture and methods of leavening batters of cereal-legume mixtures were developed in India and Indonesia, whereas acid fermented vegetables (pickles) and bread leavened by yeast were developed in Korea and Egypt, respectively, (Vijayalakshmi *et al.* 1997). Fermented foods are widely used today around the world and most are prepared by microbial action on one or more components, causing changes in their physical, biochemical and nutritional qualities. Yeasts that contribute to many of these fermentation processes have also been used in the brewing industries for centuries. They are also used in the production of industrial alcohol, glycerol and other polyhydroxyl alcohols. They also produce proteins, vitamins, pigments and flavoring compounds and can contribute useful mechanical qualities to food. Yeasts also contribute significantly to recommended daily allowances for calories, proteins, calcium, phosphorous and iron, and vitamins B, C, and niacin in the human diet (Vijayalakshmi *et al.* 1997).

2.7.2 Yeast in animal feeding.

Yeasts are used as microbial biomass protein or single cell protein (SCP) to supplement (up to 50%) animal diets (Phaff *et al.*, 1978). The most widely used yeast is *Candida utilis* (Torula yeast), which serve as a valuable protein source for the poultry, livestock and aquaculture industries (Phaff *et al.*, 1978). Bakers' inactive dried yeast is used widely in the health-food industry as a source of vitamins. *Candida humicola, Candida tropicalis and Lipomyces lipoferis* have also been used as lipid yeast for SCP because of their high concentrations of lipids, most of which are made up of unsaturated fatty acids (Raitsina and Eudokimova, 1977). Martin *et al.* (1993), using *Candida utilis* as a source of protein for rainbow trout *Onchorynchus mykiss*, reported good growth and high apparent protein digestibility (APD) for fish fed diet in which the largest of the fish meal component has been replaced with yeast biomass. Pigmented yeasts belonging to the genera *Phaffia, Rhodotorula and Sporobolomyces* sp. have also been tested as sources of carotenoids for the aquaculture, poultry and pharmaceutical industries (Johnson and An, 1991).

2.7.3 Yeast in therapeutics.

Yeasts are a potential source of wide variety of therapeutic products, from vitamins to heterologous proteins. Glucan, a component of the yeast cell has been tested for use as "biological response modifier" and immunostimulants for the

prevention of fish diseases (Anderson, 1992; Rao *et al.*, 1992) and diseases in humans such as cancer (Azuma, 1987). Several types of yeasts are today being used as vehicles for the production of heterologous proteins such as interferon, hepatitis B surface antigen (used in vaccine production), epidermal growth factor which is used in hastening wound healing in corneal transplants, human insulin production and many others (Vijaylakshmi *et al.* 1997).

2.8 Occurrence of glucans in yeast.

The yeast cell wall constitutes 15-25% of the dry weight of the cell. Polysaccharides account for 80 – 90% of the wall, followed by smaller amounts of protein and lipid. Glucan and mannans are the main polysaccharides, with chitin occurring in minute quantities (McWilliam 1970, Phaff, 1971). The bulk of studies done on the chemistry of cell walls of yeast have been concentrated on *Saccharomyces cerevisiae* and it is only recently that other species have attracted attention. Depending on the culture condition, the glucan content of the cell wall of *S. cerevisiae* is in the range of 30–60% (Ramsey and Douglas, 1979; Bacon *et al.*, 1969; Jayatissa and Rose, 1976) and is composed of three types of glucan. Bacon *et al.* (1969) first reported that the predominant glucan was a β-(1→6)-linked glucan. Other authors have reported the presence of an alkali-insoluble acetic acid-insoluble β-(1 →3) glucan and an alkali-soluble β-(1→3) glucan (Manners *et al.* 1973). The occurrence of glucan has been reported in other yeasts such as *Candida albicans*,

Schizosaccharomyces species, *Rhodotorula glutinis* (Arai *et al.*, 1978; Arai and Murao, 1978), and *Pichia polymorpha* (Villa *et al.* 1980). However, in *R. glutinis* and *P. polymorpha*, the determination was made largely on the basis of the presence of glucose in wall hydrolysates and no structural details are available.

Since glucans are in demand as immunostimulants, it is the objective of the present study to isolate and determine the chemical structure of the glucans from the cell wall of the new yeast isolate.

2.9 The taxonomy of the red yeast.

2.9.1 The Genus *Rhodotorula*.

The genus *Rhodotorula* was created to accommodate pigment producing asporogenous yeast (Harrison, 1928) which synthesize red or yellow carotenoids. This genus belongs to the family Cryptococcaceae (Kreger van-Rij, 1984) and sub-family Rhodotoruloideae (Lodder and Kreger van-Rij, 1954). Members are spheroidal, ovoidal or elongate in shape and reproduce by multilateral budding. Strains of some species form pseudo- or true hyphae, some may form arthrospores (Kreger van-Rij, 1984). As described by Harrison (1928), members do not assimilate inositol, do not form starch-like substances and lack fermentative ability. Many strains have a mucous appearance due to capsule formation, but others are pasty or dry and wrinkled (Fell *et al.*, 1984).

2.9.2 Sexuality in *Rhodotorula*

The members of the genus reproduce vegetatively and do not form ascospores or ballistopores. Banno (1967) discovered the sexual state in strains of *R. glutinis* and this led to the creation of basidiomycetous genus *Rhodosporidium* and the teliospore forming species, *Rhodosporidium toruloides*. Many of the members of *Rhodotorula* have been described as being imperfect forms of *Rhodosporidium* Banno (Banno, 1967). Strains have opposite mating types characterized by dikaryotic mycelium with clamp connections and chlamydospores.

2.9.3 The species *Rhodotorula rubra.*

This is a red yeast with cells varying from short ovoidal to elongate in shape and size. They may occur singly, in pairs or in clusters and all are able to assimilate sucrose, trehalose, raffinose, D-xylose, ribitol and succinic acid (Fell *et al.*, 1984), while some strains are also able to assimilate galactose, maltose, cellobiose, L-arabinose, D-ribose, L-rhamnose, D-mannitol and citric acid (Kreger van-Rij, 1984). Analysis of the carbohydrate content of the whole cell hydrolysate revealed fucose and mannose as the dominant sugars while mannitol and arabinitol may occur in minute quantities (Weijman and Rodrigues de Miranda, 1988b). Members of the species have a G + C content of 60-63.5 mol. % (Nakase and Komagata, 1971; Marmur and Doty, 1962), an ubiquinone Q10 system and do not require biotin and P aminobenzoic acid for growth (Yamada and Kondo, 1973). Several *Rhodotorula*

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

2.9.4 Pigment formation in *Rhodotorula rubra*.

R. rubra has been successfully cultivated on a wide range of carbohydrate sources. Martin *et al.* (1993a) optimized such parameters as pH, aeration rate, nutrient requirements and concentration of carbon sources and pigment production in this species in peat extracts. They reported that *Rhodotorula rubra* growth and pigment syntheses are both optimal at 22°C, and at a pH of 5.5. Sangha (1994) reported production of high concentrations of pigment when the organism was grown in molasses and malt extract. Fromegeot and Tchang (1938) studied the effects of glycerol on carotenogenesis in *R. rubra* and concluded glycerol is effective in promoting carotenogenesis in this organism. However, for *Rhodotorula* sp. No.100, Simpson *et al.* (1971) reported that glycerol was less effective than glucose, while phenol, resorcinol and Kojic acid stimulated the production of β-carotene in the above-mentioned strain. 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 torulene, β-carotene and γ-carotene (Wittmann, 1957). Numerous nitrogen sources have also been shown to affect pigment production in *R. rubra*. In the Rh-100 strain of *Rhodotorula*, valine, leucine and asparagine produced the best yields of carotenoids (Simpson *et al.*, 1971), while on organic and inorganic

sources of nitrogen, the highest yield was recorded for ammonium nitrate (Wittman, 1957).

Unlike *P. rhodozyma*, in which carotenoid production occurs in the exponential phase of growth (Johnson and Lewis, 1979; An *et al.*, 1989), carotenoid production in *R. rubra* mainly occurs in the stationary phase (Goodwin, 1972; 1959). Light is important for the regulation of carotenoid biosynthesis in a wide variety of microorganisms and Simpson *et al.* (1971) showed that *R. rubra* is no exception to this phenomenon.

Environmental factors such as light, temperature, oxygen and carbon dioxide concentrations have been reported to have an effect on carotenogenesis (Bramley, 1985). Other controlling factors that have been reported include regulation of oxidative metabolism, kinetic control of individual biosynthetic enzymes, compartmentalization of enzymes and substrates, repression of regulatory genes by light and developmental processes (Tada, 1989; Tada *et al.*, 1990).

2.10 Analysis and identification of carotenoids.

1. Disruption and extraction: Extraction of carotenoids involves the disruption and homogenization of the biological material to release the pigments before pigment extraction can be carried out. Fresh plants and animal tissue are usually homogenized before or preferably during extraction directly in the organic solvent with an electric blender. Other means of homogenization may include mechanically grinding the

dried material into fine powder or using the 'Ultra-Turrax' or 'Polytron Homogenizer' for soft materials in small quantities.

For microbial cells such as yeast, the cells may be either broken by passage through a French press at high pressures or by shaking with beads in a Braun MSK Homogenizer before extraction of pigments with suitable solvents. Ultrasonic disintegration may also be used for some microbes especially if the amount of cells being used is comparatively small.

Chemicals as well as enzymes can also be used to digest the cell walls of yeast and other microorganisms. For example, Okagbue and Lewis (1984) used 2N HCl followed by mild heat treatment to hydrolyze the cell wall of *Phaffia rhodozyma* rendering the pigments extractable. Gentles and Haard (1991), Acheampong (1993) and Martin *et al.* (1993b) used the enzyme 'Funcelase' (glucanase) to digest the cell wall of *P. rhodozyma* in order to extract and quantify the pigments present in the yeast. Similarly, Okagbue and Lewis (1985) used a mixed culture of *Bacillus circulans* WL-12 and *P. rhodozyma* to render the yeast pigment extractable by the bacteria enzyme complex.

Extraction of the pigment from the disrupted biological material is usually accomplished with water-miscible organic solvents, most commonly acetone. The choice of solvent usually depends on the biological material, its pre-treatment, the nature of the carotenoid, and whether the objective is partial or complete extraction (Schiedt and Liaaen-Jensen (1995).

2. Isolation and identification of carotenoids.

Different forms of chromatography are used in the isolation and identification of various classes of carotenoids. Other procedures that lend themselves to the identification of carotenoids include ultra-violet/visible spectroscopy, infra-red spectroscopy (IR), mass spectroscopy (MS), nuclear magnetic resonance (NMR), resonance raman (RR) spectroscopy and circular dichromism. Only few of these methods will be discussed briefly below.

I) Column chromatography.

Column chromatography is the most important and efficient method for the separation of carotenoids (Davies, 1976). Most of the procedures are based on the principle of adsorption chromatography. Adsorbent are usually packed in columns in the form of a slurry in the initial solvent to be used and pigments to be separated are loaded onto the column and eluted with the mobile phase. Among the adsorbents traditionally used are basic materials such as MgO, $Ca(OH)_2$, and $CaCO_3$, which have been extensively used in carotenoid analysis. Other materials such as sugar, silica and alumina are also used in the separation of various classes of carotenoids.

II) Thin layer chromatography (TLC).

Thin layer chromatography is an invaluable technique for the qualitative analysis and identification of carotenoids. In carotenoid analysis, it has been used for the preliminary examination of carotenoid mixtures as a means of choosing suitable column systems, purification of carotenoids on a small scale and partial identification of carotenoids by their adsorption affinities (Davies, 1976). The adsorbents

commonly used are MgO, silica gel and plates with bonded phases such as surface - modified silica layers with a chemically bonded hydrocarbon chain (C_2 to C_{18}) (Schiedt, 1995). Different developing systems are used depending on the polarity of the pigment being analyzed.

III) High performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC) is another chromatographic method used for the separation of carotenoids. Because it is very efficient, gives an excellent separation of pigments in a short time, is highly sensitive and generates a great deal of information on the pigments being analyzed (Pfander and Riesen, 1995), HPLC has become the method of choice for carotenoid analysis. HPLC analysis can be carried out in either a normal-phase or reverse-phase modes but in recent years reverse-phase HPLC has become the method of choice. Silica and bonded nitrile columns are commonly used for normal-phase whereas alumina columns are used in reverse-phase HPLC (Taylor *et al.* 1990).

IV) HPLC-MS.

Mass spectrometry (MS) using different ionization techniques is one of the most powerful methods for the identification of organic compounds and has been used for structural identification of carotenoids (Vetter *et al.* 1971; Taylor *et al.* 1990). MS can be used in conjunction with the traditional HPLC separations in a single procedure to identify carotenoids. However, the high cost of instrumentation and maintenance has made it unavailable to some laboratories (Taylor *et al.* 1990).

v) Ultra-violet-visible spectroscopy.

The quantitative determination of carotenoids by spectrophotometry is based on the ability of the pigments to absorb visible, and in few cases, UV light. Spectrophotometer can be used to determine the concentration of pure carotenoid, or to estimate the total carotenoid in a mixture or natural extracts (Scheidt and Liaaen-Jensen, 1995). The characteristic maximal absorption peaks in the visible spectrum also gives valuable information, which will ultimately result in the identification of the carotenoid. The characteristic absorption spectrum is defined by the number of double bonds occurring in a particular carotenoid, various additional structural features and the type of solvent used (Vetter, 1971).

CHAPTER 3

INVESTIGATIONS INTO THE TAXONOMY OF *Rhodotorula rubra* TP1 USING CELLULOSE ACETATE ELECTROPHORESIS OF ENZYMES, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS

3.1 Introduction.

A red yeast contaminating homemade yogurt was isolated and tentatively identified as *Rhodotorula rubra* TP1 (Hari *et al.*, 1992). This new isolate displayed certain characteristics of the Basidiomycetes; it showed a lamella cell wall structure characteristic of Basiodiomycetes and a positive urease test. In addition, the isolate was reported to produce ascospores, a characteristic not usually associated with *Cryptococcacae* to which most of the pigment-producing yeasts belong. If the structures reported in the new isolate are ascospores, then it will be the first time a carotenoid producing yeast has been associated with ascospore production.

Even though some carotenoid producing yeasts such as *Saitoella complicata* (Goto *et al.*, 1987) and some species of *Taphrina* and *Protomyces* (van Eijik and Roeymans, 1982) have been shown to have Ascomycete affiliation, none of them have actually been shown to produce ascospores. In the case of the new isolate, previous biochemical tests including assimilation of various carbohydrates indicated

that it belongs to the genus *Rhodotorula* (Hari *et al.*, 1992). Due to the uncertainty surrounding the phylogenetic affinity of this isolate, it was the objective of this study to confirm both the production of ascospores and the biochemical and nutritional characteristics of the isolate, and hence to clarify the phylogenetic affinity of the isolate. As part of the identification process, the study also evaluated the usefulness of cellulose acetate electrophoresis as a tool in yeast taxonomy and also employed numerical analysis of the electrophoretic mobility of enzymes as a tool for identifying the new yeast isolated from yogurt.

Protein electrophoresis is a useful taxonomic tool that has been used to discriminate between yeast species, and in some cases, strains. For example, Martini and Vaughan-Martini (1990) used the electrophoresis of 11 isofunctional enzymes to establish 13 species in the genus *Kluyveromyces*. Similarly, Yamazaki and Komagata (1981) clarified the taxonomic relationship between strains in the genera *Rhodotorula* and *Rhodosporidium* by comparing the electrophoretic mobility of seven enzymes fructose-1,6-diphosphate aldolase (FA), 6-phosphogluconate (6PGDH), malate dehydrogenase (MDH), hexokinase (HK), phosphoglucomutase (PGm), glucose-6-phosphate dehydrogenase (G6PDH) and glutamate dehydrogenase (GDH). Similarly, Holzschu (1981) used the banding patterns of 14 metabolic enzymes to determine the evolutionary relationships among 400 strains of cactophilic strains of *Pichia* and separated a number of apparent similar species.

3.2 Materials and methods.

Organisms: The organism used for the sexuality studies was *R. rubra* TP1. The following organisms were used as controls in the enzyme, biochemical and physiological characterization: *Rhodotorula rubra* ATCC 9449, *Rhodotorula graminis* ATCC 16727, *Rhodotorula minuta* ATCC 10658, *Phaffia rhodozyma* ATCC 24202, *Rhodosporidium toruloides* ATCC 10657, *Cryptococcus macerans* ATCC 2194, *Rhodotorula glutinis* and *Saccharomyces cerevisiae* (Culture Collection, Dept. of Biology, Memorial University of Newfoundland, St. John's, NF.)

Chemicals: Trizma base (Tris[hydoxymethylaminoethane]), glucose and all other carbon sources were purchased from Sigma Chemical Company, St. Louis, Missouri. YM broth, plain agar, Chritensen urea agar, yeast nitrogen base, yeast carbon base, bactopeptone, yeast extract and malt extract were obtained from DIFCO Laboratories, Detroit, Michigan. Sodium acetate, potassium nitrate, acetic acid and methanol were purchased from Fisher Scientific Ltd., Fair Lawn, New Jersey.

3.2.1 Induction of sporulation.

The following media used in the previous study (Hari *et al.*, 1992) were used in an attempt to induce sporulation in the new isolate.

1. Aqueous agar (van der Walt and Yarrow, 1984) constituting 2% agar.
2. Yeast extract glucose agar (van der Walt and Yarrow, 1984) constituting 0.5% powdered yeast extract, 2% glucose and 2% agar.

3. Fowell's acetate agar (Fowell, 1952). This medium was made up of 0.5% sodium acetate trihydrate and 2% agar.

4. Malt extract agar (van der Walt and Yarrow, 1984). The composition for this medium was 5% malt extract and 3% agar.

3.2.1.2 Method for the induction of sporulation.

A 10-mL aliquot of a pre-sporulation medium containing 5% dextrose, 1% yeast extract and 2% bactopeptone was inoculated with the yeast and incubated at 30° C for 48 hours with occasional shaking . Cells were centrifuged, washed 3 times with deionized water and resuspended in 0.5-mL deionized water. A loopful was used to inoculate sporulation medium and incubated at 25° C for 5 days. Cells were observed under a microscope for sporulating cells.

3.2.2 Biochemical characterization of *Rhodotorula rubra* TP1.

3.2.2.1 Assimilation of carbon compounds.

Wickerham's (1951) inoculation medium was used for this study. A 10-fold concentrated solution of yeast nitrogen base was prepared by dissolving 6.7 g of yeast nitrogen base and 5 g of the appropriate carbon compound in 100 mL deionized water. The solution was filter-sterilized and aliquots of 0.5 mL were pipetted into clear, plugged, sterile 16-mm tubes containing 4.5-mL sterile deionized water. The tubes were then inoculated with 0.1 mL of inoculum prepared as described by

Wickerham (1951). Test tubes containing only the yeast nitrogen base solution were similarly inoculated and used as controls. After inoculation, the tubes were incubated for 3 weeks at 28°C with occasional shaking and examined at weekly intervals.

3.2.2.2 Assimilation of nitrogen compounds.

Wickerham's (1951) liquid medium was used. The medium was prepared in a 10-fold concentration by dissolving 11.7g yeast carbon base together with 0.78 g potassium nitrate in 100 mL of deionized water and then filter-sterilized. The final medium was prepared by transferring aseptically 0.5 mL aliquots of the sterile 10-fold concentrated into 4.5 mL of sterilized deionized water in 16 mm plugged tubes. Blanks tubes were prepared and used as controls. The nitrogen assimilation tubes received the same inoculum as was used in the carbon assimilation tests and were incubated at the same temperature. After the tubes were incubated for one week, a second set of test tubes was inoculated with one loopful from the first and results recorded after one week of incubation.

3.2.2.3 Urease test.

Production of urease was determined by inoculating the culture in Christensen urea agar and the result were recorded after two days.

3.2.2.4 Diazonium blue B (DBB) test.

The method of Hagler and Ahearn (1981) was used. This was done to

determine the ascomycetes or basidiomycetes affiliation of the new isolate.

3.2.3 Electrophoresis of enzymes.

3.2.3.1 Cultivation of organisms.

The culture medium used was YM broth at a concentration of 40 g per L of deionized water and a pH of 7.2. The yeasts were grown in 2 L Erlenmeyer flasks containing 500 mL medium, incubation was for 5 days at 22°C with shaking. The cells were harvested by centrifugation in a Sorvall RC-5C *Plus* centrifuge (Dupont-Sorvall Instruments, Newark, Connecticut, U.S.A.) at 10,000 x g for 20 minutes at a temperature of 4°C. The harvested cells were washed three times with deionized water, freeze-dried and stored at -85°C until they were used.

3.2.3.2 Enzyme preparation.

Cells were suspended into a thick slurry in 0.05 M Tris-HCl buffer (pH 7.8) and then broken in a French Press (SLM Instruments Inc., Urbana, Illinois) at a pressure of 40,000 psi. Broken cell debris was removed by centrifugation at 20,000 x g at 4°C. The supernatant fluid was collected and stored at -85°C in aliquots of 5 mL in glass scintillation vials until required for use.

3.2.3.3 Cellulose acetate electrophoresis.

A type Super Z-12 application kit and Titan III cellulose acetate plates

(Helena Laboratories) were used for horizontal electrophoresis. Plates were pre-soaked in Tris-glycine buffer (pH 8.5) prior to application of enzyme extracts. Electrophoresis was carried out at 2 mA/plate for 20 - 30 minutes in Tris-glycine buffer in at room temperature.

3.2.3.4 Enzyme staining procedures.

Because of the important roles they play in the metabolism of glucose by yeasts, the following eight enzymes were studied. 6-phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.41), glucose-6-phosphate dehydrogenase (G6PDH; EC1.1.1.49), phosphoglucomutase (PGM; EC 5.4.2.2), Mannose-6-phosphate isomerase (MPI; EC 1.1.1.37), isocitrate dehydrogenase (IDH; EC 1.1.1.42), hexokinase (HK; EC 2.7.1.1), malate dehydrogenase (MDH; EC 1.1.1.37) and glucose-6-phosphate isomerase (GPI). Specific stains as described by (Herbert and Beaton, 1993) were used to visualize the above mentioned enzymes

After electrophoresis, the plates were removed from the chamber and placed on a levelled glass surface. Molten agar at 60°C was added to the stain mixture and poured immediately over the plates. The plates were then incubated in the dark until the isozyme bands could be seen clearly. Plates were then washed 2 - 3 times under running tap water and the bands fixed by immersing plates in a mixture of acetic acid: methanol: deionized water (1:4:10) for about 10 minutes. The plates were dried overnight, photographed and the distance moved by individual isozyme bands measured. Three replicate plates were prepared for each yeast isolate, each replicate

representing a separate homogenate of each of the various yeast isolates. The migration rates of the isozyme were compared by computing the relative electrophoretic mobility (μ) (Lehninger, 1979). Isozyme bands among isolates were considered to be the same if their μs were within 10% of one another. This margin of error was chosen because in the analyses the observed variation in the three replicates of the same isozyme was always found to be within 10% of one another.

3.2.3.5 Numerical analysis.

Similarity was calculated for each isozyme by the following formula (Sneath and Sokal, 1973).

$$S\% = \frac{2N_{AB}}{N_A + N_B} \times 100$$

Where S: Similarity value

N_{AB}: The number enzyme bands with identical μ

N_A: The number of enzyme bands of yeast species A.

N_B: The number of enzyme bands of yeast species B.

The overall similarity between species was found by averaging the similarity values for the individual enzyme bands.

3.3 Results.

3.3.1 Sexuality in *R. rubra* TP1.

Fig. 3.0 depicts a photograph of the morphological characteristics of the new isolate *R. rubra* TP1 grown in different sporulation media. This study was repeated several times over a period of one year. The cells of *R. rubra* TP1 vary from short ovoidal to elongated, single, in pairs and short chains. Some of the cells can be seen budding. Unlike a previous report (Hari *et al.*, 1992) there was no evidence of the production of any structures resembling ascospores or teliospores on any of the media tested. As a result no further tests were done on the cells as may have been necessary should ascospores have been produced.

3.3.2 Biochemical characteristics.

3.3.2.1 Assimilation pattern of carbon compounds.

The nutritional and biochemical characteristics of the new isolate and other yeast genera as illustrated by the growth on different carbohydrates are depicted in Table 3.0. Both the new isolate and *R. rubra* ATCC 9449 were found to utilize a wide range of carbohydrates including, galactose, trehalose, melizitose and glycerol succinic acid, soluble starch and α-methyl-D-glycoside. They however lacked the ability to assimilate inositol.

3.3.2.2 Assimilation of nitrate, urease and DBB tests.

The new isolate and *R. rubra* were both found to be nitrate negative, urease positive and gave a positive DBB reaction.

3.3.3 Isozyme analysis

Figs. 3.1, 3.2 and 3.3 show the electrophoretic patterns of the new isolate *R. rubra* TP1 and the other yeast isolates used as control. Table 3.1 presents the data on the electrophoretic mobility (μ) values for eight enzymes of the yeasts tested. The overall similarity values determined from the various isozymes are given in Table 3.2. From Fig 3.1 to 3.3, it can be seen that the new isolate had zymograms that were identical to those of some of the controls whereas some of the control organisms have their own characteristic zymograms.

3.3.3.1 Mannose-6-phosphate dehydrogenase (MPI).

These isozyme banding patterns were the same for *R. rubra* TP1, *R. rubra* ATCC 9449 and *R. minuta*. Each of these three isolates possessed double bands of MPI activity that migrated cathodally (Fig. 3.1A). According to the electrophoretic mobility (μ) measurements (Table 3.1), the same cathodal bands 1 and 7 were common to these three isolates. *R. graminis* on the other hand, possessed two cathodal bands one of which (band 1) was identical to that of the new isolate but the other band (band 6) was electrophoretically different. Similarly some of the other

isolates possessed bands that were electrophoretically distinct from those of the new isolate. For example, *S. cerevisiae* had three bands, one that migrated anodally (band -1) and two other cathodal bands (bands 2 and 3) that had different electrophoretic mobility from those of the new isolate (Table 3.1); *R. glutinis* possessed two cathodal bands (3 and 5); *P. rhodozyma* had two cathodal bands (4 and 6); *Cr. macerans* had one cathodal band (band 4) and *Rhodosp. toruloides* had one cathodal band at position 6.

3.3.3.2 Glucose-6-phosphate isomerase (GPI).

R. rubra TP1 had identical zymograms and electrophoretic mobilities (µs) with *R. rubra* ATCC 9449, *R. minuta* and *R. graminis* The zymogram consisted of two bands (1 and 4) that migrated toward the cathode (Fig 3.1B and Table 3.1). *R. glutinis* had two cathodal bands (bands 1 and 4) and band 4 was identical to that possessed by *R. rubra* TP1, *R. rubra* ATCC 9449, *R. minuta, R. graminis*, *Cr. macerans* and *R. toruloides*. *Cr. macerans* had two extra bands, bands 2 and 5. *P. rhodozyma* had one band (band 2) with the same electrophoretic mobility as that of *Cr. macerans* (Fig. 3.1B and Table 3.1). *S. cerevisiae* had three discernible bands one of which migrated anodally (band -1) and the other two bands, 1 and 3 migrated towards the cathode. Band 3 was similar to that of *R. glutinis* (Fig. 3.1B and Table 3.1).

3.3.3.3 Malate dehydrogenase (MDH).

The MDH isozyme banding patterns of *R. rubra* TP1, *R. rubra* ATCC 9449, *R. minuta* and *Rhodosp. toruloides* were identical (Fig. 3.1C and Table 3.1). These isolates have common cathodal bands at 2 and 3 with the same μ. *R. glutinis* had a cathodal band at 3 similar to those of the above-named isolates and an additional band at position 1 which was also identical to one of the bands of *Cr. macerans*. *Cr. macerans* also shared a common band at position 4 with *S. cerevisiae*. There was a band at position 5 that was unique to *S. cerevisiae* (Fig. 3.1C and Table 3.1).

3.3.3.4 Hexokinase (HK)

The hexokinase isozyme banding patterns for the various yeast isolates are depicted in Fig 3.2A. *R. rubra*, *S. cerevisiae*, *R. glutinis*, *P. rhodozyma* and *Cr. macerans* all had the same cathodal bands at position 1. The respective electrophoretic mobilities are also similar (Table 3.1). In addition, *S. cerevisiae* had two additional bands one of which moved cathodally at position 2 and the other anodally at position -1. *P. rhodozyma* also had an additional band but no anodal band whereas *Cr. macerans* had an additional band at position 3. *Rhodosp. toruloides* had no band and *R. glutinis* had one band that moved cathodally at position 2. Thus, *R. rubra* TP1, *R. rubra* and *R. graminis* were found to be the same with respect to this isozyme in that they all possessed single bands at position 1.

3.3.3.5 Isomerase dehydrogenase (IDH).

This enzyme produced identical banding patterns and μs for *R. rubra* TP1, *R. rubra, R. minuta, R. graminis* and *Cr. macerans*. These isolates had cathodal bands at position 2 and 5 (Fig. 3.2B and Table 3.1). *S. cerevisiae* also had a band at position 2 but it differed from these isolates in that it had two extra cathodal bands at positions 1 and 6 and no band at position 5. *Rhodosporidium toruloides* shared a common band at position 6 with *S. cerevisiae* and had another band at position 3, which was not present in *S. cerevisiae*. *R. glutinis* and *P. rhodozyma* on the other hand had identical bands at position 4 and therefore differed from the other isolates in this respect.

3.3.3.6 6-phosphogluconate dehydrogenase (6PGDH).

R. rubra TP1, *R. rubra* ATCC 9449, *R. minuta*, and *R. graminis* had identical zymograms and electrophoretic mobility values for 6PGDH (Fig 3.2 C and Table 3.1, respectively). Each of these four isolates possessed an anodal band (band -1) and two cathodal bands (bands 2 and 3). *P. rhodozyma* and *Cr. macerans* on the other hand, had one band each (band -1) which were identical, moved anodally and similar to the anodal band of *R. rubra* TP1. *S. cerevisiae* and *R. glutinis* each had a cathodal band at position 3, which were also identical to band 3 of *R. rubra* TP1. In addition, *S. cerevisiae* had an additional band at position 1. *Rhodosp. toruloides* had two bands at position 2 and 3 that were identical to the bands possessed by *R. rubra* TP1.

3.3.3.7 Phosphoglucomutase (PGM).

With the exception of *S. cerevisiae* which possessed five bands and *Cr. macerans* which possessed two bands, all the other isolates had only single bands. This is the only isozyme for which *R. rubra* TP1 and *R. rubra* did not share common banding patterns. *R. rubra* TP1 had a single band of activity that migrated towards the cathode (Fig. 3.3A). *R. rubra, R. minuta, R. graminis, R. glutinis, Rhodosp. toruloides* and *P. rhodozyma* all had a single cathodal band of activity but the electrophoretic mobility was greater in *R. rubra* TP1 than in any of these isolates. *Cr. macerans* possessed a band at 7 whose electrophoretic mobility was identical to that of *R. rubra* TP1, however, it had an additional band at 3 that was not present in *R. rubra* TP1. *S. cerevisiae* was unique with respect to this isozyme in that it possessed as many as 5 isozyme bands which none of the other isolates possessed.

3.3.3.8 Glucose-6-phosphate dehydrogenase (G6PDH).

Isozymes of six isolates, *R. rubra* TP1, *R. rubra* ATCC 9449, *R. minuta, R. glutinis, P. rhodozyma* and *R. graminis* all possessed single bands of activity that migrated to the cathode (Fig. 3.3B). According to the μ values (Table 3.1), these bands (band 1) were identical for these isolates. *Cr. macerans* and *Rhodosp. toruloides*, on the other hand, possessed no bands and *S. cerevisiae* possessed a band at position 3.

3.3.4 Similarity of isozymes and their of electrophoretic mobility.

It is clear from above that the new isolate produced different zymograms for each of the enzymes studied, and all zymograms except PGM were identical to those produced by *R. rubra*. Of the eight yeast isolates used as control, *R. rubra* ATCC 9449, *R. minuta* and *R. graminis* produced enzyme banding patterns identical to the new isolate for GPI, 6PGDH, HK, IDH and MDH. In addition, the electrophoretic pattern of GPI was identical for the new isolate, *R. rubra* and *R. minuta*. All the yeasts tested produced identical patterns for G6PDH except *S. cerevisiae*, which produced a unique zymogram pattern for this enzyme. Thus, except for PGM, the new isolate and *R. rubra* ATCC 9449 produced zymograms that were identical.

The overall similarity values calculated from the electrophoretic mobilities of the various enzymes studied shows that the new isolate and *R. rubra* ATCC 9449 had the highest similarity, 0.88, followed by *R. minuta* which had a 0.67 similarity with the new isolate and the least is *P. rhodozyma*, which has a 0.13 similarity with the new isolate, the rest of the isolates have similarity values in between the highest and the lowest values (Table 3.2). The 0.88 similarity value shared by the new isolate and *R. rubra* suggests that the two may belong to the same species.

3.4 Discussion.

The morphological, biochemical and physiological characteristics of the new yeast isolate were re-investigated in this study. Both the organism and *R. rubra* ATCC 9449 were found to be nitrate negative. They were also found to exhibit identical utilization patterns for the various carbon sources that are usually used in the identification of yeast (Fell *et al.*, 1984). For example, both organisms were found to utilize such carbon sources as melizitose, raffinose and maltose but were unable to utilize erythritol, melibiose and inositol. According to Fell *et al.* (1984), key characteristics used in the identification of species in the genus *Rhodotorula* are utilization patterns of nitrate, melibiose, maltose, melizitose, raffinose and erythritol. *Rhodotorula rubra* utilizes raffinose, melizitose and maltose but unable to utilize nitrate, melibiose and erythritol (Fell *et al.* 1984). The fact that the new isolate had utilization patterns identical to those suggested by Fell *et al.* (1984) strongly suggests that the organism may be a variant strain of *R. rubra*. Furthermore, *R. rubra* is reported to be urease positive and also gives a positive DBB reaction. In this study both the new isolate and *R. rubra* ATCC 9449 were found to be urease positive and also reacted positively to DBB test. This, therefore, confirms that the organism is a variant strain of *R. rubra*.

Attempts were made to induce sporulation in the new yeast isolate by employing different types of sporulation media over an extended period of time. It is however, reported here that all those attempts to reproduce sporulation in the new isolate were not successful.

In a comprehensive study of this new isolate, Hari *et al.* (1992) reported that the new isolate showed a lamellar structure, a feature characteristic of the Basidiomycetes (Kreger-van Rij). These findings, coupled with the biochemical and physiological characteristics of the new isolate reported in this study supports its basidiomycetous affiliation. It will therefore be remarkable if this isolate produces ascospores as opposed to teliospores that are produced by some basidiomycetous yeast. There is no known pigment-producing yeast that produces ascospores even though two strains of yeasts belonging to the family *Cryptococcaceae* formerly identified as *Rhodotorula glutinis* are now considered to have ascomycetous affinity. These two strains have now been reclassified into a new genus *Saitoella* with a single species *Saitoella complicata* (Goto *et al.* 1987). It should be pointed out that reclassification of these organisms was not based on the production of ascospores because they do not produce any, but rather on their negative Diazonium blue B (DBB) reaction and a cell wall ultrastructure typical of ascomycetous yeasts. A perfect state of a *Rhodotorula* species was first reported by Banno (1963) who observed the formation of teliospores in strains of *R. glutinis* after conjugation of two opposite mating type strains. He described the genus *Rhodosporidium* and the species *Rhodosporidium toruloides* to accommodate this teleomorph. Since then numerous *Rhodosporidium* species have been described by other workers (Fell, 1970; Newell and Fell, 1970; Newell and Hunter, 1970; Fell *et al.*, 1973; Fell and Tallman, 1980). If the new isolate was a teleomorph of *Rhodotorula*, then one would expect that it would produce teliospores. The argument could then be made that the new isolate is

a closely related and undescribed taxon of *Rhodosporidium*. However, the evidence gathered in this and the previous study by Hari *et al.* (1992) does not support this argument. For example, if the isolate is an undescribed taxon of *Rhodosporidium* then one would expect the new isolate to share homology with *Rhodosporidium* in terms of carbohydrate assimilation patterns and other biochemical analysis. However, it was observed in this study that the new isolate differed from *Rhodosp. toruloides* in a number of biochemical tests including the assimilation of soluble starch, succinic acid, galactose and α-methyl-D-glucoside. Furthermore, Hari *et al.* (1992) reported that the new isolate had a lamella cell wall structure that is peculiar to basidiomycetous yeasts. Finally, attempts to induce sporulation in the new isolate using conditions identical to those reported by Hari *et al.* (1992) were not successful. Considering all these facts, it would be imprudent to consider the new isolate, *R. rubra* TP1 as a closely related and undescribed taxon of *Rhodosporidium* as suggested by Hari *et al.* (1992).

Cellulose acetate electrophoresis proved effective in discriminating between the different yeast isolates studied. Overall, the isozyme profile of this isolate contains elements of various species of *Rhodotorula*. Of the eight enzymes studied, seven of them, MPI, GPI, 6PGDH, MDH, IDH, G6PDH and HK, yielded zymograms that were identical for both the new isolate and *R. rubra* ATCC 9449. Only one enzyme, PGM, yielded zymograms different for these two isolates. *R. minuta* also shared identical zymograms with the new isolate for four of the enzymes, GPI, MDH, MPI, 6PGDH and G6PDH while *R. graminis* shared identical zymograms with the

new isolate for GPI, MDH, G6PDH, 6PGDH and HKS. *R. glutinis* and *P. rhodozyma* had only one zymogram identical to that of the new isolate; *Cr. macerans*, *S. cerevisiae* had none and *Rhodosp. toruloides* had only one enzyme MDH. Overall, *R. rubra* had the highest similarity with the new isolate, 0.88 for all the enzymes studied.

The fact that one enzyme, PGM, produced a different zymograms for the two isolates *R. rubra* TP1 and *R. rubra* ATCC 9449, suggests that the use of a single enzyme will not be sufficient for the identification of an organism, but rather a combination of enzymes would permit such a task. According to Yamazaki and Komagata (1981), identical enzyme mobility is not always a proof of structural similarity. These authors contend that differences in mobility are likely to be reflections of primary structural differences. As a result they recommend that in differentiating between different strains of yeast electrophoretic mobility of combinations of enzymes should be examined in order to be able to accurately identify them. In the present study, 8 different enzymes were studied and it was observed that the new isolate and *R. rubra* ATCC 9449 had identical profiles for seven of the eight enzymes studied. It was also observed that the new isolate had only one identical enzyme (MDH) with *Rhodosporidium*. Furthermore, the new isolate had only 0.52 similarity with *Rhodosporidium* for the electrophoretic mobility of the enzymes studied. In contrast *R. rubra* ATCC 9449 had 0.88 with the new isolate.

These findings, coupled with the fact that no structures resembling ascospores

or teliospores were found in this study strongly suggests that the new isolate and *R. rubra* ATCC 9449 are closely related. The new isolate, may therefore, on the basis of the evidence presented so far be confirmed as a strain of *R. rubra.*

Cellulose acetate electrophoresis proved effective in clarifying the taxonomic relationship of the new isolate. Other electrophoretic procedures, such as polyacrylamide gel electrophoresis (Yamazaki and Komagata, 1981; Sidenberg and Lachance, 1983) have been used in the taxonomy of yeast but this is the first time that cellulose acetate electrophoresis has been used in yeast taxonomy. The data presented here indicate the usefulness of cellulose acetate electrophoresis of enzymes as an aid to the classification of yeasts. Cellulose acetate is relatively inexpensive technique, minimal amount of samples are required, gel run times can be as short as 20 to 30 minutes, and therefore, large sample sizes can be processed in a relatively short period of time.

Table 3.0 Nutritional and biochemical characteristics of *Rhodotorula rubra* TPI and other yeast isolates.

Test Compound	Growth								
	TPI	Rr	Rl	Pr	Cm	Rg	Rm	Rt	Sc
Inositol	-	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+	-
Sorbitol	+	+	w	+	w	w	+	+	w
Trehalose	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	-	+	+
Cellobiose	w	w	+	+	+	w	+	+	-
Melizitose	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	w	+	-
Melibiose	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	-
D-Xylose	+	+	+	+	+	-	w	+	-
Maltose	+	+	+	+	+	+	+	+	+
Rhamnose	-	-	+	-	-	-	-	w	-
Galactose	+	+	w	+	+	+	w	+	-
KNO_3	-	-	w	-	+	+	-	+	-
Galatin liquefaction									
Soluble starch	+	+	+	+	+	+	+	-	-
Succinic acid	+	+	+	+	+	+	-	-	-
Erythritol	-	-	+	+	w	-	-	w	-
Ribitol	+	+	-	-	+	-	-	-	-
Citric acid	+	+	-	-	-	-	-	+	-
Methyl-D-glucose	+	w	-	-	-	+	-	w	-
	+	+	-	-	+	-	-	-	w

Note: w = weak growth, + = growth, - = no growth. TPI = *R. rubra* TPI, Rr = *R. rubra* ATCC 9449, Pr = *P. rhodozyma*, Cm = *Cr. macerans*, Rg = *R. graminis*, Rl = *R. glutinis*, Rt = *Rhodosp. toruloides*, Sc = *S. cerevisiae*.

Table 3.1: Mean[1] electrophoretic mobility (cm^2/sec./v) of isozymes of enzymes in various yeast isolates.

No of bands[a]	TP1[b]	Rr	Sc	Rm	Rg	Rl	Pr	Cm	Rt
			MDH						
1	-	-	-	-	-	$5.80x10^{-5}$	-	$5.90x10^{-5}$	-
2	$6.82x10^{-5}$	$6.82x10^{-5}$	-	$6.82x10^{-5}$	$7.24x10^{-5}$	-	$6.83x10^{-5}$	-	$6.84x10^{-5}$
3	$1.30x10^{-4}$	$1.37x10^{-4}$	-	$1.30x10^{-4}$	$1.37x10^{-4}$	$1.39x10^{-4}$	-	-	$1.29x10^{-4}$
4	-	-	$1.76x10^{-4}$	-	-	-	-	$1.76x10^{-4}$	-
5	-	-	$2.64x10^{-4}$	-	-	-	-	-	-
			IDH						
1	-	-	$5.65x10^{-5}$	-	-	-	-	-	-
2	$8.53x10^{-5}$	$8.53x10^{-5}$	$8.50x10^{-5}$	$8.58x10^{-5}$	$8.54x10^{-5}$	-	-	$8.56x10^{-5}$	-
3	-	-	-	-	-	-	-	-	$1.47x10^{-4}$
4	-	-	-	-	-	$2.17x10^{-4}$	$2.18x10^{-4}$	-	-
5	$2.80x10^{-4}$	$2.78x10^{-4}$	-	$2.80x10^{-4}$	$2.76x10^{-4}$	-	-	$2.83x10^{-4}$	-
6	-	-	$3.70x10^{-4}$		-	-	-	-	$3.80x10^{-4}$
			G6PDH						
1	$2.19x10^{-4}$	$2.19x10^{-4}$	-	$2.12x10^{-4}$	$2.24x10^{-4}$	$2.24x10^{-4}$	$2.30x10^{-4}$	-	-
2	-	-	$3.86x10^{-4}$	-	-	-	-	-	-

Table 3.1 contd. Mean electrophoretic mobility (cm^2/sec./v) of isozymes of enzymes in various yeast isolates.

No of bands	TPI	Rr	Sc	Rm	Rg	Rgl	Pr	Cm	Rt
			MPI						
-1	-	-	5.21×10^{-5}	-	-	-	-	-	-
1	7.44×10^{-5}	7.44×10^{-5}	-	7.44×10^{-5}	7.44×10^{-5}	-	-	-	-
2	-	-	1.49×10^{-4}	-	-	-	-	-	-
3	-	-	2.08×10^{-4}	-	-	2.08×10^{-4}	-	-	-
4	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	2.70×10^{-4}	2.23×10^{-4}	2.31×10^{-4}	-
6	-	-	-	3.13×10^{-4}	2.93×10^{-4}	-	2.95×10^{-4}	-	2.99×10^{-4}
7	3.35×10^{-4}	3.35×10^{-4}	-	-	-	-	-	-	-
			GPI						
-1	-	-	4.28×10^{-5}	-	-	-	-	-	-
1	6.11×10^{-5}	6.11×10^{-5}	5.89×10^{-5}	6.11×10^{-5}	5.50×10^{-5}	-	-	-	-
2	-	-	-	-	-	-	1.96×10^{-5}	1.96×10^{-5}	-
3	-	-	1.53×10^{-4}	-	-	1.52×10^{-4}	-	-	-
4	1.83×10^{-4}	1.83×10^{-4}	-	1.83×10^{-4}	1.83×10^{-4}	1.83×10^{-4}	-	1.73×10^{-4}	1.73×10^{-4}
5	-	-	-	-	-	-	-	2.38×10^{-4}	-
			6PGDH						
-1	5.05×10^{-5}	5.07×10^{-5}	-	5.21×10^{-5}	5.05×10^{-5}	-	5.21×10^{-5}	5.21×10^{-5}	-
1	-	-	-3.37×10^{-5}	-	-	-	-	-	-
2	1.60×10^{-4}	1.68×10^{-4}	-	1.70×10^{-4}	1.68×10^{-4}	-	-	-	1.66×10^{-4}
3	2.27×10^{-4}	2.36×10^{-4}	2.10×10^{-4}	2.34×10^{-4}	2.27×10^{-4}	2.20×10^{-4}	-	-	2.20×10^{-4}

Table 3.1 contd. Mean electrophoretic mobility ($cm^2/sec./v$) of isozymes of enzymes in various yeast isolates.

No of bands	TP1	Rr	Sc	Rm	Rg	Rl	Pr	Cm	Rt
			PGM						
1	-	-	$8.73x10^{-5}$	-	-	-	-	-	-
2	-	-	$1.53x10^{-4}$	-	-	-	-	-	-
3	-	-	$1.82x10^{-4}$	-	-	-	-	-	-
4	-	-	$1.97x10^{-4}$	-	-	-	$2.04x10^{-4}$	$2.04x10^{-4}$	-
5	-	-	$2.18x10^{-4}$	-	-	-	-	-	-
6	-	$2.48x10^{-4}$	-	$2.55x10^{-4}$	$2.48x10^{-4}$	$2.55x10^{-4}$	-	-	$2.39x10^{-4}$
7	$2.77x10^{-4}$	-	-	-	-	-	-	$2.83x10^{-4}$	-
			HKS						
-1	-	-	$6.04x10^{-5}$	-	-	-	-	-	-
1	$7.54x10^{-5}$	$7.54x10^{-5}$	$7.46x10^{-5}$	$7.60x10^{-5}$	$7.54x10^{-5}$	-	$7.50x10^{-5}$	$7.50x10^{-5}$	-
2	-	-	$1.58x10^{-4}$	-	-	$1.51x10^{-4}$	$1.61x10^{-4}$	-	-
3	-	-	-	-	-	-	-	$1.96x10^{-4}$	-

[1]Values are the mean of three replicate plate, each plate representing a separate homogenate of the individual yeast isolate.
[a]Bands were numbered cathodal (+) or anodal (-) in increasing numerical order relative to the distance that they migrated away from the origin. Bands among isolates were considered the same if their μ were within 10% of each other. Dashes indicate absence of bands. [b]TP1 = *R. rubra* TP1, Sc = *S. cerevisiae*, Rm = *R. minuta*, Rg = *R. graminis*, Rl = *R. glutinis*,, Cm = *Cr. macerans*, Rt = *Rhodosp. toruloides*.

Table 3.2 Similarity matrix based on the mean electrophoretic mobility (μ) of eight isozymes of enzymes used in this study.

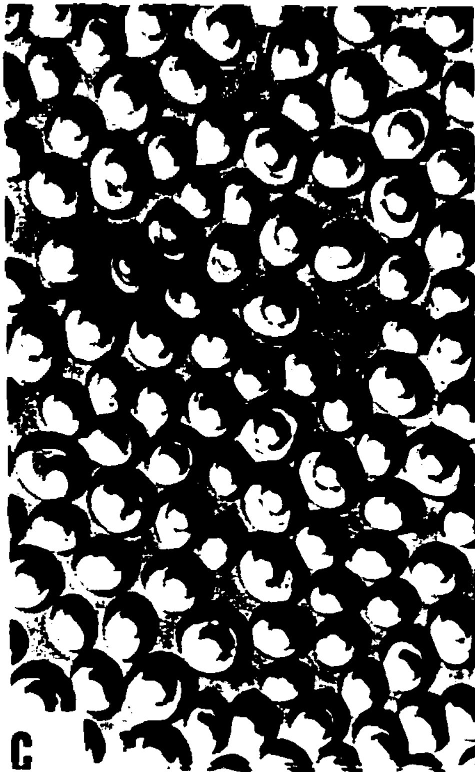
	TP1	Rr	Sc	Rm	Rg	Rl	Pr	Cm	Rt
TP1[1]									
Rr	0.875								
Sc	0.050	0.050							
Rm	0.667	0.854	0.232						
Rg	0.488	0.613	0.210	0.696					
Rl	0.413	0.538	0.256	0.471	0.596				
Pr	0.133	0.133	0.103	0.338	0.163	0.175			
Cm	0.258	0.258	0.350	0.192	0.113	0.150	0.271		
Rt	0.517	0.642	0.146	0.454	0.584	0.500	0.083	0.230	

[1] TP1 = New isolate, *R. rubra* TP1; Rr = *R. rubra*; Rl = *R. glutinis*; Rg = *R. graminis*; Rm = *R.. minuta*; Cm = *Cr. macerans*; Rt = *Rhodosp. toruloides*

Fig. 3.0. Cell morphology of *Rhodotorula rubra* TP1 grown in various sporulation media. A: aqueous agar, B: yeast extract glucose agar, C: Fowell's acetate agar, D: malt extract agar.

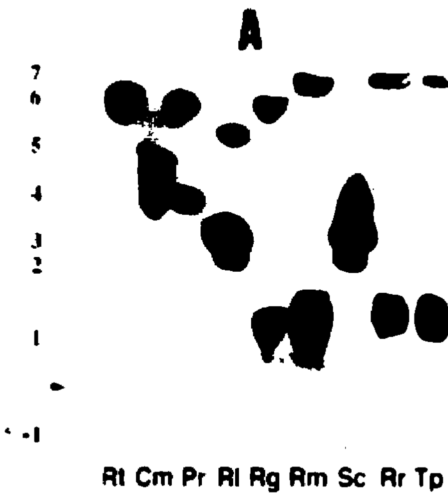
A

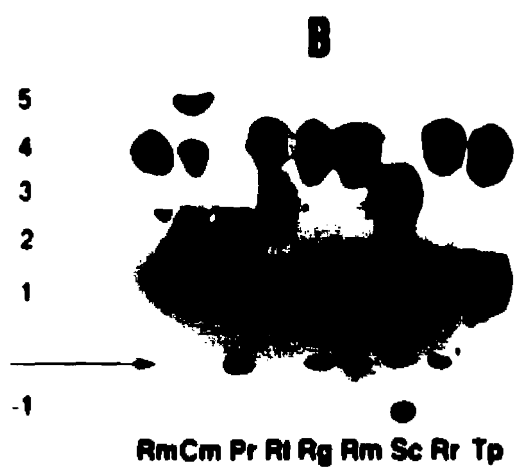
B


C

D

Fig. 3.1 Electrophoregrams of enzymes in nine yeast isolates studied. Rm = *R. minuta*, Rl = *R. glutinis*, Cm = *Cr. macerans*, Rg = *R. graminis*, Rr = *R. rubra*, TP1 = *R. rubra* TP1, Rt = *Rhodosp. toruloides*, Sc = *S. cerevisiae*. A = Mannose-6-phosphate-isomerase (MPI), B = Glucose-6-phosphate isomerase (GPI), C = Malate dehydrogenase (MDH).


A
7
6
5
4
3
2
1
-1
Rt Cm Pr Rl Rg Rm Sc Rr Tp


B
5
4
3
2
1
-1
RmCm Pr Rl Rg Rm Sc Rr Tp

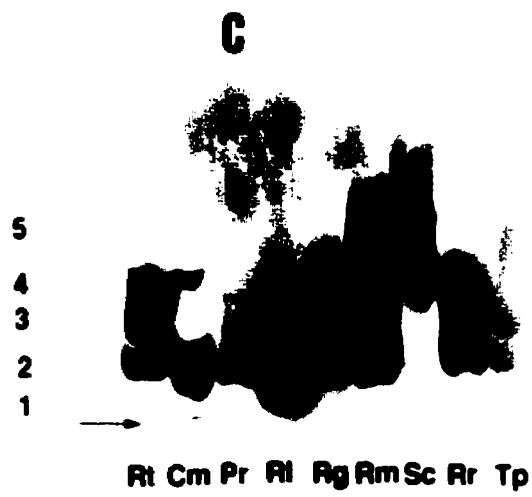
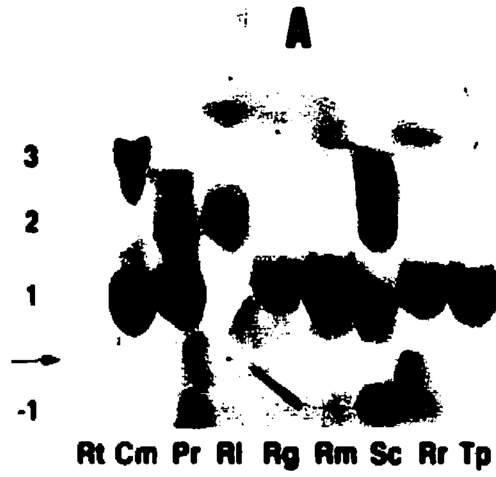
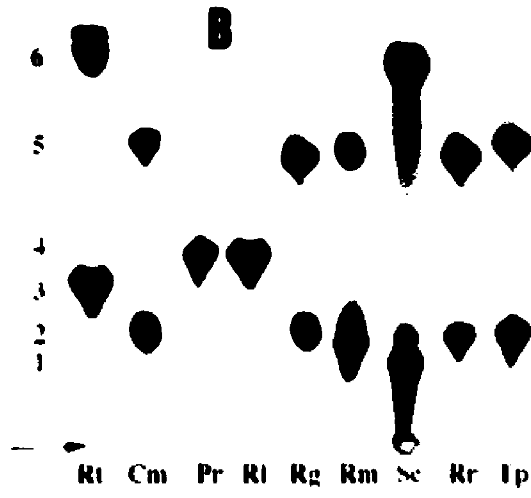

C
5
4
3
2
1
Rt Cm Pr Rl Rg Rm Sc Rr Tp

Fig. 3.2 Electrophoregrams of enzymes in nine yeast isolates studied. Rm = *R. minuta*, Rl = *R. glutinis*, Cm =*Cr. macerans*, Rg = *R. graminis*, Rr = *R. rubra*, TP1 = *R. rubra* TP1, Rt = *Rhodosp. toruloides*, Sc = *S. cerevisiae*. A = Hexokinase, B = Isocitrate dehydrogenase (IDH), C = 6-phosphogluconate dehydrogenase (6PGDH).


A
3
2
1
-1
Rt Cm Pr Rl Rg Rm Sc Rr Tp


B
6
5
4
3
2
1
Rt Cm Pr Rl Rg Rm Sc Rr Tp

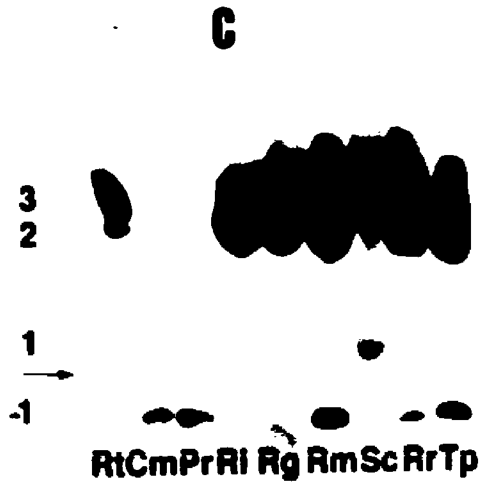
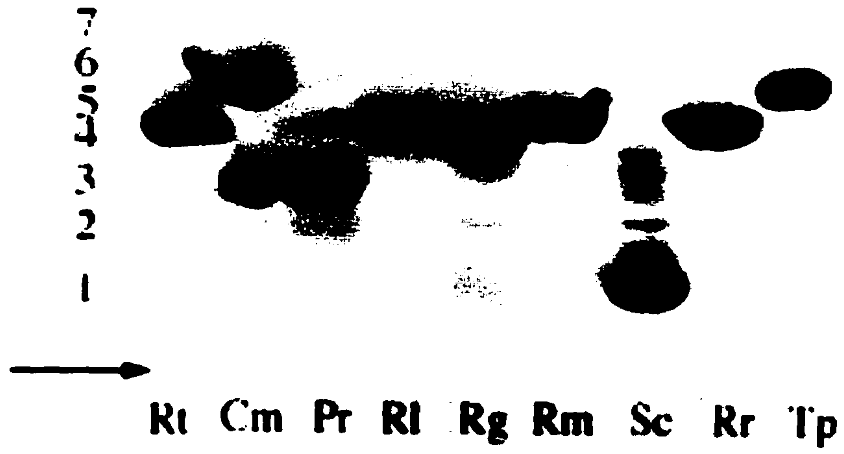
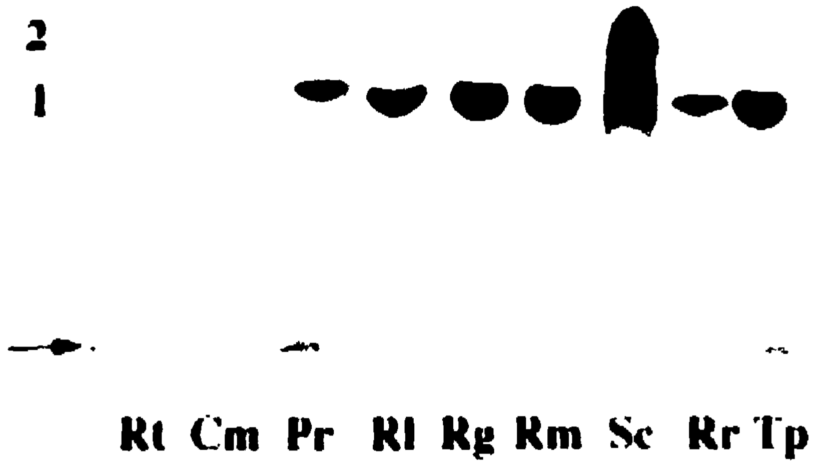

C
3
2
1
-1
RtCmPrRl Rg RmScRrTp

Fig. 3.3 Electrophoregrams of enzymes in nine yeast isolates studied. Rm = *R. minuta*, Rl = *R. glutinis*, Cm =*Cr. macerans*, Rg = *R. graminis*, Rr = *R. rubra*, TP1 = *R. rubra* TP1, Rt = *Rhodosp. toruloides*, Sc = *S. cerevisiae*, A = Phosphoglucomutase (PGM), B = Glucose-6-phosphate dehydrogenase (G6PDH).


A
7
6
5
4
3
2
1
Rt Cm Pr Rl Rg Rm Sc Rr Tp


B
2
1
Rt Cm Pr Rl Rg Rm Sc Rr Tp

CHAPTER 4

THE USE OF CELLULAR FATTY ACID IN THE TAXONOMY OF *Rhodotorula rubra* TP1.

4.1 Introduction.

Fatty acyl lipids such as triacylglycerol and polyisopropenoid ether lipids are produced by a wide range of microorganisms that include filamentous fungi, yeast and bacteria (Ratledge, 1994). In bacterial taxonomy, analyses of these lipids by gas-liquid chromatography (GLC) have already been established and provided commendable and convenient characters for classification and identification of many coryneform and actinomycetes genera (Goodfellow and Minnikin, 1982; Shaw, 1974; Collins *et al.*, 1982; Bousfield *et al.*, 1983). Abel *et al.* (1963) and Yamakawa and Ueta (1964) were the first to describe the use of GLC analysis of fatty acids as identification tool. Since then cellular fatty acid (CFA) analysis by gas-liquid chromatography has emerged as the method of choice in bacterial taxonomy, due to its high degree of accuracy and rapidity (Larsson *et al.*, 1989; Veys *et al.*, 1989). Data from these studies have shown that total cellular fatty acid analysis provides useful information for rapidly distinguishing between closely related bacteria.

The production of long chain fatty acid have been reported in several yeast isolates (Ratledge, 1994; Augustyn *et al.*, 1992; Blignaut *et al.*, 1996) and the use of

the fatty acid components as a tool in yeast taxonomy has become a common practice. Several workers (Kock *et al.*, 1985; Kock, 1988; Blignaut *et al.*, 1996; Moss *et al.*, 1982; Viljoen *et al.*, 1988; 1989; Augustyn *et al.*, 1991, 1992; Marumo and Aoki, 1990, van der Westhuizen *et al.*, 1991) have reported that cellular fatty acid data are useful for distinguishing between various strains, species and genera of yeast. GLC method combined with multivariate statistical analysis ensures an objective analysis of the ensuing chromatogram and a good linear discrimination between species (Marumo and Aoki, 1990; Blignaut *et al.* 1996; Smit, 1991; O'Donnell, 1980).

The fatty acids important in bacterial identification are in the range of C16 to C20. In yeasts Ratledge (1994) reported that C16 to C18 fatty acids predominate, namely C16:0 (palmitic), C18:0 (stearic), C18:1 (oleic), C18:2 (linoleic) and C18:3 (linolenic). Total cellular fatty acids ranging from C14 to C20 have also been detected in ascomycetes, basidiomycetes and other imperfect yeast (Augustyn, 1992; van der Westhuizen *et al.*, 1987, 1991; Cottrell *et al.*, 1985, Kock and Lategan, 1986; Smit *et al.*, 1987).

This report describes the application of discriminant analysis to gas-liquid chromatography results of methylated fatty acids (FAME) for the identification of a new yeast isolate, *Rhodotorula rubra* TP1 and various species of other yeast.

4.2 Materials and Methods.

Chemicals: Glucose, borontrifluoride, lauric acid and fatty acid standards were purchased from Sigma Chemical Company, St. Louis, Missouri; yeast nitrogen base (YNB) was from DIFCO Laboratories, Ltd., Detroit, Michigan; potassium hydroxide, hexane (Optima™ grade) and methanol (Optima™ grade) were obtained from Fisher Scientific Ltd., Fair Lawn, New Jersey

4.2.1 Organisms.

The organism used in this study was *Rhodotorula rubra* TP1. In addition, *Rhodotorula rubra* ATCC 9449, *Rhodotorula glutinis* and *Saccharomyces cerevisiae* (from the Culture Collection of Dept. of Biology, Memorial University of Newfoundland, St. John's, Newfoundland, Canada) *Rhodosporidium toruloides* ATCC 10657, *Rhodotorula minuta* ATCC 10658, *Rhodotorula graminis* ATCC 16727, *Cryptococcus macerans* ATCC 24194 and *Phaffia rhodozyma* ATCC 24202 were used as controls.

4.2.2 Inoculum preparation.

The method described by Koch *et al.* (1985) was used with slight modification. A loopful of each yeast isolate was grown in 50 ml of sterile medium consisting of 80 g/L glucose and 6.7 g/L yeast nitrogen base in a 250 Erlenmeyer Flask at 22°C for 24 hours. A 20 mL aliquot of this pre-inoculum was used to inoculate 500 mL of the same medium in a 2 L Erlenmeyer flask and grown to

stationary phase (ca. 4 days) at 22°C in a Psychrotherm Controlled Environment Incubator (New Brunswick Scientific Ltd., New Brunswick, New Jersey). Cells were harvested by centrifugation at 10,000 x g in a Sorval RC-5C *Plus* centrifuge (Sorvall Instruments-Dupont Ltd., Newark, DE.), washed several times (ca. 3 times) in a sterile physiological saline and then freeze dried. Freeze-dried cells were kept at -85°C until used.

4.2.3 Extraction of fatty acid methyl esters.

Approximately 0.12 g of freeze-dried cells was weighed into clean test tubes with teflon-lined screw caps. Tubes had previously been heated at 200°C for 24 hours in an oven to get rid of any lipid contaminants. A 5 mL aliquot of 15% KOH in 50% methanol plus 30 µL of 6% lauric acid internal standard in methanol was then added to each tube. The mixture was boiled at 100°C for an hour in a water bath, allowed to cool and the pH of the mixture adjusted to 2 with concentrated HCl solution. The resulting suspension was methylated by the addition of 3 mL of 14% borontriflouride in methanol solution, followed by flushing with nitrogen gas and heating in a boiling water bath in sealed tubes for 15 minutes with occasional shaking. The methyl esters were then extracted 2 times with 10 mL aliquots of hexane. The pooled hexane extracts (20 mL) were blown dry by passing a stream of nitrogen gas through them and the resulting residue were taken up in 1 mL hexane.

4.2.4 Gas chromatographic analysis.

Analysis of the methylated fatty acids was performed on a Perkin-Elmer Gas Chromatograph equipped with dual flame ionization detectors. A 2 µL aliquot of extract was injected onto a 30 m x 0.75 mm i.d. Supelco Wax 10 capillary column. The instrument was run under the following conditions: injection temperature 170°C; detector temperature 250°C; initial column temperature 145°C, then ramped to 225°C at 3°C/min; held at 225°C for 10 min and ramped again to 240°C at a rate of 3°/min. (Blignaut *et al.*, 1996). Nitrogen was used as a carrier gas at a flow rate of 3.9 mL/min; hydrogen and air were supplied at flow rates of 30 mL/min and 300 mL/min., respectively.

The resultant peaks were identified by comparing the retention times of the test samples with those obtained from a series of chromatographically pure standards. All species were cultivated in triplicate and each replicate analyzed once. The individual concentrations of each fatty acid determined from the three chromatograms obtained were used as data input for all statistical analyses.

4.2.5 GC-MS Conditions.

To confirm the identity of the fatty acids, the extracts were run on a GC-MS. The instrument consisted of a HP 5970 Mass Selective Detector coupled with a model 5890 Gas Chromatograph (Hewlett-Packard, Palo Alto, California) and a model 300 Data System from the same manufacturer. The column was a CP-Sil-5 CB, WCOT fused silica with a length of 25 m, inside diameter of 0.25 mm, outside diameter of

0.39 mm and a film thickness of 0.12 µm (ChromPack, The Netherlands). Chromatographic conditions were the same as those used in the GLC analysis. Mass spectra of the eluted fatty acids were identified by comparison with the mass spectra of standards run in parallel and those from literature.

4.2.6 Statistical analyses.

Stepwise discriminant analysis, 'jackknifing' procedure and canonical correlation (variate) analysis were used to examine the relationship between the test yeasts. These programs are available in the BMDP statistical computing package (Biomedical Computer Programs, University of California, Los Angeles, California). The BMDP 7M program was run on the Unix System at Memorial University of Newfoundland, St. John's, Newfoundland, Canada. The program was run using four different datasets that have been titled Applications 1 to 4 below.

Application 1 (6 variables): comprised all the FA data for all the yeast isolates employed in this study (Section 5.2.1).

Application 2 (6 variables): included only isolates that have more than 1% 18:3 and less than 30% 18:2, and these isolates were *R. rubra* TP1, *R. rubra*, *R. glutinis*, *R. minuta*, *Cr. macerans* and *Rhodosp. toruloides*.

Application 3 (6 variables) included only *R. rubra* TP1, *R. rubra*, *R. glutinis* and *R. minuta*.

Application 4: only *S. cerevisiae* was not considered and only 4 variables, C16:0, 16:1, 18:0 and 18:1 were used.

These combinations were done to determine whether or not any one particular fatty acid, major or minor, was important in discriminating between the individual groups.

4.3 Results.

The fatty acid composition of microorganisms is believed to be influenced by cultural conditions and the growth medium (Boulton and Ratledge, 1983; Ratledge, 1988); rigid control should therefore be exercised over these conditions in order to obtain reproducible results (Blignaut *et al.*, 1996). The cellular fatty acids of the yeast isolates utilized in this study were therefore determined using rigidly standardized methods developed by Koch *et al.* (1985). The fatty acid profiles (mean relative percentage of fatty acid) of all the yeast are presented in Table 4.0. It was observed that the yeast isolates contained predominantly saturated and unsaturated fatty acids with 16 to 18 carbon atoms. Visual examination of the fatty acid profiles of the various isolates indicated that *R. rubra* TP1, *R. rubra* and *R. glutinis* have similar fatty acid profiles whereas the rest of the isolates have their own unique fatty acid profiles. Overall, all the yeast studied contained large amounts of 18:1 (40-70%) but differ considerably in the composition of the other fatty acids.

The results presented in Table 4.0 were subjected to stepwise discriminant analysis (SDA), 'jackknifing' procedure and canonical variate analysis using different dataset (Applications) and groupings in an attempt to classify the new isolate.

The stepwise discriminant analysis is an iterative technique that allows the selection of the valid discriminant variables from the redundant or non-informative

ones (Anderson, 1958) using the minimum number of peaks. At each step of the analysis, the peak is selected for which the ratio of the variation between the species to the average variation within a species is a maximum. This ratio is called the F ratio and its multivariate generalization is the Wilks Lambda criterion (Kshirsagar, 1972; Rao, 1965). This procedure is repeated until either all the variables are included or the addition of any of the remaining variables does not increase the Wilks Lambda criterion (Jennrich and Sampson, 1981). At each stage, an approximate F value, which tests the significance of the improvement in discrimination, is determined. Thus an organism would be allocated to the species for which it achieves the highest F score.

The 'jackknifing' or 'leaving one out' procedure (Lachenbauch and Mickey, 1968) on the other hand, is used at each step of the discriminant analysis to test the stability of the discriminant functions obtained. Each strain is removed in turn for analysis and the discriminant functions are recomputed. The strains are then re-allocated to the species for which its discriminant score is maximized. Comparison of the percentage of correct allocations before and after 'jackknifing' gives an estimate of the stability of the discriminant functions (Lachenbauch and Mickey, 1968).

The F-value used for the calculation of the classification functions for the SDA in applications 1 to 4 are presented in Table 4.1. From Table 4.1, it can be seen that the F-values rate the fatty acids in order of decreasing importance in their use in the discrimination process. Careful analysis of these values for the various applications suggests that all of the fatty acids are important in the identification

process.

The results of the application of stepwise discriminant analysis with 'jackknifing' to the fatty acid data are depicted in Table 4.2. From the 'jackknife' classification matrix, it is clear that all the isolates except *R. rubra* TP1 and *R. rubra* ATCC 9449 were classified correctly during application 1 and all subsequent applications. In application 1, only 1 replicate of *R. rubra* TP1 was correctly identified as *R. rubra* TP1 whereas the 2 remaining replicates were identified as *R. rubra*. This finding was also true for *R. rubra* ATCC 9449 (Table 4.2). In application 2, none of the *R. rubra* TP1 replicates were identified as such, in fact all were identified as *R. rubra* whereas 1 replicate of *R. rubra* was identified as *R. rubra* TP1 and the rest as *R. rubra*. In applications 3 and 4, 2 replicates of *R. rubra* TP1 were identified as *R. rubra* and only 1 as *R. rubra* TP1 whereas 2 replicates of *R. rubra* were identified as such and only one as *R. rubra* TP1.

The canonical variates analysis is a descriptive method that gives the best separation among all the isolates studied. For each isolate, a set of new coordinates expressed as linear combination of the original variables (canonical variate axes) is determined. These are given by the principal components of the group means calculated with the Mahalanobis D^2 generalized distance (Anderson, 1958; Jennrich and Sampson, 1981). Plots of the first and second canonical variables for the various applications are depicted in Figs. 4.0 to 4.3. Fig. 4.0 shows that organisms were distributed among 8 clusters. The new isolate, *R. rubra* TP1 and *R. rubra* ATCC 9449 were clustered together whereas the rest of the organisms were distributed into

their own unique clusters. It can also be seen that the cluster containing *R. rubra* TP1 and *R. rubra* was close to *R. glutinis, Cr. macerans* and *Rhodosp. toruloides.* This indicates that these organisms may be related. Similarly, the organisms in the other plots (Figs. 4.1 to 4.3) appeared to be well separated except the cluster of *R. rubra* TP1 and *R. rubra* ATCC 9449. These results corroborated those achieved with the SDA in which over 66% of *R. rubra* TP1 chromatographic peaks were allocated to *R. rubra* whereas 100% of the chromatographic peaks of all the other isolates were allocated to their original groups. These plots therefore strongly suggest that *R. rubra* TP1 and *R. rubra* cannot be differentiated from each other and should therefore be considered as belonging to the same species.

4.4 Discussion.

Traditionally, yeast systematists have used morphological and physiological characteristics in determining the relationship between various isolates (van Unden and Beckley, 1970; Meyer *et al.*, 1984). However, to accurately assess the relatedness of various types of yeast, other criteria such as cellular composition of fatty acids (Viljoen *et al.*, 1987), the degree of DNA relatedness (Lethbak and Stenderup, 1969; Nakase and Komagata, 1971b), electrophoretic comparison of enzymes (Yamazaki and Komagata, 1981) and other tests are desirable.

Cellular fatty acid composition analysis was used to determine the relationship between the new yeast isolated from yogurt, tentatively identified as *R. rubra* TP1 and eight other yeast isolates. The data obtained was subjected to 'jackknifing',

stepwise discriminant and canonical variate analyses. It was found that the new isolate, *R. rubra* TP1 and *R. rubra* ATCC 9449 clustered together when subjected to canonical variate analysis. The two also demonstrated the best similarity as evident by the similarity matrix of the 'jackknifing' procedure. Out of three replicates of *R. rubra* TP1 fatty acid profile subjected to analysis, 2 were identified as *R. rubra* (66.7% homology). Although some of the other yeast isolates showed a FA profile similar to that of TP1, none of them were actually identified as belonging to *R. rubra* TP1 when subjected to the 'jackknife' procedure. This is a strong indication that they are not as closely related to *R. rubra* TP1 as *R. rubra* ATCC 9449 does.

Examination of the fatty acid data in Table 4.0 reveals that the new isolate *R. rubra* TP1, *R. rubra* and *R. glutinis* have high content of 18:1 (over 60%), followed by medium amount 16:0 (over 13%) and low amounts of 18:0, 18:2, 18:3 and o16:1 (2-7%) in each case. The percentage compositions of the various fatty acids determined for *R. rubra* TP1 and *R. rubra* ATCC 9449 are in agreement with those reported for *Rhodotorula rubra* by other workers. In a review on the occurrence of fatty acids in yeast, Ratledge (1994) pointed out that yeast generally exhibit conservative patterns of fatty acyl distributions with the vast majority producing only C16 and C18 fatty acids. The principal saturated fatty acid, according to him, is always palmitic acid (16:0), whereas the principal unsaturated fatty acid is oleic acid (18:0) except for a few species where 16:1 is dominant. The concentration of 18:2 may occasionally equal that of 18:1 but 18:3 is usually a minor component. Rattray (1988) on the other hand presented a data on the fatty acid from various yeasts

isolates which indicated that C18:0 acids are the dominant fatty acids in many yeast species. The data also suggests that C18:1 is the most common mono-unsaturated and predominant fatty acid in most yeasts while 16:1 may be entirely absent from the cellular fatty acid profiles of some yeast. Rattray (1988) indicated further that C16:1 is the major fatty acid in some members of *Saccharomyces* including *S. cerevisiae* which can have a C16:1 concentration of up to 63% of the total fatty acid present. He reiterated further that C16:0 is generally more abundant than C18:0 which occasionally may be absent altogether. However, in a study to determine the value of fatty acid analysis in the identification of oral yeasts, Blignaut *et al.* (1996) reported that *R. rubra* has a C18:1 concentration of 68.8% which is in agreement with the concentrations of the same fatty acid obtained for *R. rubra* TP1 and *R. rubra* in this study. Similarly, The fatty acid data published for two strains of *R. rubra* by Ratledge and Evans (1989) and Zuyaginsteva and Pitryuk (1975) indicated that C18:1 was the dominant fatty acid, 56.9 and 81.2% for the two strains, respectively. Furthermore the concentrations for the other fatty acids published by these same authors were similar to those obtained for *R. rubra* and *R. rubra* TP1 in this study. Based on the results obtained in this study it can be concluded that *R. rubra* TP1 and *R. rubra* are closely related if not the same species.

GLC analysis of fatty acids has been employed in the identification of several yeast isolates. Augustyn (1989) pointed out that it was possible to differentiate between a large number of *Saccharomyces cerevisiae* by means of cellular fatty acid analysis. Other workers (Augustyn *et al.*, 1991, 1992, 1996; van der Westhuizen *et*

al., 1991, Viljoen *et al.*, 1989, Smit *et al.*, 1988) have also used cellular fatty acid analysis as a tool for the identification of various yeasts isolates. Gangopadhyay *et al.* (1979), studying the cellular long-chain fatty acid composition of the anamorphic genera, *Candida, Cryptococcus* and *Torulopsis*, reported substantial differences in the CFA profile which resulted in the grouping of the yeasts into 38 distinct isolates. Similarly, Gunaskeran and Hughes (1980) reported that 85 different strains representing 7 *Candida* species, each generated a distinctive fatty acid profile characterized by the presence or absence of certain fatty acids when grown in specific medium. Using reference strains, they classify 70 yeast isolates into 5 species and concluded that the sensitivity and speed of long-chain fatty acid analysis by gas chromatography provides advantages over conventional physiological and morphological analytic methods.

Cellular lipid content, however, is influenced by cultural conditions, sporulation, the age of culture, growth factors, pH, temperature and amount of oxygen available to organisms during growth (Smit, 1991; Lechevalier and Lechevalier, 1988; Hunter and Rose, 1972). It has also been determined that major changes in the fatty acid composition occur during the exponential and early stationary phase but no significant changes occur during the stationary growth phase (Viljoen *et al.*, 1986; Smit, 1991). As a result a standardized and growth condition for fatty acid analysis of Kock *et al.* (1985) was used in this study to ensure stability in the fatty acid composition of the organisms.

Application of stepwise discriminant analysis to GLC data for discrimination

between closely related yeast species have been reported (Augustyn *et al.*, 1991, 1992). The application of discriminant and canonical variate analyses to GLC data in this study was able to separate the taxonomically different species. The procedure however, suggested that *R. rubra* TP1 and *R. rubra* are not separable and therefore belong to the same species. These results illustrate that discriminant and canonical variate analyses of the GLC chromatogram of fatty acids is a valuable tool in the identification of yeasts. The fact that the different applications used were able to separate the taxonomically different organisms into separate clusters indicates that all the fatty acids should be considered equally important when attempting to differentiate between yeast strains, species and genera.

Comparison of the relative percentages of the various fatty acids of the new isolate and *R. rubra* ATCC 9449 show some variation in the relative amounts, however, these variations are much less than those obtained when the new isolate is compared to the other yeast isolates used as controls. According to Augustyn *et al.* (1991), the cellular fatty acid profile of a type of particular yeast can be considered as defining a specific, uniquely shaped "envelope" in space around a point representing 0% for all fatty acids involved. The shape and volume of the "envelope" defining a species will then vary within limits imposed by the variation in relative percentages of all the fatty acids in various strains of that species. Point distortions of this envelope, they contended, will sometimes occur as a result of particular strains having unusually high or low relative percentage for a specific fatty acid.

Table 4.0 Mean[1] relative percentages of 6 fatty acids in 9 yeast isolates

YEAST TYPE	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
R. rubra TP1	13.22	3.00	6.03	69.69	5.40	3.00
R. rubra	13.40	2.69	5.93	70.12	4.97	3.29
S. cerevisiae	10.65	54.55	3.69	30.67	0.00	0.00
P. rhodozyma	2.74	11.94	13.55	40.55	30.29	0.61
R. glutinis	13.97	6.54	6.13	62.47	5.96	3.70
R. graminis	12.66	1.51	6.67	50.53	22.54	5.98
R. minuta	9.78	11.58	3.32	43.92	28.10	3.06
Cr. macerans	15.79	2.11	11.57	54.26	13.85	1.89
Rhodosp. toruloides	17.24	3.83	3.18	58.94	11.73	4.64

[1]Values are mean of three determinations.

Table 4.1. Fatty acids and F-values used for the calculation of classification functions in order of decreasing importance.

Application 1		Application 2		Application 3		Application 4	
Fatty acids	F-value	Fatty acids	F-value	Fatty acids	F-value	Fatty acids	F-value
C16:1	2469.60	C18:2	413.78	C18:2	620.04	C16:1	297.98
C18:2	504.92	C18:0	126.22	C16:1	40.31	C18:1	172.41
C18:0	89.97	C16:1	67.51			C18:0	50.85
C16:0	25.22	C18:1	15.55			C16:0	20.07
C18:3	10.74						

Table 4.2 'Jackknife' classification matrix for yeast isolates included in this study

Application 1

Yeast Isolate	Percent Correct	TP1	Rr	Sc	Pr	Rl	Rg	Rm	Cm	Rt
TP1[1]	33.3	1[2]	2	0	0	0	0	0	0	0
Rr	33.3	2	1	0	0	0	0	0	0	0
Sc	100.0	0	0	3	0	0	0	0	0	0
Pr	100.0	0	0	0	3	0	0	0	0	0
Rl	100.0	0	0	0	0	3	0	0	0	0
Rg	100.0	0	0	0	0	0	3	0	0	0
Rm	100.0	0	0	0	0	0	0	3	0	0
Cm	100.0	0	0	0	0	0	0	0	3	0
Rt	100.0	0	0	0	0	0	0	0	0	3

Application 2

Yeast Isolate	Percent Correct	TP1	Rr	Rl	Rm	Cm	Rt
TP1[1]	0.0	0[2]	3	0	0	0	0
Rr	66.7	1	2	0	0	0	0
Rl	100.0	0	0	3	0	0	0
Rm	100.0	0	0	0	3	0	0
Cm	100.0	0	0	0	0	3	0
Rt	100.0	0	0	0	0	0	3

Table 4.2 contd.

Application 3

Yeast isolate	Percent Correct	TP1	Rr	Rl	Rm
TP1[1]	33.3	1[2]	2	0	0
Rr	66.7	1	2	0	0
Rl	100.0	0	0	3	0
Rm	100.0	0	0	0	3

Application 4

Yeast Isolate	Percent Correct	TP1	Rr	Rl	Rg	Rm	Cm	Rt
TP1[1]	33.3	1[2]	2	0	0	0	0	0
Rr	66.7	1	2	0	0	0	0	0
Rl	100.0	0	0	3	0	0	0	0
Rg	100.0	0	0	0	3	0	0	0
Rm	100.0	0	0	0	0	3	0	0
Cm	100.0	0	0	0	0	0	3	0
Rt	100.0	0	0	0	0	0	0	3

[1]TP1 = *R. rubra* TP1, Rr =*R. rubra* ATCC 9449, Sc = *S. cerevisiae*, Pr = *P. rhodozyma*, Rl = *R. glutinis*, Rg = *R. graminis*, Rm = *R. minuta*, Cm = *Cr. macerans*, Rt = *Rhodosp. toruloides*. [2]Number of individual replicates classified as a particular yeast species.

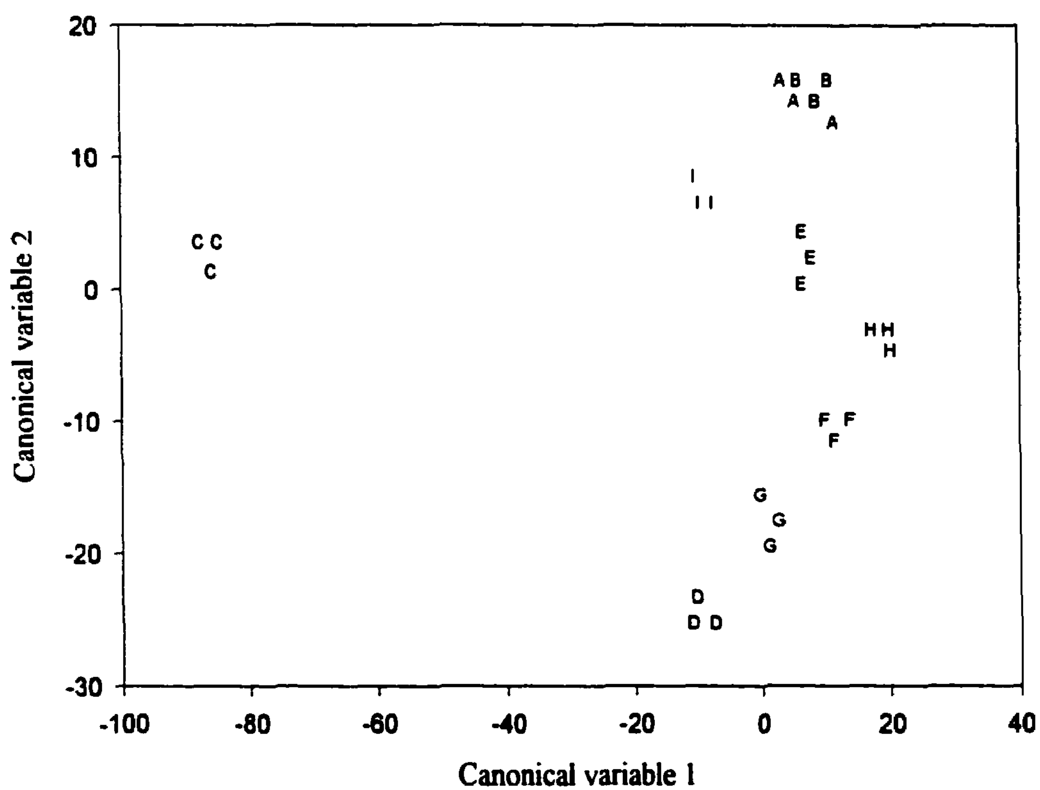


Fig. 4.0 Plot of the first and second canonical variables for Application 1 (A: *R. rubra* TP1; B: *R. rubra* ATCC 9449; C: *S. cerevisiae*; D: *P. rhodozyma*; E: *R. glutinis*; F: *R. graminis*; G: *R. minuta*; H: *Cr. macerans*; I: *Rhodosp. toruloides*).

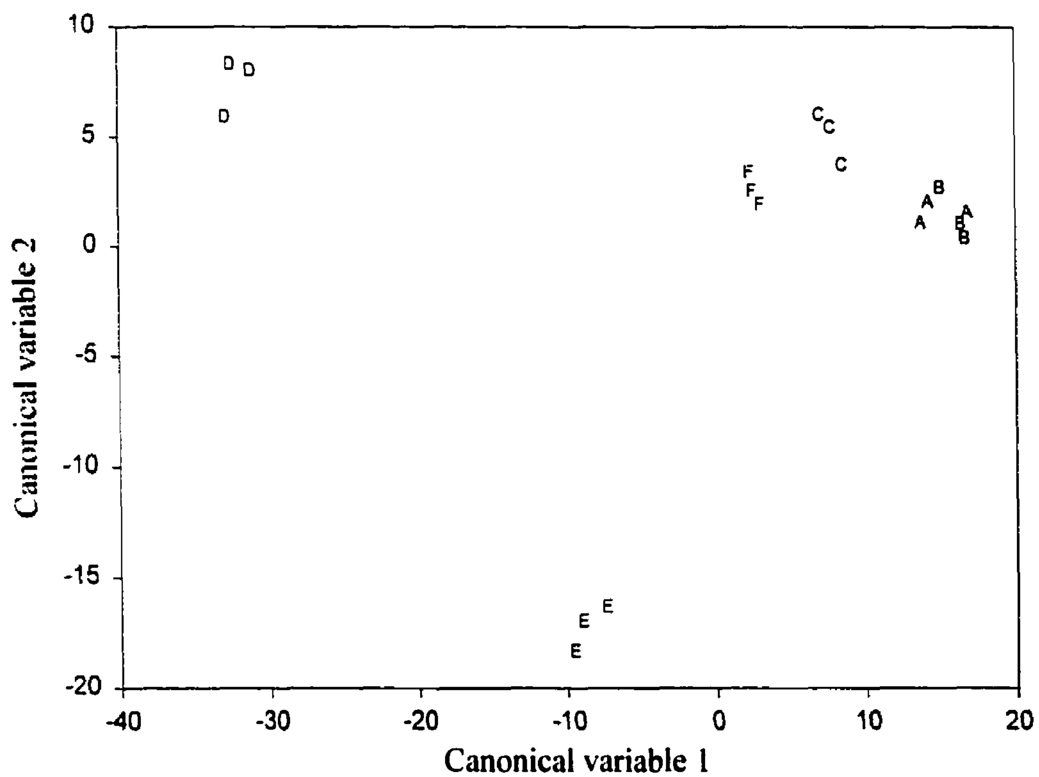


Fig. 4.1 Plot of the first and second canonical variables for Application 2 (A: *R. rubra* TP1; B: *R. rubra* ATCC 9449; C: *R. glutinis*; D: *R. minuta*; E: *Cr. macerans*; F: *Rhodosp. toruloides*)

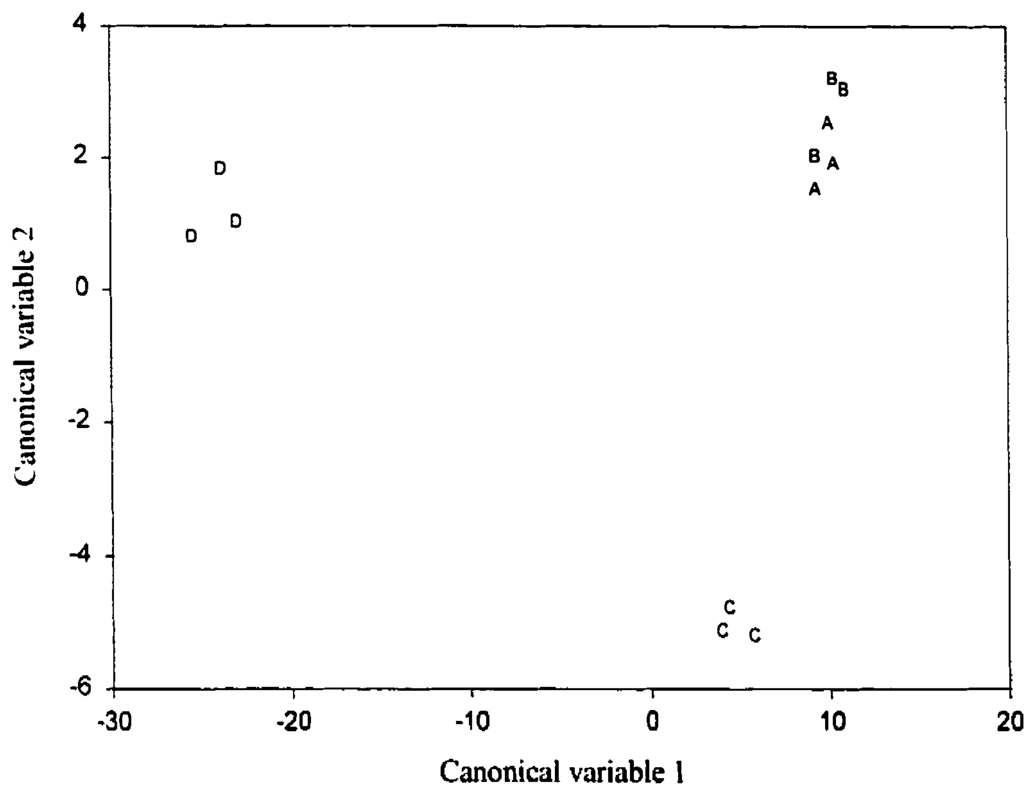


Fig. 4.2 Plot of first and second canonical variables for Application 3 (A: *R. rubra* TP1; B: *R.rubra* ATCC 9449; C: *R. glutinis*; D: *R. minuta)*

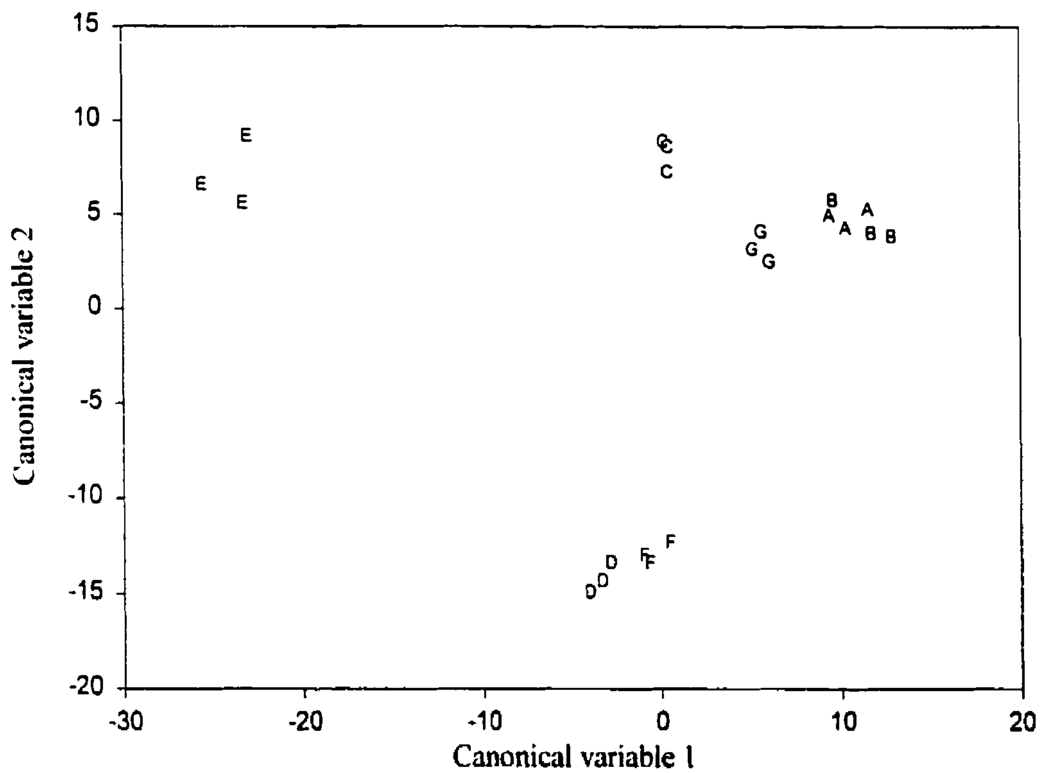


Fig. 4.3 Plot of the first and second canonical variables for Application 4 (A: *R. rubra* TP1; B: *R. rubra* ATCC 9449; C: *R. glutinis*; D: *R. graminis*; E: *R. minuta*; F: *Cr. macerans*; G: *Rhodosp. toruloides*)

CHAPTER 5

MOLECULAR PHYLOGENY OF *Rhodotorula rubra* TP1 AS SUGGESTED BY THE PARTIAL SEQUENCES OF 18S RIBOSOMAL DNA AND INTERGENIC TRANSCRIBED SPACER

5.1 Introduction

Ribosomal DNAs (rDNA) sequence comparison provides information that is routinely used in establishing the relationships and phylogenies among different organisms. It has also been used to estimate the evolutionary distances among a large variety of organisms (Field *et al.* 1988; Woese, 1987; Yamada *et al.* 1994; James *et al.* 1998; Fell *et al.* 1998, 1999, Sigita *et al.*, 2000). Ribosomal DNA appears particularly well suited as a general indicator of evolutionary relationships because of its occurrence in all species and largely conservative structure and function (Fox *et al.* 1980).

In yeast taxonomy, analyses of partial rDNA sequences have been used for the phylogenetic evaluation among various genera and among species within the same genera (Peterson and Kurtzman, 1991; James *et al.* 1998, Barns *et al.* 1991; Guého *et al.* 1989; Montrocher *et al.* 1998; Fell *et al.*, 1999). In most fungal species, the four different ribosomal DNA (rDNA) genes (5S, 5.8S, 18S and 28S) occur in tandem repeat units that are arranged head-to-tail to form a single cluster. The 18S and 28S are separated from each other by the internal transcribed spacer (ITS) and intergenic

spacers (IGS) which are non-coding regions except for the short 5.8S and 5S genes. Both the rDNA genes and the spacer regions are amplified by the polymerase chain reaction (PCR) using oligonucleotide primers homologous to specific sites of the rDNA and sequences present at the end of the rDNA genes (White *et al.* 1990). Analysis of the sequences obtained through PCR amplification permits the determination of the phylogenetic distances between different yeast species and groups (Lachance *et al.* 1990; Kurtzman, 1992, Kurtzman and Robnett, 1991).

The present work reports the determination of the phylogenetic affinity of the new strain of yeast isolated from yogurt as revealed by the 18S rDNA genes and the internal transcribe spacer (ITS) sequences.

5.2 Materials and Methods

Chemicals: Trizma base, isopropanol, EDTA, mercapethanol, CTAB, isoamyl alcohol, deionized formamide and Na_2EDTA were purchased from Sigma Chemical Company, St. Louis, MO. NaCl, chloroform, borate and HCl were obtained from Fisher Scientific Ltd., Fair Lawn, New Jersey.

5.2.1 Growth of microorganisms.

The organisms used were *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* ATCC 24202. They were cultivated in YM broth at a temperature of 22°C in an orbital shaker for 5 days. Cells were harvested by centrifugation at 10,000 x g, washed three times with sterile deionized water and then freeze-dried. Freeze-dried cells were stored at -20°C until needed for use.

5.2.2 Ribosomal DNA extraction.

Ribosomal DNA (rDNA) was extracted from the organisms using a modified CTAB PCR miniprep protocol of Zolan and Pukkila (1986). About 0.4g of cells from the freeze-dried cultures were mixed with approximately 700 µL of 1X CTAB extraction buffer (700 mM NaCl, 50 mM Tris-HCl {pH 8.0}, 10 mM EDTA, 1% CTAB, 0.2% mercapoethanol). The mixture was homogenized in a mortar with a tube pestle and samples were incubated in a water bath at 60°C for about an hour. After incubation, the DNA was extracted with chloroform-isoamyl alcohol (24:1), precipitated with cold isopropanol, washed twice with 500 µL of 70% ethanol and

dried in a vacuum oven. Pellets were re-suspended in 50 µl of deionized water and heated to 60° C for several minutes to dissolve. Horizontal gel electrophoresis was used to determine the quality of DNA. The samples were then diluted 1:10 or 1:20 with sterile distilled deionized water, then 1 µL of the diluted samples were used as templates for the PCR reaction.

5.2.3 DNA amplification.

The polymerase chain reaction (Mullis and Faloona, 1987) was used to amplify a portion of the ribosomal DNA from the DNA extracts using various rDNA specific primers which amplify conserved regions of the 18S and ITS-1 region of the ribosomal DNA. The primers used were ITS5-GGAAGTAAAAGTC CGTAACAAGG (White *et al.* 1990), ITS9mun-TGTACACACCGCCCGTCG, ITS10mun-GGAACCTTTCCCCACTTC, NS12mun-TGGTTTCTAGGACCGCCGT NS7mun-GAGGCAATAACAGGTCTGTGATGC, NS11mun-GCAAATTACCCAA TCCCGAC (All sequences are written 5' to 3'). Letter designations follow the convention in White *et al.* (1990). The designation mun stands for Memorial University of Newfoundland. Amplification reactions were performed in 100 µL volumes containing PCR premix (1.5 mM $MgCl_2$, 10 mM Tris-HCl {pH 8.3}), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, 2 mM each of dATP, dCTP, dGTP, dTTP, 10 µM each of oligonucleotide primers, 0.5 units of Taq DNA polymerase (Promega Corp., Madison, WI), sterile distilled deionized water and approximately 1 µL of diluted DNA extract. One drop of mineral oil was placed on top of each

mixture to prevent evaporation. Two control tubes were also prepared and included with the reaction tubes. Amplification of samples was carried out in a Perkin-Elmer Cetus DNA Thermal Cycler using the following parameters: initial denaturation at 94°C for 2 minutes, all other denaturation steps afterwards were held at 94° C for 1 minute. Annealing temperature was at 46°C for 1 minute, 55 sec ramp to 72° C, followed by 35 cycles consisting of 72° C for 1 min. 30 seconds (extension temperature) and a final extension step of 72°C for 5 minutes A 5 µL of the PCR products were subjected to electrophoresis on 2% agarose gel in TBE Buffer (0.4 M Tris-borate pH 8, 1 mM EDTA). The gel was stained with ethidium bromide and exposed to UV light on Ultraviolet Transilluminator (Ultra-Violet Products Inc., San Gabriel, CA) to visualize PCR products and to check whether amplification was successful.

5.2.4 Purification of PCR products for sequencing.

Amplified DNA was purified using Wizard™ Magic PCR Preps DNA Purification Kit (Promega Corp, Madison, WI) following the manufacturer's instructions. Quantification of purified DNA was carried out by measuring absorbance at 260 nm on a Spectronic UV-Visible Light Spectrophotometer. DNA concentration was determined by assuming that 1 O.D unit is equivalent to 50 µg/mL DNA (Maniatis *et al*, 1982).

5.2.5 Automated DNA sequencing.

The optimum concentration of DNA solution was determined from the spectrophotometer reading and dried under reduced pressure. Automated sequencing required the drying down of 3.2 pmol. of DNA. Primers ITS5, 9, 10 and NS11, 12 and 13 were used to amplify a portion of the single-stranded rDNA for sequencing. All primers were used in separate reactions. Sequencing reactions were carried out with a Taq DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the conditions recommended by the manufacturer. The reactions were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler using the following cycle sequencing parameters: 95°C for 2 minutes, 95°C for 1 minute, 60°C for 1 minute (35 cycles) and 72°C for 1 minute 30 seconds.

After cycle sequencing the reaction mixture was passed through a Sephadex G-50 Spin column to remove any excess primers and unincorporated dye. The eluted DNA were then dried under reduced pressure and resuspended in 5 µL of 5:1 mixture of deionized formamide and 50 mM Na_2EDTA and then loaded onto a 6% polyacrylamide gel attached to an ABI 373A Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA). Electrophoresis was carried out at 32 watts constant power for 11 hours and DNA sequence data was collected using ABI collection and analysis software version 1.0.2 (Perkin Elmer, Inc.).

5.2.6 Sequence and phylogenetic analyses.

DNA sequences were edited using ABI Sequence Navigator DNA Sequence

Editor version 1.0.1 (Perkin Elmer Inc.). Comparison of complimentary strands were done using ABI Sequence Navigator (Perkin Elmer Inc.), sequences were then aligned with Clustal W (Thompson *et al.* 1994) and similarity of sequences was calculated. Alignment of sequences in a publishable format was obtained with Boxshade (Macbox version 2) (Brown, 1998), the aligned sequences were then used to generate a distance matrix by using the Phylogenetic Analysis Using Parsimony (PAUP version 3.1) of Swafford (1993). Sequences of the 18S genes and ITS region of various types of yeast were obtained from the National Center for Biotechnology Information (NCBI) GenBank Data Base (National Library of Medicine, Bethesda, Maryland) and analyzed. The organisms and their accession numbers are: *Saitoella complicata* (Accession # D12530), *Rhodotorula glutinis* (Accession # AB026018), *Rhodotorula mucilaginosa* (Accession # AB026017), *Rhodotorula* sp. SY-100 (Accession # AB026011), *Rhodotorula* sp. SY-101 (Accession # AB026012), *Rhodotorula* sp. SY-103 (Accession # AB026013), *Rhodotorula laryngis* (Accession # AF 190014), *Filobasidiella neoformans* var. *neoformans* (Accession # AB034643), *Cryptococcus aerius* (Accession # AB032666) and *Saccharomyces cerevisiae* (Accession # D89886).

Maximum Parsimony [MP] (heuristic search algorithm, tree-bisection-and-reconnection, with random addition and delayed-character transformation optimization) network was obtained by bootstrap majority consensus tree analysis of 100 replicates and a phylogram grouping yeasts with the most similar sequences were generated.

5.3 Results.

To determine the phylogenetic affinity of the new isolate, several primers were targeted to selected regions of the 18S genes and the ITS region of the ribosomal DNA and sequenced by the Taq DyeDeoxy Termination cycle. With the exception of ITS 9, we had difficulty aligning sequences in the ITS region among closely related taxa in the genus *Rhodotorula* and other distantly related groups. The sequences of the various portions of the 18S genes and ITS region that are not presented here are depicted in Appendix A.

5.3.1 Partial base sequences in positions 1 through 360 of 18S and ITS subunits.

The partial base sequences of the new yeast isolate, *R. rubra* TP1, *P. rhodozyma* and *R. rubra* ATCC 9449 examined in this study were aligned in position 1 through 360 (360 basepair) with sequences of other yeast isolates obtained from the GenBank and the results are depicted in Fig. 5.0. As can be seen from the figure, there were several deletions and insertions at various positions which makes the alignment somewhat ambiguous. However, when *R. rubra* TP1 was compared to *R. rubra* ATCC 9449, the insertions were minimal and in most cases occur at the same positions for both organisms. *Rhodotorula rubra* ATCC 9449 had base substitutions at position 182 (C to A), 190 (G to C), 226 (C to T), 243 (A to C), 278 (A to T), 280 (A to T), 289 (A to G), 302 (A to T), 313 (A to T) and 324 (A to T) when compared to the new isolate. Similarly, several of the isolates used as controls exhibited base

substitutions (differences) at various positions when compared to the new isolate, *R. rubra* TP1. *R. rubra* ATCC 9449 had the least number of base substitutions, 10 (about 2.8%), followed by *Rhodotorula* sp. SY-100 and SY-101 which had 11 base substitutions (3.1%) each. *P. rhodozyma* had 24 (7%) which was the highest, followed by *Rhodotorula* sp. SY-103 with 16 (4.4%) and *R. glutinis* with 15 (4.2%).

Percent similarities (= maximum homology, %) were calculated for the respective pairs of the isolates using Clustal W (Thompson *et al.* 1994) and the results are depicted in Table 5.0 together with the pairwise evolutionary distance estimates. The highest percent similarity (93) was obtained between the new isolate and *R. rubra* ATCC 9449. The second highest (89) was between the new isolate and *Rhodotorula* species SY-100 and SY-101.

Based on the partial base sequence analysis, a majority rule bootstrap consensus tree was obtained using the Maximum Parsimony (MP) heuristic search algorithm, tree-bisection-and-reconnection with 10 random addition and delayed-character-transformation optimization. Bootstrap analyses (Felsentein, 1985) were performed by means of the heuristic search algorithm with 10 random taxon additions and the tree-bisection-and-reconnection option in each of 100 replicates. The resulting bootstrap phylogenetic trees are depicted in Figs. 5.1 and 5.2. The phylogenetic tree depicted in Fig. 5.1 was generated with only seven isolates whereas in Fig. 5.2, *Cryptoccocus aerius, Rhodosporidium toruloides, Filobasidiella neoformans* var. *neoformans* and *Saccharomyces cerevisiae* were used as outgroups. As shown in both figures, *R. rubra* TP1 and *R. rubra* ATCC 9449 constituted a single

cluster supported by a bootstrap value of 100 and a pairwise evolutionary distance estimate of 0.041. This pairwise evolutionary distance estimate was the lowest among all the pairwise evolutionary distance estimated between *R. rubra* TP1 and any of the isolates. The second lowest pairwise evolutionary distance estimate was between the new isolate and *Rhodotorula* species species SY-100 and SY-101. *P. rhodozyma* was linked to the *R. rubra* TP1 and *R. rubra* ATCC 9449 cluster with an evolutionary distance of 0.119 and 0.125 for the new isolate and *R. rubra* ATCC 9449, respectively. In the case of Fig. 5.2, *R. rubra* TP1 and *R. rubra* formed a single cluster that was linked to *P. rhodozyma.* The species of *R. glutinis* and *Rhodosp. toruloides* clustered together and this cluster was linked to cluster formed by *Filobasidiella neoformans* var. *neoformans and Cr. aerius.* Similarly, *R. mucilaginosa* and *R. laryngis* clustered together. *Rhodotorula* species SY-100, 101 and 103 formed a single cluster whilst *Rhodosp. toruloides* and *R. glutinis* clustered together and the cluster was linked to *S. cerevisiae.*

5.3.2 Partial base sequences in position 481 through 802 of 18S subunit.

The partial rDNA base sequences of *R. rubra* TP1, *R. rubra* ATCC 9449, and *P. rhodozyma* were aligned in positions 481 through 802 (320 bp) with those of other isolates obtained from the GenBank. The alignment is shown in Fig. 5.3. With the exception of *R. rubra* ATCC 9449 which had exactly the same alignment as the new isolate, all the other isolates used as controls had base substitutions and deletions at various positions when compared with the new isolate.

The percent similarity determined for *R. rubra* TP1 and *R. rubra* ATCC 9449 was 100 (Table 5.1) and those between *R. rubra* TP1 and the other isolates were as follows: *P. rhodozyma*, 80; *R. glutinis*, 89; *R. graminis*, 75; *S. complicata*, 69; and *Rhodosp. toruloides*, 78. The percent similarity between *R. rubra* ATCC 9449 and the other isolates were identical to those between *R. rubra* TP1 and the other isolates.

Based on the sequence data obtained, a majority rule consensus tree produced by bootstrapping using MP analysis was generated and is depicted in Fig. 5.4. The pairwise evolutionary distance estimates are also depicted in Table 5.1. It can be seen that the new isolate and *R. rubra* ATCC 9449 had a pairwise distance of 0.000 indicating that the two isolates are closely related. According to the topology of the phylogenetic tree (Fig. 5.4), *R. rubra* TP1 is closely related to *R. rubra* ATCC 9449. The two constituted a single cluster supported by a bootstrap value of 100 and was linked to *S. complicata*, *R. glutinis*, *Rhodosp. toruloides* and *P. rhodozyma* at 0.159, 0.104, 0.058 and 0.165 evolutionary distances apart, respectively.

5.4 Discussion.

The eukaryotic genome is made up of nuclear RNA genes that are organized into a cluster of tandem repeats that includes a small subunit gene, (16S to 18S), a large subunit gene (26S to 28S) and the 5.8S gene. In between these genes are two non-coding regions referred to as the internal transcribed spacers (ITS-1 and ITS-2). It is generally thought that the 18S region are more highly conserved than the ITS

region which have higher rates of divergence. Also the length of the 18S and the 26S rRNA gene are essentially identical in all species while the length of the ITS region are species specific (White *et al.*, 1990). For example in *Candida globrata* (V70498), *Candida kefyr* (V70502) and *Saccharomyces cerevisiae* (Z75722), the length of the ITS-2 region have been reported to be approximately 230 to 240 basepairs long; those of *Candida guiliermandii* (V70499) and *Candida famata* (V70500) are approximately 190 basepairs long; those of *Candida albicans* (L07796), *Candida tropicalis* (L11349), *Candida parapilosa* (L11352) and *Candida viswanathii* (V70501) are approximately 130 to 140 basepairs long; and those of *Candida lusitaniae* (V70503) and *Candida rugosa* (V70506) are only 70 to 90 base pairs long (Lott *et al.* 1998).

The internal transcribed spacer, the 18S, 26S and 5.8S subunits have been sequenced from various yeast species. Yamada *et al.* (1994) examined the partial base sequences of 18S and 26S rRNAs of several species of the teleomorphic genera *Dekkera* and the anarmophic genus *Brettanomyces* and concluded that *D. bruxellensis* (type species) (and *B. bruxellensis*, type species) and *D. anomala* (and *B. anomalus*) were related phyogenetically.

In this study the partial base sequences of the 18S rDNA and ITS region of the new isolate were compared with those of other isolates to determine the phylogenetic affiliation of the new isolate. The ITS primers used in this study made use of the conserved regions of the 18S rRNA genes to amplify the non-coding ITS-1 region between them and the 5.8S genes whereas the NS11 targeted portions of the 18S

rDNA genes. It was observed that the total length of the region amplified by the ITS9mun primer was approximately 520-525 basepairs long for *R. rubra* TP1 and *R. rubra* ATCC 9449. This suggests that the two organisms may be closely related. Also the phylogenetic analyses performed indicated that the new yeast isolate, *R. rubra* TP1 is phylogenetically identical to *R. rubra* ATCC 9449. Compared to *R. rubra* TP1, *R. rubra* ATCC 9449 had the lowest base substitution (9-0 and 0-0) for the partial base sequences 1 through 360 and 481 through 802, respectively. The percent similarities calculated for the DNA sequences were also the highest for these two isolates (93 and 100 for positions 1 through 360 and position 481 through 802, respectively). The new isolate and *R. rubra* ATCC 9449 clustered together when MP analyses were performed either on the partial base sequences of the 18S rDNA or a combined sequences of the 18S and ITS. The *R. rubra* TP1 and *R. rubra* ATCC 9449 cluster was closely linked to *P. rhodozyma* in all the analysis. This was surprising because coming from different genera, the two were expected to be well separated. However, the genus *Rhodotorula* is considered to be phenotypically most similar to *Phaffia* (Goto *et al.*, 1987). Differential characters among the two genera are: fermentation (positive for *Phaffia*) and formation of starch-like compounds (positive for *Phaffia*) (Miller *et al.*, 1976; Kreger-van Rij and Veenhuis, 1971). These two genera have the same ubiquinone system, Q-10, positive DNase and DBB tests, basidiomycetous type of cell wall and similar DNA base composition which fall in the "grey zone" (Miller *et al.*, 1976; Goto *et al.*, 1987; Kreger-van Rij and Veenhuis, 1971). The fact that the two genera share these common characteristics suggests a

closer relationship between the two. Also the close evolutionary relationship between the two is in keeping with the fact that they shared a number of common features observed in this study. From Table 3.0 (Chapter 3), it can be observed that both the new isolate, *R. rubra* ATCC 9449 and *P. rhodozyma* had identical nutritional and biochemical characteristics. For example all three were nitrate negative and were unable to utilize inositol. Unfortunately, no prior literature reference is available concerning rDNA sequence analysis of *R. rubra* for comparative purposes.

The teliospore-forming basidiomycete, *Rhodosp. toruloides* and its closet relative, *R. glutinis* clustered together and were closely related to the ascomycete, *S. cerevisiae*. This was also surprising since it was expected that the basidiomycetous yeasts would be well separated from the ascomycetous yeasts. The use of a large data set comprising a large number of ascomycetous yeasts would probably have resulted in a better separation of the ascomycetous and basidiomycetous yeasts. However, as indicated earlier, we encountered a number of difficulties trying to align the sequences of the basidiomycetous yeast with those of the ascomycetous yeasts, hence the small data set used. Aside this, other researchers have shown that the ascomycetes and the basidiomycetes share a common ancestor and therefore form a monophyletic group. For example, Van de Peer *et al.* (1991) applied a matrix optimization method developed by De Soete (1983) to construct additional evolutionary trees and reported that the ascomycetes and the basidiomycetes share a common ancestor. Similarly, Van de Peer *et al.* (1992) using a complete small

subunit rRNA sequences to infer the relationship between several basidiomycetous yeasts, and to resolve the evolutionary position of the basidiomycetous among the fungi, concluded that the ascomycete and basidiomycete together form a monophyletic cluster. These findings are in accordance with the results of this study and therefore supports the phylogenetic tree depicted in Fig. 5.2.

In view of the positive DBB reaction and the cell wall ultrastructure typical of basidiomycetous yeasts (Hari *et al.*, 1992), we consider that the new isolate is of a basidiomycetous origin. The close relationship between the new isolate and *R. rubra* ATCC 9449 observed in this molecular study is supported by previous biochemical studies based on nutritional requirements, isozyme analysis and the cell wall fatty acid composition reported earlier on in this thesis. In conclusion, similarities in 18S rDNA and ITS-1 sequences have demonstrated that the new isolate, *R. rubra* TP1 is a strain of *Rhodotorula rubra* and should therefore be considered as such.

Table 5.0. Matrices of the overall percent similarity and evolutionary distance estimates from portions of the 18S subunit and ITS-1 region for *Rhodotorula rubra* TP1 and other yeast isolates[a].

	1	2	3	4	5	6	7
1. *R. rubra* TP1	-	93	86	84	1	89	85
2. *R. rubra* ATCC 9449	0.041	-	85	83	88	88	84
3. *P. rhodozyma*	0.119	0.125	-	74	79	79	75
4. *R. glutinis*	0.076	0.096	0.158	-	93	93	93
5. *Rhodotorula* sp. SY-100	0.056	0.064	0.126	0.042	-	100	95
6. *Rhodotorula* sp. SY-101	0.056	0.064	0.126	0.042	0.000	-	95
7. *Rhodotorula* sp. SY-103	0.068	0.088	0.150	0.053	0.031	0.031	-

[a]The lower-left half of the matrix gives evolutionary distance estimates, the upper-right half gives the percent similarity based on the comparison of the aligned sequences

Table 5.1. Matrices of the overall percent similarity and evolutionary distance estimates from portions of the 18S subunit for *Rhodotorula rubra* TP1 and other yeast isolates[a].

	1	2	3	4	5	6	7
1. *R. rubra* TP1	-	100	80	89	75	69	78
2. *R. rubra* ATCC 9449	0.000	-	80	89	75	69	78
3. *P. rhodozyma*	0.165	0.165	-	81	68	61	70
4. *R. glutinis*	0.104	0.104	0.166	-	81	71	80
5. *R. graminis*	0.071	0.071	0.153	0.065	-	87	96
6. *S. complicata*	0.159	0.159	0.233	0.129	0.118	-	86
7. *Rhodosp. toruloides*	0.058	0.058	0.134	0.026	0.022	0.124	-

[a]The lower-left half of the matrix gives evolutionary distance estimates, the upper-right half gives the percent similarity based on the comparison of the aligned sequences

Fig.5.0. Aligned sequences of a 360 basepair portion of the small subunit (18S) rDNA gene and the 5' internal transcribed spacer (ITS). Dashes indicate there are no bases present at those positions. TP1 = *Rhodotorula rubra* TP1; Rr = *Rhodotorula rubra* ATCC 9449; Pr = *Phaffia rhodozyma;* glutinis = *Rhodotorula glutinis;* SY-100 = *Rhodotorula* sp. SY-100; SY-101 = *Rhodotorula* sp. SY-101; SY-103 = *Rhodotorula* sp. SY-103.

```
                      10        20        30        40        50
RSY-101   --------------CCGATT-AATGGCTTAGTGAGGCCTCCGGATTGGCT   35
SY-100    --------------CCGATT-AATGGCTTAGTGAGGCCTCCGGATTGGCT   35
glutinis  --------------CCGATT-AATGGCTTAGTGAGGCCTCCGGATTGGCT   35
SY-103    --------------CCGATT-AATGGCTTAGTGAGGCCTCCGGACCGGCT   35
TP1       ---TCCTCCTACTCCCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCT   47
Rr        ---TCTTCCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCT   47
Pr        CCCCTTATCCTATCCCGATT-GATGGCTTAGTGAGGCCTCCGGATTGGCT   49

                      60        70        80        90       100
RSY-101   ATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGG   85
SY-100    ATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGG   85
glutinis  ATTGGGAGCTCGCGAGAGCACCTGACTGCTGAGAAGTTGTACGAACTTGG   85
SY-103    ATTGGGAGCTCGCGAGAGCACCCGACTGCTGGGAAGTTGTACGAACTTGG   85
TP1       ATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGG   97
Rr        ATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGG   97
Pr        ATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGG   99

                     110       120       130       140       150
RSY-101   TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC-TGC  134
SY-100    TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC-TGC  134
glutinis  TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC-TGC  134
SY-103    TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAAC-TGC  134
TP1       TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  147
Rr        TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGC  147
Pr        TCATTTAGAGGAAGTAAAAGTCGCCACAGGGTTTCCGTGGGTGAACCTGC  149

                     160       170       180       190       200
RSY-101   GGAAGGATCATTAGTGAACATAGGACGTCCAACTTAACTTGGAGTCCGAA  184
SY-100    GGAAGGATCATTAGTGAACATAGGACGTCCAACTTAACTTGGAGTCCGAA  184
glutinis  GGAAGGATCATTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAA  184
SY-103    GGAAGGATCATTAGTGAATATAGGATGTCCAACTTAACTTGGAGTCCGAA  184
TP1       GGAAGGATCATTAGTGAATATAGGACGTCCACCTTAACTCGGAGTCCGAA  197
Rr        GGAAGGATCATTAGTGAATATAGGACGTCCAACTTAACTGGGAGTCCGAA  197
Pr        GGAAGGATCATTAGCGAATATAGGACGTCCAACTTTACTCGGTGTCCGAC  199

                     210       220       230       240       250
RSY-101   CTCTCACTTTCTAACCCTGTGCATTTGTT---TGGGATAGTAACTC-TCG  230
SY-100    CTCTCACTTTCTAACCCTGTGCATTTGTT---TGGGATAGTAACTC-TCG  230
glutinis  CTCTCACTTTCTAACCCTGTGCATCTGTTAATTGGACTAGTAGCTCTTCG  234
SY-103    CTCTCACTTTCTAACCCTGTGCATTTGTT---TGGGATAGTAGCCTCTCG  231
TP1       CTCTCACTTTCTAACCCTGTGCACTCGTT---TGGGATAGTAACTC-TCG  243
Rr        CTCTCACTTTCTAACCCTGTGCACTTGTT---TGGGATAGTACCTC-TCG  243
Pr        CTCTCACTGTCTCACCCTGTGCACTCGTG---TGGGATAGTAACTC-TCG  245
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                    260       270       280       290       300
RSY-101    CAA-GAGAGCG-AACTCCTATTCACTTATAAACACAA-AGTCTATGAATG  277
SY-100     CAA-GAGAGCG-AACTCCTATTCACTTATAAACACAA-AGTCTATGAATG  277
glutinis   ------GAGTG-AACCGCCATTCACTTATAAACACAA-AGTCTATGAATG  276
SY-103     ------GGGTG-AACTCCTATTCACTTATAAACACAA-AGTCTATGAATG  273
TP1        CAA-GAGAGCG-AACTCCTATTCACTTATTAACACAA-AGTCTATGAATG  290
Rr         CAA-GAGAGCG-AACTCCTATTCACTTTTAAACACAA-GGTCTATGATTG  290
Pr         CCATGAGAGCGCAACTCCTGTTCACTTATTCGCACAACGGTCTATGATTG  295

                    310       320       330       340       350
RSY-101    TATTTAAT-TTTATAACAAAA-TAAAACTTTCAACAA-CGGATCTCTTGG  324
SY-100     TATTTAAT-TTTATAACAAAA-TAAAACTTTCAACAA-CGGATCTCTTGG  324
glutinis   TATACAAA-TTTATAACAAAA-CAAAACTTTCAACAA-CGGATCTCTTGG  323
SY-103     TATTTAA--TTTATAACAAAA-TAAAACTTTCAACAA-CGGATCTCTTGG  319
TP1        TATTAACT-CTTATAACAAACCTAAAGCTTTCAACAAACGGATCTCTTGG  339
Rr         TTTTACCT-CTTTTAACAAAC--TAAAGGTTCAACAA-CGGATCTCTTGG  336
Pr         TGTTAACCCCATTTGCCAAAC-TGGAGGTTTCAATTAACGGATCTCTTGG  344

                    360       370       380       390
RSY-101    CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA-----           360
SY-100     CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA-----           360
glutinis   CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAC----           360
SY-103     CTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA           360
TP1        GTCTCGCATCGG-GAAAAA-------AAA------------           360
Rr         GTCTCGAATGGA--AAAAA-------AAA----AAAA----           360
Pr         GTCTCGGCTGG-----GAA-------AA-------------           360
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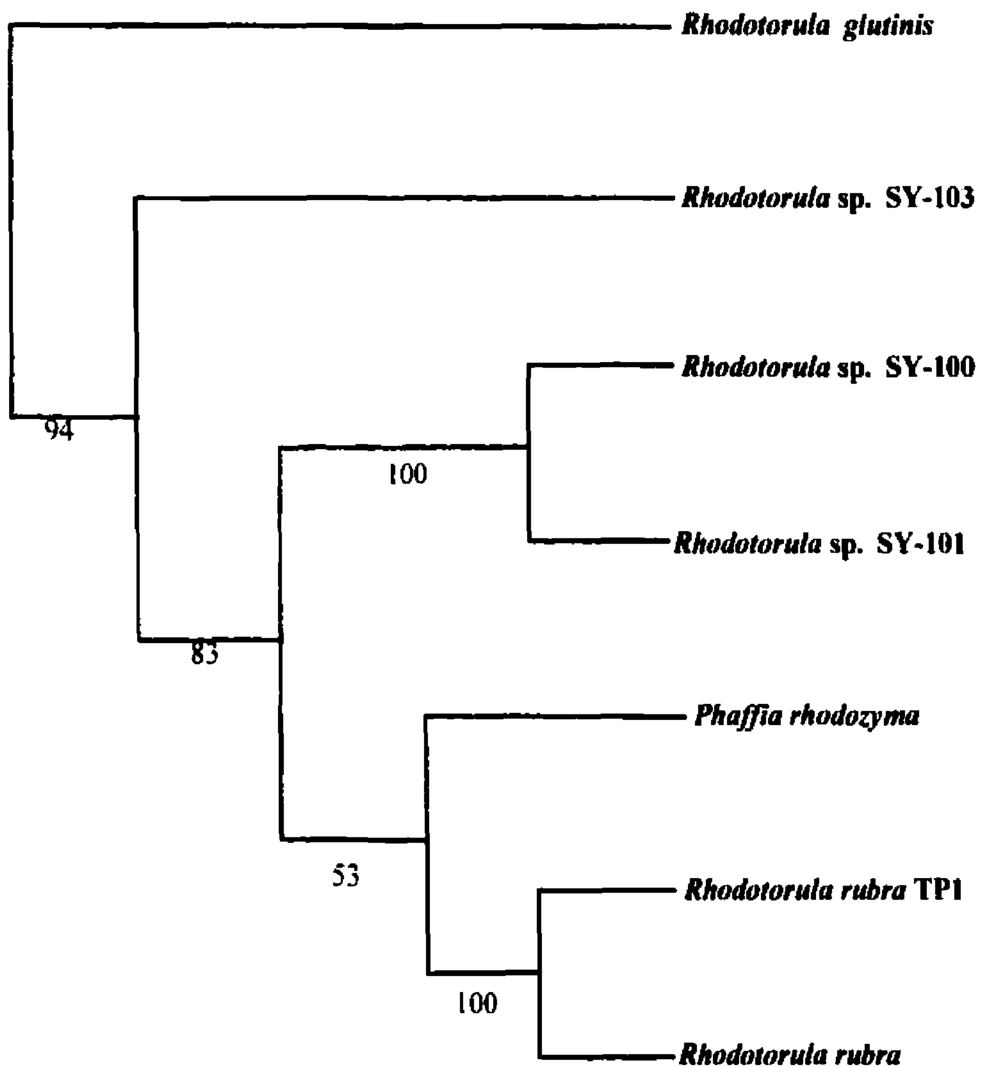

Fig. 5.1 Boostrap majority-rule consensus tree constructed from a cladistic analysis of the partial sequences of position 1 through 360 of the small subunit rDNA and the 5' internal transcribed spacer region of *Rhodotorula rubra* TP1 and 6 other yeast isolates using100 times resampling bootstrapping in PAUP (Swafford, 1993). The numerals represent the percentages of sampling of bootstrap supporting the interna branches.

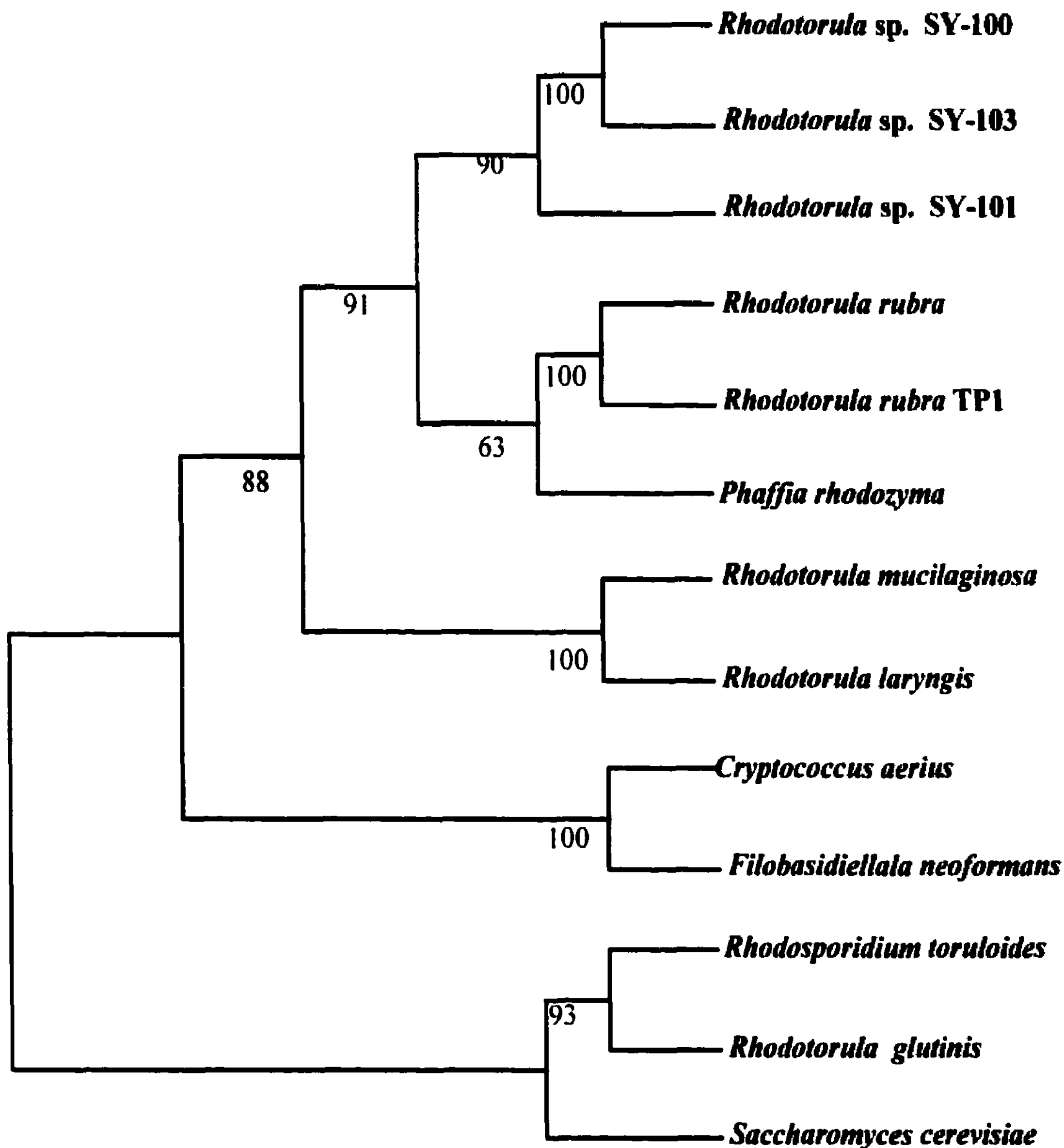

Fig. 5.2 Boostrap majority-rule consensus tree constructed from a cladistic analysis of the partial sequences of position 1 through 360 of the small subunit (18S) rDNA and the 5' internal trancribed spacer (ITS) region of *Rhodotorula rubra* TP1 and 12 other yeast isolates using 100 times resampling bootstrapping in PAUP (Swafford, 1993). The numerals represent the percentages of samplings of bootstrap supporting the internal branches.

Fig. 5.3 Aligned sequences of a 320 basepair portion of the small subunit (18S) rDNA gene. Dots indicate there are no bases present at those positions. TP1 = *Rhodotorula rubra* TP1; Rr = *Rhodotorula rubra* ATCC 9449; Pr = *Phaffia rhodozyma;* glutinis = *Rhodotorula glutinis;* Saitoella = *Saitoella complicata;* Rhodosp. = *Rhodosporidium toruloides;* graminis = *Rhodotorula graminis*

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                490       500       510       520       530
graminis    AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC   50
glutinis    ..GATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC   48
Saitoella   AGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC   50
Rhodosp     AGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGC   50
TP1         ..GATCANTTGGAGGGCAAGTCTGGNGCCAGCAGCCGAGGGAATTCCAGC   48
Rr          ..GATCANTTGGAGGGCAAGTCTGGNGCCAGCAGCCGAGGGAATTCCAGC   48
Pr          ..GATCAATTGGNGGGGAAGTCTGGTGGCAGCAGTCGNGGTAATTCCAGC   48

                540       550       560       570       580
graminis    TCCAATAGCGTATATTAAAGTTGTTGCCGTTAAAAAGCTCGTAGTCGAAC  100
glutinis    TCCAATAGCGTATATTAAAGTTGTTGCCGTTAAAAAGCTCGTAGTCGAAC   98
Saitoella   TCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAC  100
Rhodosp     TCCAATAGCGTATATTAAAGTTGTTGCCGTTAAAAAGCTCGTAGTCGAAC  100
TP1         TCCAATAGCGTATATTAAATTTGTTGCCGTTAAAAAGCTCGTAGTCGAAC   98
Rr          TCCAATAGCGTATATTAAATTTGTTGCCGTTAAAAAGCTCGTAGTCGAAC   98
Pr          TCCAATAGCGTATATTAAAATTGTTGGCGATAAAAAGCTCGTCGTCGAAC   98

                590       600       610       620       630
graminis    TTCGGG.TCCTG.TCCGCCGGTCCGCCNNCTTGGNGNGNACTTGTTGGAT  148
glutinis    TTCGGG.TCCTG.TCCGCCGGTCCGCCTTCTTGGTGTGTACTTGTTGGAT  146
Saitoella   CTTGGG..CCTGGTCGGCCGGTCCGCCT.CACGGTGTG.ACTGACCCGAC  146
Rhodosp     TTCGGG.CTCTG..CAGCCGGTCCGCCTTCTTGGTGTGTACTTGTTTGGT  147
TP1         TTCGGG.CTCTG.TCAGTCGGACCGCCTTCTTGGTGTGTACTTGTATGAC  146
Rr          TTCGGG.CTCTG.TCAGTCGGACCGCCTTCTTGGTGTGTACTTGTATGAC  146
Pr          TTCGGG.CTCTG.TCAACCGGACCGCCTTCTTGGTGTGTACTTGTTTGGC  146

                640       650       660       670       680
graminis    GGGACCTTACCTCCTGGTGAACA.GCG.ATGTCCTTTACTGGGTGT.CGT  195
glutinis    GGGACCTTACCTCCTGGTGAACA.GCG.ATGTCCTTTACTGGGTGT.CGT  193
Saitoella   CGGGCCTTTCCTTCTGGCTAACC..CGTATGCCCTTTACTGGGTGTGCGG  194
Rhodosp     GGAGCCTTACCTCCTGGTGAACA.GCG.ATGTCCTTCACTGGGTGT.CGT  194
TP1         GGAGCCTTACCTCCTGGTGAACG.GCG.ATGTCCTTTACTGGGTGT.CGT  193
Rr          GGAGCCTTACCTCCTGGTGAACG.GCG.ATGTCCTTTACTGGGTGT.CGT  193
Pr          GGAGGCTAACCTCCTGGTGAACG.GGG.ATGTACCATACTGGGTGT.CGC  193

                690       700       710       720       730
graminis    TGCAAACCAGGACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC  245
glutinis    TGCAAACCAGGACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC  243
Saitoella   .GAGAACCAGGACTTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCA  243
Rhodosp     TGCAAACCAGGACGTTTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC  244
TP1         CGCAAACCAGGACTATTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC  243
Rr          CGCAAACCAGGACTATTACTTTGAAAAAATTAGAGTGTTCAAAGCAGGCC  243
Pr          CGCAAACCAGGACTACAACTTTGAGGAAGTTCGAGTGTTCAAAGCAGACC  243
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                   740       750       760       770       780
graminis   TTTGCCCGAATACATTAGCATGGAATAATAGAATAGGACGCGCG.TTCCC  294
glutinis   TTTGCCCGAATACATTAGCATGGAATAATAGAATAGGACGCGCG.TTCCC  292
Saitoella  TTTGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGTG.GTTCT  292
Rhodosp    TTTGCCCGAATACATTAGCATGGAATAATAGAATAGGACGCGCG.TTCCC  293
TP1        TTTGCCCGAATACATAAGCATGGAATAATAAAATAGGACGCGCG.TTCCC  292
Rr         TTTGCCCGAATACATAAGCATGGAATAATAAAATAGGACGCGCG.TTCCC  292
Pr         TTCGGCCGAGTACATTAGCATGGGATAATAATATAGGTCG.GCTATTCCC  292

                   790       800
graminis   AT.TTTGTTGGTTTCTGA....  311
glutinis   AT.TTTGTTGGTTTCTGAGATC  313
Saitoella  AT.TTTGTTGGTTT........  305
Rhodosp    AT.TTTGTTGGTTTCTGA....  310
TP1        AT.TCCGTTGGTGTCTGAGATC  313
Rr         AT.TCCGTTGGTGTCTGAGATC  313
Pr         ATCTTGGATGGACTCTGATATT  314
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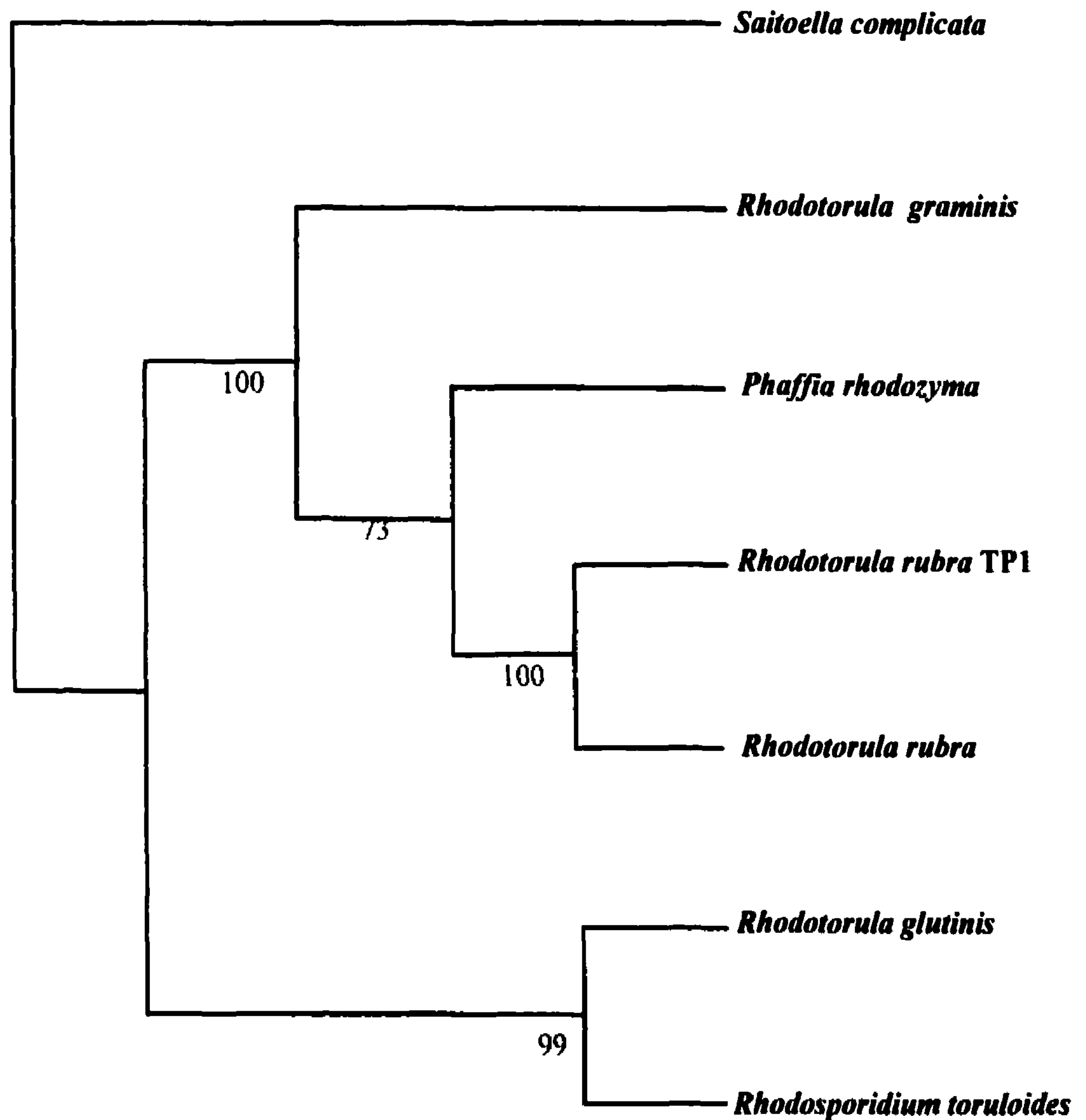


Fig. 5.4 Boostrap majority-rule consensus tree constructed from a cladistic analysis of the partial sequences of position 481 through 802 of the small subunit (18S) rDNA of *R. rubra* TP1 and 6 other yeast isolates using 100 times resampling bootstrapping in PAUP (Swafford, 1993). The numerals represent the percentages of sampling of bootstrap supporting the internal branches.

CHAPTER 6

ISOLATION OF PIGMENT HYPER-PRODUCING MUTANTS AND IDENTIFICATION OF PIGMENTS PRODUCED BY THE MUTANTS AND PARENTAL STRAIN OF *Rhodotorula rubra* TP1.

6.1 Introduction

Carotenoids are widespread in nature occurring among plants, animals, bacteria and fungi. Among the yeast, carotenoids usually encountered include β-carotene, lutein, zeaxanthin, cryptoxanthin, torulene, torularhodin (Ciegler, 1965; Goodwin, 1992) and astaxanthin (Andrewes *et al.*, 1976). In the genus *Rhodotorula*, it has been established that β-carotene, γ-carotene, torulene and torularhodin occur with torulene usually predominating (Ciegler, 1965). The presence of carotenoids in this genus makes it commercially attractive for use as feed in the aquaculture and food industries.

Rhodotorula rubra TP1 is a new strain of yeast isolated from contaminated home made yogurt (Hari *et al.*, 1992). Preliminary studies done with this yeast indicates that it might be a good source of pigment for salmonids (Sangha, 1994). However, the chemical nature of the pigments produced by this isolate could not be determined. Considering the potential for further development of this product in the

aquaculture industry, it is important that the exact nature of the pigments produced by this yeast be determined. Furthermore, like other yeasts that are being tested for use as a source of pigment in the aquaculture industry, the pigment concentration in this new isolate is low. The market potential of this new isolate will be greatly enhanced if mutants that are able to produce the red pigments in quantities far exceeding the amount produced by the parental strain could be isolated.

The present study was therefore undertaken to characterize and determine the concentration of the pigment produced by this new isolate. The report also describes the use of various mutagens to screen for pigment hyper-producing mutants. Yellow pigments such as β-carotene are also in demand for use in the poultry and pharmaceutical industries (Johnson *et al.*, 1980). The other focus of the mutation studies was, therefore, to produce mutants that are blocked in the production of the red pigment torularhodin and torulene but which produce β-carotene and other yellow pigments that may be of commercial and industrial value.

6.2 Materials and methods.

Chemicals: Sodium sulfate, acetic acid, acetone (Optima™ grade), petroleum ether (Optima™ grade), Florisil™, hexane, magnesium sulfate (anhydrous) magnesium oxide, Hyflo Super Cel were purchased from Fisher Scientific, Ltd., Fair Lawn, N.J. Toluene, β-carotene standard, β-ionone, citric acid, micanozole, 2-methyl-imidazole, antimycin A, ethyl methane sulfonate (EMS), thenoyltrifluoroacetone (TTFA) N-methyl-N-nitrosoguanadine (NTG) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co., St. Louis, MO. YM broth and YM agar were from DIFCO Laboratories, Detroit, Michigan; silica gel 60 F_{254} were purchased from EM Science, Darmstadt, Germany. Ketoconazole was obtained from ICN Biomedicals, Aurora, OH. Some of the β-carotene standards and torularhodin were gifts from Hoffman La Rouche, Basel, Switzerland. Phytoene and phytofluene standards were prepared from freshly ripened tomatoes by a modified method of Britton (1995).

6.2.1 Quantification and identification of pigments produced by *Rhodotorula rubra* TP1.

6.2.1.1 Growth of organisms for pigment analysis.

The organism was grown in YM broth in a 14 L fermenter with 10 L working volume (New Brunswick Scientific Co., Edison, New Jersey). Aeration rate, pH and

agitation speeds were controlled. A turbine-type impeller provided agitation and air was delivered by an external source through a sterilized air filter and a hollow agitator shaft. An automatic pH controller fitted with a pump module was used to control the pH. The growth conditions were agitation rate of 100 revolutions per minute (r.p.m.), aeration rate of 1 volume of air per volume of medium per minute (v.v.m), temperature of 22°C and incubation period of 5 days. Light was provided by an external light source.

6.2.1.2 Growth of organism in MVA-supplemented medium

The yeast was grown in a 500 mL YM broth in a 2 L Erlenmeyer flask supplemented with [2R]-(2-^{14}C) MVA (680,000 dpm). The organisms were cultured in a Psychrotherm Environment Controlled Incubator (New Brunswick Scientific Ltd, New Brunswick, New Jersey) at a temperature of 22°C, agitation speed of 150 rpm for 5 days. Cells were harvested by centrifugation at 10,000 x g, washed several times with deionized water before being subjected to pigment analysis. Pigments were radio assayed by liquid scintillation counting in a Beckman Model LS-3150T Liquid Scintillation Spectrophotometer with a Beckman liquid scintillation fluid (Beckman Scientific Instruments, Irvine, California, U.S.A.). Quenching corrections were made by automatic colour quench compensation (Bramley *et al.* 1974).

6.2.1.3 Estimation of total pigment concentration.

For this portion of the work, advice was sought from Dr. K. L. Simpson (University of Rhode Island). Freshly harvested cells (ca. 3g dry weight) were suspended into a thick slurry in deionized water and ruptured in a French Press (SLM Instruments, Inc., Urbana, Illinois) at a pressure of 40,000 psi. The ruptured cells were centrifuged at 10,000 x g in a Sorval RC-5B *Plus* centrifuge (Sorvall Instruments-Dupont Ltd., Newark, DE.) for 20 minutes and the resulting pelleted cells were extracted with 100 mL aliquots of acetone. The acetone extracts were pooled and mixed together with petroleum ether. The petroleum ether phase containing the pigment was collected and saved. The acetone phase was further extracted with petroleum ether until colorless, and the collected petroleum ether phase was filtered through a glass wool to remove any suspended particles, and then dried over sodium sulfate. The carotenoid concentration in the petroleum ether was estimated by measuring the abosrbance at 465 nm in a Shimadzu Ultraviolet 260 Recording spectrophotometer (Shimadzu Corp., Kyoto, Japan). The total carotenoid content was calculated using 1 % extinction coefficient of 2000 and a formula given by An *et al.* (1989). The formula is given as:

$$TC = \frac{V \times A_{465} \times 100}{20 \times W}$$

Where: TC = Total pigment concentration

V = Total volume of petroleum ether extract collected

W = dry weight of yeast used

A_{465} = absorbance at 465 nm

6.2.1.4 Separation and identification of individual pigments.

For the identification of the pigments column chromatography, thin layer chromatography, high performance liquid chromatography (HPLC) and light spectroscopy were used to separate and identify the pigments.

6.2.1.4.1 Column chromatography.

The petroleum ether extract was evaporated to dryness by passing through it a stream of nitrogen gas. The slurry of the extracted pigment (about 0.5g wet weight) was re-dissolved in petroleum ether and the petroleum ether solution was dried over anhydrous sodium sulfate and stored at -85°C overnight. Open column chromatography was employed for pigment separation. The column consisted of MgO-Hyflo Super Cel® chromatographic media at a ratio of 1:2 {w/w} (Fisher Scientific Ltd, Fair Lawn, New Jersey) in a glass column (2.6 x 30 cm). The column was initially developed with 1% acetone in petroleum ether (v/v), and then washed with 15% acetone in petroleum ether, the eluant was collected and saved. Torularhodin (red band) remained on the top of the column and the other less polar pigments were eluted. The column was dried and the torularhodin band was cut from the column. The torularhodin was eluted from the column with 10% acetic acid in

petroleum ether (v/v). The solution was then washed free of the acetic acid with deionized water and dried over anhydrous sodium sulfate and stored at -85 °C.

The other less polar pigments were combined with petroleum ether in a separatory funnel and the two phases were resolved with deionized water. The petroleum ether phase was collected, concentrated with a stream of nitrogen and the pigments were applied onto a fresh MgO-Hyflo Super Cel® column (1.6 x 60 cm, 1:2 w/w). The chromatogram was developed with 1% acetone in petroleum ether until the bands separated and eluted from the column. β-carotene was found to co-migrate with phytofluene so the fraction containing the two pigments were re-chromatographed on Florisil™ chromatographic medium (Fisher Scientific Ltd., Fair Lawn, New Jersey) and developed with 5% toluene in PE. Phytofluene was monitored with a long wavelength (366 nm) UV lamp; the pigment was seen as a blue-green fluorescent band moving just ahead of β-carotene. The collected fractions were individually transferred to petroleum ether and the two phases separated with deionized water. The petroleum ether phase was collected, dried over sodium sulfate and evaporated to dryness with a stream of nitrogen before being stored at -85 °C.

All the separated bands were re-dissolved in petroleum ether and the absorbance measured by spectrophotometer. The maximum absorbance was determined by scanning from 600 to 300 nm in a Shimadzu Ultra Violet 260 Recording Spectrophotometer (Shimadzu Corp. Kyoto, Japan). Identification of the individual pigments was done by comparison of their absorption maxima with those of standard carotenoids reported by other researchers (Davies, 1976; Bauerfeind,

1981). The concentrations of the individual pigments were calculated using the method provided by An *et al* (1989) and the following 1% extinction coefficients (Simpson *et al.*, 1964; Bauerfiend, 1981): β-carotene:2600; torulene:2680; torularhodin:1932; phytoene:850, and phytofluene:1540.

6.2.1.4.2 Thin layer chromatography (TLC)

Thin layer chromatography was performed with silica gel 60 F_{254} thin layer plates (EM Science, Darmstadt, Germany). The plates were developed with 10% toluene in petroleum ether for 60 minutes, which separated all the pigments with torularhodin remaining at the origin. The separated bands were located by colour and by exposure to long wavelength UV light (366 nm), scrapped off and eluted with acetone; the eluted pigments were then mixed with petroleum ether and the phases resolved with deionized water. In the case of torularhodin, the pigment was eluted with 10% acetic acid in acetone and then mixed with petroleum ether. The acetic acid was washed out with deionized water and the phase containing the torularhodin was dried over sodium sulfate and then concentrated with a stream of nitrogen. The identities of the pigments were determined by comparison of their Rf values with known pigments and also by scanning from 600 to 300 nm in a spectrophotometer. The absorption spectra and maxima were then compared with those from literature.

6.2.1.4.3 High performance liquid chromatography (HPLC).

Samples were dissolved in the mobile phase (100% methanol) and filtered through 0.25 μm membrane. A 10 μL sample solution was injected onto HPLC column for the separation and identification of pigments. The HPLC system consisted of a Hewlett Packard (Palo Alto, California) HP 1050 HPLC Series coupled with a Phenomenex Partisil ODS column (100 mm x 2 mm i.d. with 5 μm packing size). The sample was eluted at a rate of 0.75 mL/min with a 15 minute linear gradient of 80 to 100% methanol in water over 30 minutes. Separation was carried out at ambient temperature. A HP 1050 series variable UV detector was used to monitor the chromatographic effluent and detection of carotenoids was made at 475 nm. Standards of β-carotene, torulene, torularhodin phytoene and phytofluene were run in parallel and the carotenoids were identified by comparison of their retention times with those of the standards.

6.3 Mutagenesis of *R. rubra* TP1 cells

Mutagenesis of *R. rubra* cells were attempted with UV light, ethyl methane sulfonate (EMS) and N-methyl-N-nitro-N-nitrosoguanadine (NTG). Methods used in this study are modified from those reported by An *et al.* (1989).

6.3.1 UV irradiation

The yeast cells were grown in YM broth to an optical density of 0.3 to 0.4 at 700 nm. The cells were then aseptically poured into a petri dish in a sterile

inoculating chamber which had an UV lamp with a maximum energy range of 220 to 280 nm. The cells were exposed to UV radiation for a period that resulted in greater than 95% kill (about 40 min), grown in the dark for 24 hours and then plated on YM agar. UV irradiation resulted in cells that were pale in colour and have the appearance of substantial loss of pigment. Attempts at isolating highly pigmented colonies were not successful hence this method was discontinued.

6.3.2 EMS mutagenesis

Twenty-four-hour culture of *R. rubra* TP1 diluted to an optical density of 0.4 at 700 nm were suspended in a sterile phosphate buffer (pH 7.0) and EMS was added to give concentrations of 2 %, 4 %, 8 % and 16 %. One mL aliquots of these suspensions were pipetted into 1.5 mL eppendorf tubes. The cells were then shaken in a rotary shaker for 20 min and then allowed to stand for 10 min after which the cells were centrifuged, washed 4 times with the same buffer and then several times with sterile deionized water. Surviving cells were grown overnight in YM broth and then several dilutions of the YM broth culture were prepared and plated on YM agar. The cells were then visually inspected for pigment hyper-producing mutants. EMS mutagenesis did not generate any colonies that were highly pigmented and, therefore, this method was not given any further consideration.

6.3.3 N-methyl-N-nitro-N-nitrosoguanadine (NTG) mutagenesis

For NTG mutagenesis, freshly grown yeast cells were washed twice in 5 mL

of sterile 0.1 M sodium citrate buffer (pH 5.5) and cells were re-suspended to an optical density of 1.5 to 2 at 700 nm. Samples of 6.7 mL of yeast suspension were pipetted into culture tubes and NTG solution (1 mg/mL in sodium citrate buffer) were added to give concentrations of 40, 60 and 100 µg/mL. The mixtures were vortexed for 30 seconds, and 1 mL aliquots were transferred to sterile eppendorf tubes and incubated for 30 min, 1, 2, 4 and 16 hours. After the incubation period, the samples were washed several times with sterile phosphate buffer (pH 7) and then transferred into 30 mL sterile YM broth which were incubated at 22°C overnight. Several dilutions were then prepared and the appropriate dilutions plated on YM agar. The concentration that gave greater than 95% kill was determined and was used in all subsequent studies.

Mutants isolated from YM agar plates and freshly grown cells from the parental strain were also plated on various selective media in an attempt to induce increased pigmentation in the yeast. The selective media were made up of YM agar into which the following inhibitors at various concentrations have been incorporated: micanozole, ketoconazole, nicotine, thenoyltrifluoroacetone (TTFA) and 2-methyl-imidazole.

To determine the effects of β-ionone on the production of pigments in both the parental strain and mutants, the organisms were grown on YM agar incorporated with β-ionone and in YM broth incorporated with various concentrations of β-ionone. Since β-ionone is insoluble in water, it was dissolved in ethanol before being added to the medium. The controls were incorporated with equal amounts of ethanol and the

cells were visually screened for pigment hyper-producing mutants.

6.4 Characterization of mutant strains.

6.4.1 Physiological characterization of mutants.

Mutants were characterised by the quality and quantity of pigmentation, assimilation of various carbons, potassium nitrate utilisation, urease test, starch formation and gelatin liquefaction. The growth curves for all the mutants were determined by growing organisms in YM broth for 5 days. Samples were withdrawn at intervals and growth measured by determining the optical density (OD) at 700 nm.

6.4.2. Quantitative determination and identification of pigments produced by mutants.

For pigment analysis, mutants were grown in YM broth in 2 L Erlenmeyer flasks at 22°C for five days. The cells were harvested by centrifugation and freeze-dried before pigment extraction. Lyophilized cells (1 g) were ruptured by mixing with 6 mL dimethyl sulphoxide (DMSO) which has been warmed up to 40°C in a 40 mL centrifuge tube. The suspension was vortexed for 1 minute and then allowed to stand in the dark for 20 minutes at room temperature. The ruptured cells were then centrifuged at 14,000 x g for 10 minutes and the supernatant decanted into a 100 mL tube, flushed with nitrogen and kept on ice. The pellet was re-extracted 3 times with 5 mL aliquots of acetone. The acetone and DMSO extracts were pooled together in a

separatory funnel and equal amounts of petroleum ether added as well as deionized water to ensure phase separation. The petroleum ether phase was removed and the aqueous layer re-extracted with petroleum ether. The petroleum ether extracts were pooled together, dried over anhydrous sodium sulfate and finally evaporated to dryness under a stream of nitrogen. The residue was re-dissolved in petroleum ether and subjected to column chromatography. The chromatographic analysis and determination of the concentration of the individual carotenoids have already been described in section 6.2.1.3.1.

6.5 Results.

6.5.1 Quantification and characterisation of pigment present in *Rhodotorula rubra* TP1.

To characterize the pigments produced by the new yeast isolate, three chromatographic methods were used, open column chromatography, thin layer chromatography and HPLC. A sample HPLC chromatogram is shown in Fig. 6.0 and the carotenoid profile is given in Table 6.0. Figs. 6.1 to 6.3 show the spectra of the various carotenoids separated by column chromatography. From the HPLC chromatogram, it can be seen that torularhodin, torulene and β-carotene were the major pigments found in the new yeast isolate *R. rubra* TP1 whereas phytoene and phytofluene were produced in small quantities. From the carotenoid profile indicated

in Table 6.0, it can be observed that β-carotene (104 μ/g yeast) was the pigment with the highest concentration of the total carotenoid and is closely followed by torulene (80 μ/g yeast) and then torularhodin (64 μ/g yeast). The absorption maxima recorded for the various carotenoids in petroleum ether are also depicted in Table 6.1. From these results, it can be seen that the characteristic absorption maxima for all the pigments are in close agreement with published results. Torularhodin had an absorption maxima of 535 compared with the reference value of 537, torulene 514, β-carotene 478, phytoene 294 and phytofluene 364. The total pigment concentration determined for the new isolate was 261 μg/g yeast on dry weight basis (Table 6.0).

The RF values determined for the individual pigments also coincided with those reported in literature (Table 6.0). Torularhodin had RF value of 0.15, torulene 0.30, β-carotene 0.50, phytoene 0.84 and phytofluene 0.62.

6.5.2 Growth of organism in media supplemented with [2-^{14}C]MVA.

To confirm the identity and the biosynthetic pathway of the carotenoids produced by the new isolate, the organism was grown in a medium supplemented with labelled MVA. The pigments were analyzed using column chromatography, light spectroscopy and liquid scintillation counting. The results of the pigment analysis and scintillation counting are presented in Table 6.2. From the table it can be inferred that MVA was incorporated into β-carotene, phytoene, phytofluene, torularhodin and torulene as evidenced by the amount of radioactivity detected in

each of these pigments. It can also be seen that β-carotene and torulene had the highest percentage of the total radioactivity, 40 and 30%, respectively whereas torularhodin has 25% radioactivity and phytoene and phytofluene have 2 and 3%, respectively. The quantities of the radioactivity detected in the individual carotenoids were consistent with the concentration of the individual pigments produced by the organism on a non-supplemented medium.

6.5.3 Isolation of mutants from *R. rubra* TP1.

6.5.3.1 Screening of colonies for pigment hyper-producing mutants

The effectiveness of UV light, EMS and NTG in generating hyper-pigmented mutants was evaluated. Most of the mutants generated by UV light and EMS were either colourless or very pale and therefore did not warrant any further analysis. NTG was found to be the best mutagen. It resulted in considerable variation in pigmentation among the colonies (Table 6.3) and repeated mutagenesis with NTG resulted in stable clones.

Mutants TP1-ntg-3, TP1-ntg-4, TP1-ntg-5 and TP1-ntg-6 were isolated after a single mutagenesis of the parent strain *R. rubra* TP1 with NTG. These mutants were found to have total pigment concentration lower than that of the parent strain. Mutant TP1-ntg-3 contained approximately 154 μg/g yeast (estimated as torularhodin) whereas TP1-ntg-4, TP1-ntg-5 and TP1-ntg-6 contained 199, 215 and 121 μg/g yeast, respectively. Compared with the parental strain, the total pigments produced by these

mutants were very low. Qualitative analysis of the pigments revealed that mutants TP1-ntg-4 and TP1-ntg-6 accumulated mainly β-carotene with only small quantities of torulene, phytoene and phytofluene (Table 6.3) whereas TP1-ntg-5 produced mainly torulene and torularhodin. Mutants TP1-ntg-4 and TP1-ntg-6 were yellow in colour and this may be due to the accumulation of β-carotene as main pigment. The absorption maxima of the various carotenoids isolated from the mutants were similar to those reported earlier for the parental strain in Table 6.1. The spectra of the various carotenoids isolated from the mutants were also similar to those depicted in Figs. 6.1 to 6.3 for the parental strain. There was one mutant, TP1-ntg-7, that was colourless and appeared to have no pigmentation at all. Since screening of colonies after a single mutagenesis was not successful in producing highly pigmented colonies, the decision was made to mutagenized the cells several times. Mutants TP1-ntg-1 and TP1-ntg-2 were isolated after repeated (2x) mutagenesis with NTG. These two mutants were found to contain total carotenoid concentration of 537 and 405 μg/g yeast, respectively (Table 6.3). These pigment concentrations were greater than that found in the parental strain which had only 261 μg/g yeast. The quality of pigments produced by these two mutants were, however, the same as those that occur in the parental strain. The dominant pigments that occur in these two mutants and the parental strain were β-carotene, torulene and torularhodin.

6.5.3.2 Physiological and biochemical characterization of mutants.

The growth rate of the parental strain and the isolated mutants were determined by growing them in YM broth for 5 days and the results are depicted in Fig. 6.8. The ability of the mutants to utilize various sources of carbon and nitrogen sources was also determined. The carbon and nitrogen utilization patterns for the parental strain and the mutants are presented in Table 6.5. It can be seen from Fig. 6.8 that both the parental strain, *R. rubra* TP1 and the isolated mutants gave approximately equal amounts of growth after 5 days of incubation. It can also be seen from Table 6.5 that the utilization patterns for the various sources of carbon for all the mutants differ somewhat from that of the parental strain. Mutants TP1-ntg-1, TP1-ntg-2 and TP1-ant-1, unlike the parental strain were found to utilize succinic acid, cellobiose, inositol and α-ketoglutaric acid as sole carbon sources.

6.5.3.3 Susceptibility of *R. rubra* TP1 to antimycin and other inhibitors

Several sterol biosynthesis inhibitors were tested in an attempt to divert all energy and resources towards the production of carotenoid (Kappeli, 1986, An *et al.*, 1989) and hence isolate resistant strains with increased pigment production. The inhibitors tested include micanozole, ketoconazole, nicotine, and 2-methyl-imidazole. The survival rate for colonies treated with these inhibitors are depicted in Figs. 6.4 and 6.5. Even though these inhibitors were able to decrease the survivability of the mutants by as much as 50% or more, none of them were able to generate colonies with increased pigment production. All of the strains generated by this procedure

showed drastic reduction in pigmentation when inspected visually. The mutants were left on these plates for three months but no mutant with increased pigment production was obtained.

In a study to determine the formation of astaxanthin in the yeast *P. rhodozyma*, Johnson and Lewis (1979) reported that high concentration of glucose or reduced oxygen availability reduced xanthophyll biosynthesis and can therefore cause the accumulation of carotene pigments. An *et al.* (1989) concluded from these findings of Johnson and Lewis (1979) that oxygenation of carotenes through the activation of molecular oxygen is rate limiting for astaxanthin synthesis in some growth environments. By analogy to other related pathways such as sterol biosynthesis, An *et al.* (1989) suggested that the source of active oxygen and oxygenating enzymes would likely be the mitochondrial respiratory chain and associated heme proteins, e.g. cytochrome P-450. This strongly suggests that treatment of organisms with inhibitors of the electron transport system would inhibit the organism's ability to oxygenate carotenes and hence would result in the accumulation of carotenes. With this in mind, we treated *R. rubra* cells with two inhibitors of the electron transport chain, thenoyltriflouroacetone (TTFA) and antimycin A in an attempt to generate colonies that produce large quantities of carotenes. The results of the susceptibility of *R. rubra* to these inhibitors are depicted in Fig. 6.6. Small concentrations of antimycin A and TTFA killed the yeast, and at TTFA concentration of 0.6 mM, the survivability of *R. rubra* was only 10% whereas antimycin concentration of 60μm reduced the population of *R. rubra* to about 11%.

Resistant colonies isolated from plates incorporated with various concentrations of TTFA were pale in colour and were therefore not given any further consideration. Colonies that grew on 20 and 40 μm antimycin A were also pale in colour, however, at 60 μm, the colonies appeared dark red. These colonies were isolated for further studies and named mutant TP1-ant-1. The results of both the quantitative and qualitative pigment analyses of mutant TP1-ant-1 are depicted in Table 6.3. This mutant was found to contain total pigment concentration of 483 μg/g and this concentration was greater than that found in the parental strain. This mutant also had increased production of torularhodin and torulene when compared to that of the parental strain.

6.5.3.4 Susceptibility of *R. rubra* TP1 and isolated mutants to β-ionone.

β-ionone, an end ring analogue of β-carotene has been reported to inhibit astaxanthin production in *P. rhodozyma* and provides little or no stimulation for β-carotene production in this same organism. However, when cells mutagenized with NTG were plated on YM agar incorporated with β-ionone, astaxanthin over-producing mutants were isolated (Lewis *et al.*, 1990). These mutants were found to produce more oxycarotenoids and smaller amounts of β-carotene than the parental strain. In *R. glutinis*, it has been reported that β-ionone stimulated the formation of phytoene, phytofluene, ζ-carotene, neurosporene and β-zeacarotene (Simpson *et al.*, 1964) whereas torulene, torularhodin and β-carotene production were suppressed.

We tested the effects of various concentrations of β-ionone in YM broth on carotenoid production in both the parental strain of *R. rubra* TP1 and one of its NTG mutants, TP1-ntg-1. We also plated cells from the parental strain on YM agar plates that have been incorporated with β-ionone. When the yeast was plated on β-ionone, mutant TP1-β-ion-1 was isolated. In YM broth incorporated with various concentrations of β-ionone, it was observed that there was a drastic reduction in growth as the concentration increased. At β-ionone concentrations of 10^{-2} and 10^{-3} M, the growth was reduced to less than 10% of that of the parental strain (Fig 6.7). The colour of the colonies also changed from red/pink to yellow and then white as β-ionone concentration increased. Analysis of the carotenoid content of the cells revealed a decrease in the concentration of the total carotenoid, from 261 μg/g in the parental strain to 194 μg/g at a concentration of 10^{-4} M (Table 6.4). Pigment analyses in *R. rubra* TP1 and TP1-ntg-1 grown in media supplemented with various concentrations of β-ionone also revealed a decrease in the concentration of individual pigments as the concentration of β-ionone increased (Table 6.4). In *R. rubra* TP1, there was a complete loss of torularhodin and phytoene at all levels of β-ionone concentrations, whereas TP1-ntg-1 had gradual reduction in all pigments as the concentration of β-ionone increased. At β-ionone concentration of 10^{-2} and 10^{-3} M, the organisms seemed to have lost the ability to produce pigments. The cells were pale in colour with no indication of the presence of pigments hence no pigment analysis was conducted in those cells. β-carotene was found to be the dominant

carotenoid at all β-ionone concentrations tested.

6.6 Discussion.

6.6.1 Identification of pigments produced by *R. rubra* TP1.

Inspection of the carotenoid composition of *R. rubra* TP1 as well as the mutagenized cells allowed the identification of most of the carotenoid previously described in other *Rhodotorula* species (Bonner *et al.*, 1946; Hayman *et al.*, 1974; Simpson *et al.*, 1964). In the present study, torulene, torularhodin and β-carotene were determined to be the major pigments produced by the new yeast isolate *R. rubra* TP1. These findings are consistent with reports on pigments produced by *R. rubra*. In *Rhodotorula* and *Rhodosporidium* species, the abundant carotenoids usually encountered are torulene and torularhodin even though several other carotenoids including β-, γ-, and ζ-carotene, phytoene, phytofluene and β-zeacarotene may also be present (Ciegler, 1965; Hayman *et al.* 1974). In a mutational study in *R. mucilaginosa*, Villoutreix (1960) reported that torulene, torularhodin, γ-carotene and β-carotene were the principal pigments of the parental strain, whereas phytoene and phytofluene were absent. Nakayama *et al.* (1954) also examined the pigments from several *Cryptococcus* and *Rhodotorula* species and concluded that depending on the cultural conditions, especially the growth temperature, the quantities of the red and yellow pigments can vary. According to these authors the concentration of the red pigments decreased at 5°C and then increased as the temperature is increased.

In a study to re-examine the pigments produced by *R. glutinis* strain 48-23T

which had been studied earlier by Nakayama *et al.* (1954), Simpson *et al.*, (1964) reported that the total carotenoid concentration, on a dry weight basis, was nearly equal at both room temperature and 5°C. The level of γ-carotene was reported to be fairly constant but there was an increase in the level of torulene and torularhodin coupled with a decrease in the levels of β-carotene when the yeast was cultured at a higher temperature (25°C). The gain in the levels of torulene and torularhodin were nearly equal to the decrease in the level of β-carotene. According to Simpson *et al.* (1964), these results suggest that γ-carotene lies at the branch point in the carotenoid biosynthesis sequence, and that intermediates can be channelled through it either to β-carotene or to the red pigments, torulene and torularhodin, depending on the growth temperature. Similarly, in *R. pallida* 62-506, it was shown that there was an increase in the level of torulene and torularhodin as the level of γ-carotene decreased. Based on these studies Simpson *et al.* (1964) concluded from that γ-carotene is converted to torulene which is in turn converted to torularhodin.

Nakayama *et al.* (1954) determined the content of individual carotenoids present in several species of *Rhodotorula* and reported the principal pigments to be torulene, torularhodin, β- and γ-carotene. Bonner *et al.* (1946) on the other hand, found four major carotenoids in *R. rubra* and several of its mutant strains. These carotenoids were identified as torulene (76%), β-carotene (11%), γ-carotene (9%) and an unidentified carotenoid (4%). Other workers (Fink and Zenger, 1934; Fromageot and Tchang, 1938; Karrer and Ratschmann, 1943) have also reported similar

carotenoid production patterns in several *Rhodotorula* species. Although all these workers agree on the presence of these three components (torulene, torularhodin and β-carotene), the data on the respective concentrations reported by them differ somewhat. These workers employed different strains of yeast and cultural conditions in their studies. Since it has been reported that pigment composition depends on the strain of yeast and particular cultural conditions (Nakayama *et al.*, 1954; Kvanikov *et al.*, 1978; Bonner *et al.*, 1946), the differences in the concentration of the various pigment composition should not be surprising. We therefore report here that the major pigments produced by this new isolate have been identified as β-carotene, torulene and torularhodin. From the results of the studies that have been elaborated above, it is reasonable to assume that the pigment identified in the new isolate *R. rubra* TP1 are consistent with the pigments found in other *Rhodotorula* species.

Mevalonic acid is known to be an obligatory intermediate in the biosynthesis of carotenoids (Bramley and Mackenzie, 1988; Goodwin, 1965). MVA is converted into MVA 5-phosphate, MVA 5-pyrophosphate, isopentinyl pyrophosphate and dimethylallyl pyrosphophate. Successive condensation reactions of isopentinyl pyrophosphate with dimethylallyl pyrophosphate, geranyl pyrophosphate and farnesyl pyrophosphate yield geranylgeranyl pyrophosphate which condenses with similar C_{20} units to form phytoene, which is the precursor of the more unsaturated carotenoids. Labelled MVA has been used in tracer experiments to determine the carotenoid biosynthetic pathway and also to confirm the identity of the pigments produced by

numerous micro-organisms (Tefet *et al.* 1970). It has been shown that labelled MVA contributes carbon to the end methyl group of β-carotene and to six other positions in the molecule (Tefet *et al.* 1970). In the formation of torularhodin, one or either end of the methyl group may be utilized in the formation of the carboxyl group. Other isotopes have been used to obtain information on the biosynthesis of carotenoids in yeast. Yamamoto *et al.* (1962) used labelled carotenoid precursors to show that the hydroxy- and oxo- groups in carotenoids are derived from gaseous oxygen. Simpson *et al.* (1963) using *R. rubra* and ^{18}O-enriched atmosphere confirmed the hypothesis that the formation of hydroxytorulene is through the direct participation of atmospheric oxygen, and that oxidation of oxotorulene to torularhodin involves the use of another oxygen molecule from the medium.

To confirm the identity and the biosynthetic pathway of the pigments produce by *R. rubra* TP1, the organism was grown in a medium supplemented with labelled MVA. Radioactivity was detected in β-carotene, torulene, torularhodin, phytoene and phytofluene. Based on the radioactivity in these compounds, the pathway for the production of pigments in the new isolate may be considered as being identical to the pathway of pigment production in *R. glutinis* postulated by Simpson *et al.* (1971) and which has already been depicted in Fig. 6.9. Simpson *et al.* (1971) reported the presence of β-zeacrotene in *R. glutinis* and therefore suggested that β-zeacarotene may lie at a branch point in the biosynthetic pathway. β-zeacarotene was not detected in this study even though it is possible that it may have been produced by the yeast

but was quickly converted to other carotenoids. The accumulation of β-zeacarotene in yeasts occurs in adverse environmental conditions, e.g. in the presence of inhibitors or in stressful environment indicating possible inefficiencies in the carotenoid biosynthetic pathway (Johnson and Lewis, 1979). The formation of β-zeacarotene in organisms may be rationalized by a hypothesis of McDermott *et al.* (1974) who postulated that the synthesis of zeaxanthin by *Flavobacterium* spp. involves an enzyme complex with two active sites. Each of these sites acts on a carotenoid 'half molecule' in synchrony with equal efficiency i.e. desaturation or cyclization of each half molecule proceeds at the same rate. Under abnormal conditions, however, the individual sites may not act in synchrony and asymetrical products such as β-zeacarotene may result. Thus under normal growth conditions, all the β-zeacorotene produced by the organism is converted to other carotenoids. It should be noted that Simpson *et al.* (1971) detected the presence of β-zeacarotene only after treating the yeast with inhibitors, the inhibitors may have therefore caused the accumulation of β-zeacarotene.

The total pigment concentration determined for the new isolate was 261 μg/g (on dry weight basis). This concentration is low and for the yeast to be industrially competitive, mutants with increased pigment production may be needed. This report therefore describes the isolation of mutants of *R. rubra* TP1 with significantly higher concentrations of pigment than the parental strain.

6.6.2 Isolation of pigment hyper-producing mutants of *R. rubra* TP1.

NTG was used to generate various strains of mutants from the parental strain, *R. rubra* TP1. The results of the mutagenic studies are depicted in Table 6.3. It can be seen that the mutant strains generated fall into several categories: (a) mutants TP1-ntg-1 and TP1-ntg-2 can be described as mutants producing the same carotenoid profile as the parental strain but with significantly higher total pigment concentration; (b) colourless mutant, TP1-ntg-7 which appeared to have lost the basic general process to pigment production. These mutants were probably inhibited during the early stages of carotenogenesis and were in all likelihood affected in the enzyme phytoene synthetase (Girard *et al.* 1994); (c) mutants TP1-ntg-3 and TP1-ntg-4 and in which β-carotene represented a larger percentage of the total carotenoid content; (d) mutant TP1-ntg-5 which produced high concentrations of torularhodin and torulene with inhibited phytoene production; and finally, (e) mutant TP1-ntg-6 which accumulated mainly β-carotene. Mutant TP1-ntg-6 was yellow in colour and was clearly inhibited in xanthophyll production, probably formation of other carotenoids are blocked at the β-carotene level.

In a study using *P. rhodozyma*, Girard *et al.* (1994) reported that yellow mutants accumulating high concentrations of β-carotene and white mutants with no carotenoids were obviously affected in distinct steps in the carotenoid biosynthetic pathway. Using protoplast fusion of a colourless mutant and β-carotene accumulating mutant of *P. rhodozyma*, they postulated that the yellow β-carotene accumulating

mutants were blocked in the oxidase step therefore unable to convert β-carotene to eichinenone. The biosynthetic pathway in all carotenogenic yeasts studied up to date follows similar routes (Goodwin, 1965) with minor variations in the final steps leading to a characteristic carotenoids. The proposed pathway for the biosynthesis of carotenoids in yeast have earlier been depicted in Fig. 2.0 to 2.2 and that for *Rhodotorula* species are depicted in Fig 2.3. In *Rhodotorula* species, γ-carotene produced in the biosynthetic pathway usually suffers two fates. First, it can undergo a cyclization reaction to yield β-carotene or the γ-carotene can undergo dehydrogenation to yield torulene. The torulene can then be oxidized to form torularhodin (Simpson *et al.* 1964). In this study, it is most likely that the β-carotene accumulating mutants isolated were impaired in the dehydrogenation step and were unable to convert γ-carotene to torulene. The net result was that most of the γ-carotene underwent cyclization to form β-carotene. The β-carotene overproducing mutants isolated in this study are therefore reminiscent of the β-carotene accumulating mutants of the yeast *R. glutinis* (Kayser and Volloutreix, 1961). Like the β-carotene accumulating mutants of *R.rubra* TP1, the mutants of *R. glutinis* accumulated β-carotene at the expense of torularhodin production.

The mechanism leading to the overproduction of carotenoids in mutants TP1-ntg-1 and TP1-ntg-2 cannot be explained at this point. However some workers have offered explanation for the overproduction of carotenoids in some organisms. For example, in a study of the β-carotenoid overproducing mutants of the alga *Dunaleilla*

bardawil, Shaish *et al.* (1991) proposed that alterations in the metabolic steps preceding GGPP was responsible for the activation of the carotenoid biosynthetic pathway. A similar mechanism has been proposed by An *et al.* (1989) who isolated astaxanthin-overproducing mutants from the yeast *P. rhodozyma* by subjecting them to increased antimycin A concentration. They tentatively attributed the carotenoid overproduction to alteration in the cytochrome b or cytochrome P-450 components. Whatever the mechanism for the overproduction of pigments in mutants isolated in this study, it is obvious that the mutation resulting in carotenoid overproduction affected the overall flow of the biosynthetic pathway. Mutant TP1-ntg-7 was colourless and appeared to have no carotenoid production. This mutant appeared to have lost the ability to synthesize carotenoids.

6.6.3 Nutritional and biochemical characteristics of mutants

Mutants TP1-ntg-1, TP1-ntg-2 and TP1-ant-1 were found to utilize succinic acid, cellobiose, inositol and α-ketoglutaric acid in contrast to the parental strain that was unable to utilize these sugars as sole carbon. The ability of these mutants to utilize these carbons may have enhanced their ability to produce pigments since these compounds have been reported to promote increase pigment production in yeast. For example, Meyer *et al.* (1993) reported an astaxanthin concentration of 1926 μg/g in *P. rhodozyma* J4-3 when grown in media containing succinic acid. They also reported significant increase in astaxanthin content with cellobiose and other carbon compounds. Similarly, Johnson and Lewis (1979) reported higher astaxanthin

content in *P. rhodozyma* UCD 67-210 grown on cellobiose, mannitol, succinic acid and sucrose. They suggested that the higher astaxanthin content obtained on succinic acid was due to direct incorporation into the tri-carboxylic acid cycle, whereas cellobiose stimulated carotenoid production because it can be utilized aerobically.

6.6.4 Susceptibility of *R. rubra* TP1 to antimycin and other inhibitors.

Antimycin has been reported to stimulate carotenogenesis in micro-organisms at low concentrations. In a study to determine the effects of antimycin on carotenoid production in *P. rhodozyma* and its mutants, An *et al.* (1989) reported significant increases in several carotenoids including astaxanthin, β-carotene and 3',4'-didehydro-β-ψ-carotene-4-one in both the parental strain and the mutants. However, the antimycin-induced mutants were found to grow slowly on various nitrogen sources and also had a reduced yield on numerous carbon sources. An *et al.* (1989) also observed that the mutants have small size and a slower growth rate when grown in YM broth. They concluded that the slower growth, reduced yield and the small size is an indication that the mutants were impaired in their ability to obtain energy from carbon sources and may have been unable to obtain equivalent amount of energy from the media as the parental strain. In our study, the antimycin induced mutant, TP1-ant-1 was not tested on carbon sources nor potassium nitrate. The growth rate was also determined in YM broth. It was observed that the mutant had reduced yield and a slow growth rate when compared to the parental strain. It is possible that this mutant was impaired in its ability to utilize energy from the

medium. However, the quantity of pigments produced by this mutant was about 185 % more than that produced by the parental strain. There was also a considerable increase in the production of the oxycarotenoids, torulene and torularhodin whereas the other mutants treated with the other inhibitors of the electron transport chain - micanozole, ketoconazole, nicotine, TTFA and 2-methyl imidazole were completely inhibited in carotenoid production and restricted in growth.

Cytochrome P-450s are a diverse class of b-type heme-containing monooxygenases that are involved in a wide variety of biotransformations (Martinis *et al.*, 1991). In *S. cerevisiae*, cytochrome P-450s are involved in demethylation, desaturation and oxygenation of sterol intermediates (Jefcoate, 1986; Waterman *et al.*, 1986). Similarly, multiple plant cytochrome P-450s are well documented in plants where they are involved in hydroxylations of carotenoids (Donaldson and Luster, 1991). Hydroxylation during carotenogenesis involves mixed function oxidase (MFO) reactions involving cytochrome P-450s (Johnson and An, 1991, Britton, 1982). Sandman and Bramley (1985) reported that *in vitro* biosynthesis of β-cryptoxanthin from β-carotene in *Aphanocapsa* membranes involved a monooxygenase reaction and that hydroxylation was dependent on oxygen and sensitive to potassium cyanide and other monooxygenase inhibitors. Astaxanthin formation in *P. rhodozyma* was inhibited by metyrapone and piperonyl butoxide, compounds that are known to inhibit mixed function oxidase reactions involving cytochrome P-450s (Johnson and An, 1991). The results of the studies enumerated above strongly suggest that cytochrome P-450s are actively involved in

carotenogenesis and therefore any compound that inhibit the functioning of cytochrome P-450s will adversely affect carotenogenesis.

Cytochrome P-450s are inhibited by a number of substituted imidazoles, pyridines, pyrimidines, miconazole and other lypophilic heterocyclic compounds (Vanden Bossche *et al.*, 1983; 1984). Similarly azole antifungal agents such as ketoconazole inhibits sterol demethylation by binding with cytochrome P-450 and cytochrome P-450 reductase (Yoshida *et al.*, 1986; Yoshida and Aoyama, 1987). This binding leads to the inhibition of the enzymatic reduction of cytochrome P-450 by NADPH in the presence of NADPH-cytochrome P-450 reductase (Aoyama *et al.* 1983, Yoshida and Aoyama, 1987). Since the reduction of cytochrome P-450 through the activity of NADPH-cytochrome P-450 reductase is critical to the hydroxylation and desaturation of carotenoids (Waterman *at al.*, 1986), inhibition of the enzymatic reduction of the cytochrome will lead to the inhibition of carotenoid synthesis. This may explain why the mutants treated with micanazole, ketoconazole, nicotine and 2-methyl imidazole in this study were unable to produce any pigments.

Unlike the above-mentioned compounds, antimycin enhances the reduction and accumulation of cytochromes b species, particularly b_{566} and b_{562} (Chance, 1958; Roberts *et al.*, 1980; Jefcoate, 1986). Accumulation of reduced cytochrome b could promote the reduction of P-450 through NADPH-cytochrome P-450 reductase, which transfers reducing equivalents from NADPH to P-450. As a result, there will be a concomitant increase in hydroxylation and desaturation reactions (Donaldson and Luster, 1991; Waterman *et al.*, 1986) which will ultimately lead to increased

production of carotenoids particularly xanthophylls. In this study it was found out that there was a significant increase in the production of the oxy-carotenoids, torulene and torularhodin in the antimycin A induced mutant, TP1-ant-1. The fact that antimycin stimulated growth and pigment production in the mutant TP1-ant-1, whereas the other inhibitors completely suppressed pigment production suggests that there was induction of cytochrome P-450 in this mutant. This finding is supported by Johnson and Schroeder's (1995) report that a yellow mutant of *P. rhodozyma*, yan-1, which produces β-carotene under normal cultural conditions, produced xanthophylls when treated with antimycin and light. Although the exact mechanism by which the antimycin induced mutants increase carotenoid production is not known, analysis of the pigments produced in this study is evidence that the mutant acquired increased ability to hydroxylate and desaturase carotenoid intermediates than the parental strain. It can therefore be concluded that antimycin induced the production of an alternate oxidase system which allowed the mutant to grow and stimulate carotenoid biosynthesis.

The hyper-pigmented strains isolated in this study would be useful as parental strains to isolate strains with further increase in pigment production. These mutants can also be used for salmon feeding trials to determine whether or not they can effectively pigment the flesh of fish. The mutants blocked in particular steps of the carotenoid biosynthetic pathway may also be used for cloning and characterization of carotenogenic genes.

6.6.5 Susceptibility of *R. rubra* TP1 to β-ionone.

β-ionone had been reported to stimulate the production of β-carotene (Lewis *et. al.*, 1990). We therefore tested the effects of various concentrations of β-ionone on carotenoid production in both the parental strain and the mutant, TP1-ntg-1. It was observed that the cells changed in color from red to yellow and white as the concentration of β-ionone increased. It was observed on further analysis that β-ionone inhibited the production of torulene, torularhodin and phytoene whilst phytofluene and β-carotene production were stimulated. The loss of torularhodin production coupled with an increase in the concentration of β-carotene and a decrease in torulene production when cells were treated with β-ionone may explain the change in the colour of the cells from red to yellowish. Reports on carotenoid produced by organisms treated with β-ionone are mixed. For example, Ninet *et al.* (1969) and Mackinney *et al.* (1954) reported that β-ionone stimulated carotenogenesis in *Blakeslea trispora* and *Phycomyces blakesleeanus*, respectively. Reyes (1963) also showed that sterol as well as carotenoid biosynthesis was stimulated to a marked degree by β-ionone. This effect was explained as being one of a negative feedback inhibition that acts at the level of the phosphorylated derivatives of mevalonic acid. In a study to determine the effects of β-ionone on carotenogenesis, Simpson *et al.* (1964), reported that β-ionone stimulated the formation of phytoene, phytofluene, ζ-carotene neurosporene, and β-zeacarotene in *R. glutinis* whereas the formation of β-carotene, torulene and torularhodin was greatly suppressed. In *R. rubra*, Uehleke and

Decker (1962) found that 250 mg/L of β-ionone inhibited the formation of carotenoids. When added to the mature cultures, β-ionone destroyed torularhodin and β-carotene.

In this study β-ionone clearly inhibited the formation of oxy-carotenoids in *R. rubra* TP1 but stimulated β-carotene production at low concentrations. The overall pigment concentration was, however, drastically reduced at all β-ionone concentrations tested. The accumulation of β-carotene by the mutants and the parental strain grown in β-ionone supplemented media is supported by studies done by other workers. For example, Lewis *et al.* (1990) reported that there was little or no stimulation of oxy-carotenoid production in *P. rhodozyma* and suggested that β-ionone inhibited xanthophyll formation by blocking the carotenoid biosynthetic pathway at the β-carotene level. β-ionone, they contended, being an end ring analogue of β-carotene, may compete for oxygenation at the C-3 and or C-4 with β-carotene and the xanthophylls. As a result, β-carotene would tend to accumulate and the xanthophylls would tend to decrease. This may clearly be the case in this study where inhibition of torularhodin and torulene production was associated with stimulation of β-carotene production.

Table 6.0 Carotenoid profile of *Rhodotorula rubra* TP1

Carotenoid	Concentration (μg/g yeast, dry wtt)	% Composition	RF value
Torularhodin	64	24.52	0.15
Torulene	80	30.65	0.30
β-carotene	104	39.85	0.50
Phytofluene	8	3.10	0.62
Phytoene	5	2.00	0.84
Total	261	-	-

Table 6.1. Comparison of spectral characteristics of pigments isolated from *Rhodotorula rubra* TP1 with literature values.

Carotenoid	Expt. value[1]	Wavelength max. (λ) Lit. value	Reference
Torularhodin	465, 500, 535	467, 501, 537	Goodwin, 1955
Torulene	454, 481, 514	454-460, 480-484, 513-518	Liaaen-Jensen, 1965
β-Carotene	425, 451, 478	425, 448-453, 475-482	Goodwin, 1955
Phytoene	283, 294	276, 286, 297	Britton, 1995
Phytofluene	331, 347, 367	330-333, 347-348, 366-368	Davies, 1965

[1]All determinations were made in petroleum ether.

Table 6.2 Production of carotenoid from labelled mevalonic acid (MVA) by *Rhodotorula rubra* TP1.

Carotenoid	Total incorporation (dpm)[1]	% Incorporation
Torularhodin	27,501	25
Torulene	33,040	30
β-carotene	44,001	40
Phytofluene	2210	2
Phytoene	3023	3

[1]dpm = doses per minute.

Table 6.3. Carotenoid composition (μg/g yeast) of *Rhodotorula rubra* TP1 and its mutant isolated in this study.

		Mutant								
Carotenoid	TP1	TP1-ntg-1	TP1-ntg-2	TP1-ant-1	TP1-ntg-3	TP1-ntg-4	TP1-ntg-5	TP1-ntg-6	TP1-β-ion-1	TP1-ntg-7
Torularhodin	64	140	110	160	-	-	84	-	-	ND[1]
Torulene	80	103	90	130	20	28	70	-	34	ND
β-carotene	104	228	151	120	114	151	34	101	168	ND
Phytofluene	8	36	28	32	12	36	16	11	-	ND
Phytoene	5	30	25	41	10	20	10	9	8	ND
Total conc.	261	537	405	483	154	199	215	121	210	ND

[1]ND = Not determined.

Table 6.4. Carotenoid composition of *Rhodotorula rubra* TP1 and its mutant TP1-ntg-1 grown in YM broth supplemented with various concentrations of β-ionone.

Carotenoid	Amount of carotenoid (μg/g yeast) in *R. rubra* TP1 and TP1-ntg-1 at various concentrations (M) of β-ionone					
	TP1					
	None (control)	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}
Torularhodin	64	-	-	-	ND[1]	ND
Torulene	80	63	38	30	ND	ND
β-carotene	104	165	179	142	ND	ND
Phytofluene	8	15	10	22	ND	ND
Phytoene	5	-	-	-	ND	ND
Total	261	243	227	194	ND	ND
	TP1-ntg-1					
Torularhodin	140	80	65	55	ND	ND
Torulene	103	65	52	48	ND	ND
β-carotene	228	190	187	195	ND	ND
Phytofluene	36	22	10	8	ND	ND
Phytoene	30	10	5	-	ND	ND
Total	537	367	319	306	ND	ND

Table 6.5 Nutritional and biochemical characteristics of *R. rubra* TP1 and isolated mutants.

Test Compound	Growth						
	TP1	TP1-ntg-1	TP1-ntg-2	TP1-ntg-3	TP1-ntg-4	TP1-ntg-5	TP1-ant-1
Inositol	-	+	+	+	-	-	+
Arabinose	+	+	+	+	+	+	+
Sorbitol	+	+	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+
Raffinose	+	+	+	+	-	-	+
Cellobiose	w	+	+	+	+	-	+
Melizitose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
Melibiose	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-
Galactose	+	+	w	w	+	-	+
KNO_3	-	-	-	w	w	-	w
Gelatin liquefaction	+	+	+	+	+	-	-
Soluble starch	+	+	+	+	+	+	+
Succinic acid	-	+	+	w	w	+	+
Erythritol	+	-	w	w	+	+	w
Ribitol	+	+	+	+	w	+	+
Citric acid	+	+	+	-	+	w	-
α-ketoglutaric acid	-	+	+	w	w	w	+

w = weak growth, - = no growth, + = growth. TP1 = *Rhodotorula rubra* TP1.

Table 6.5 contd.

Test Compound	Growth TP1	 TP1-mtg-6	 TP1-mtg-7	 TP1-β-ion-1
Inositol	-	-	w	-
Arabinose	+	+	w	+
Sorbitol	+	-	+	+
Trehalose	+	+	+	+
Raffinose	+	+	+	+
Cellobiose	w	+	+	+
Melizitose	+	+	+	+
Sucrose	+	+	+	+
Mannitol	+	+	+	+
Melibiose	-	-	-	w
Lactose	-	-	-	+
Urease	+	+	+	+
D-Xylose	+	+	+	+
Maltose	+	+	+	+
Rhamnose	-	-	-	-
Galactose	+	w	w	-
KNO_3	-	-	-	w
Gelatin liquefaction	+	+	+	+
Soluble starch	+	+	+	+
Succinic acid	-	+	+	-
Erythritol	+	-	w	w
Ribitol	+	+	+	+
Citric acid	+	+	+	-
α-ketoglutaric acid	-	+	+	w

w = weak growth, - = no growth, + = growth. TP1 = *Rhodotorula rubra* TP1.

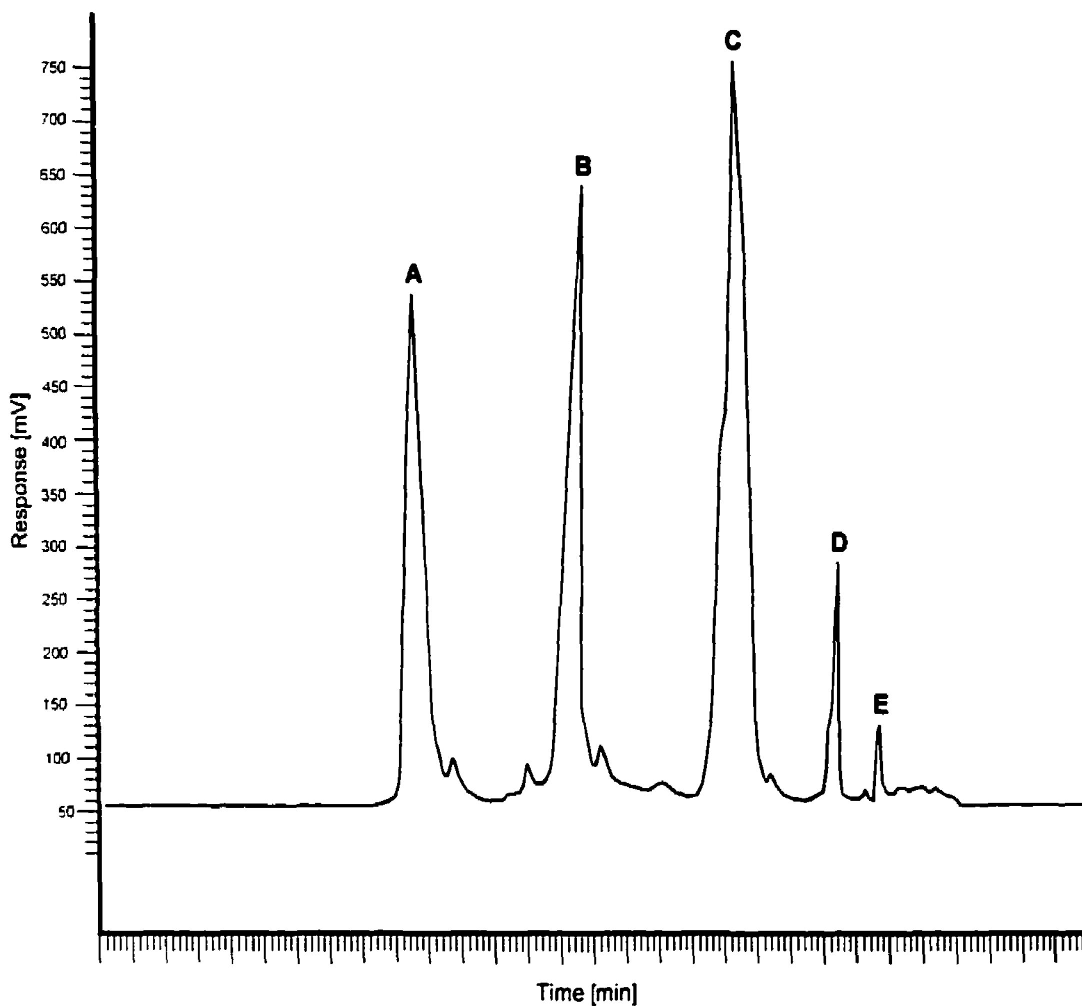
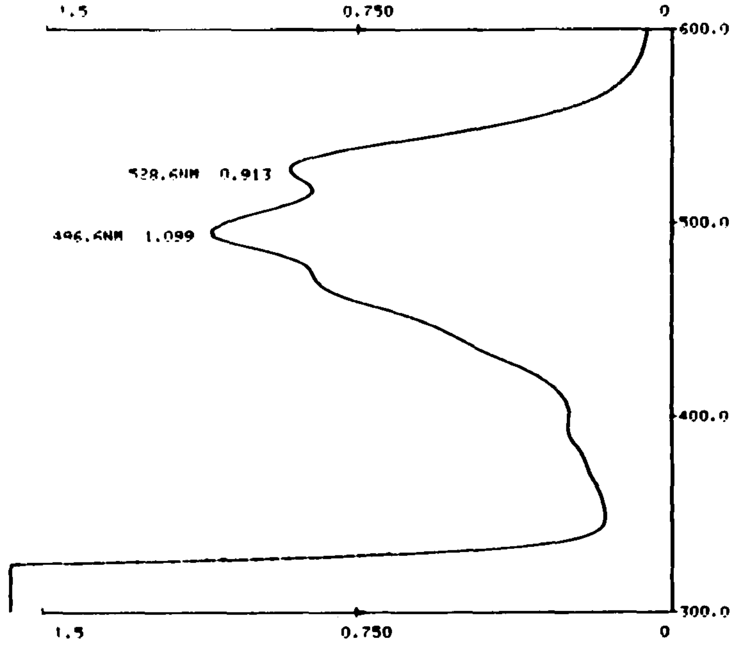


Fig. 6.0 HPLC spectrum of the carotenoid composition of *R. rubra* TP1. A = torularhodin, B = torulene, C = β-carotene, D = phytofluene, E = phytoene.

Fig. 6.1 Spectrophotometric spectra of various carotenoids isolated from *Rhodotorula rubra*TP1. Top = torularhodin, bottom = torulene.


1.5
0.750
0
600.0
500.0
400.0
300.0
528.6NM 0.913
496.6NM 1.099

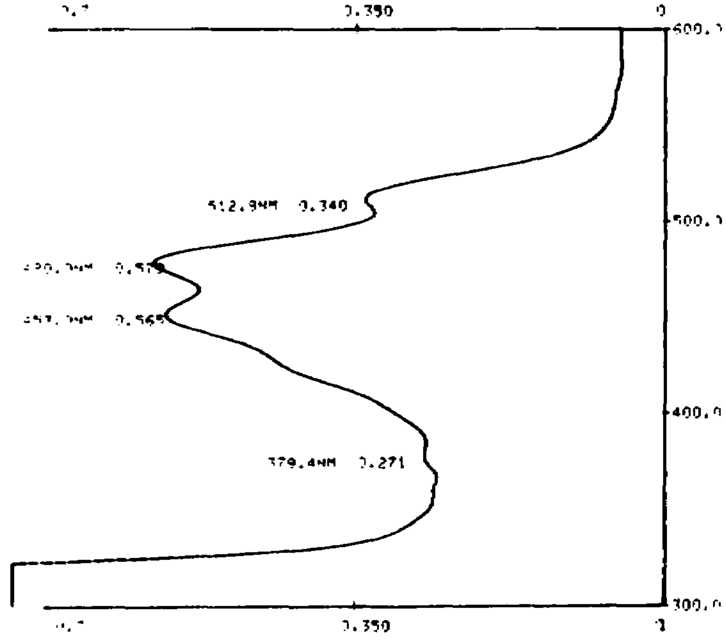
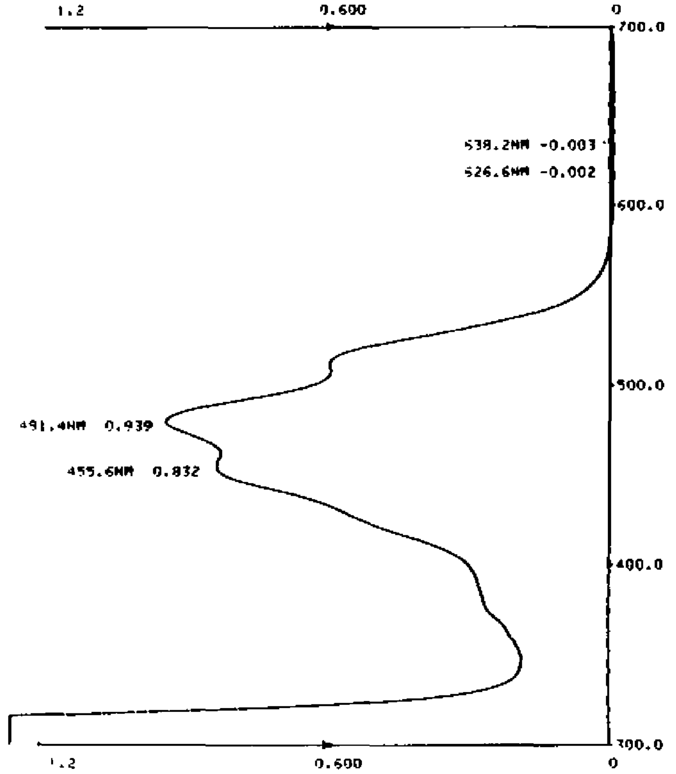

0.350
0
400.0
512.9NM 0.340
379.4NM

Fig. 6.2 Spectrophotometric spectra of various carotenoids isolated from *Rhodotorula rubra* TP1. Top = β-carotene, bottom = phytoene.


1.2
0.600
0
700.0
538.2NM -0.003
526.6NM -0.002
600.0
500.0
491.4NM 0.939
455.6NM 0.832
400.0
300.0

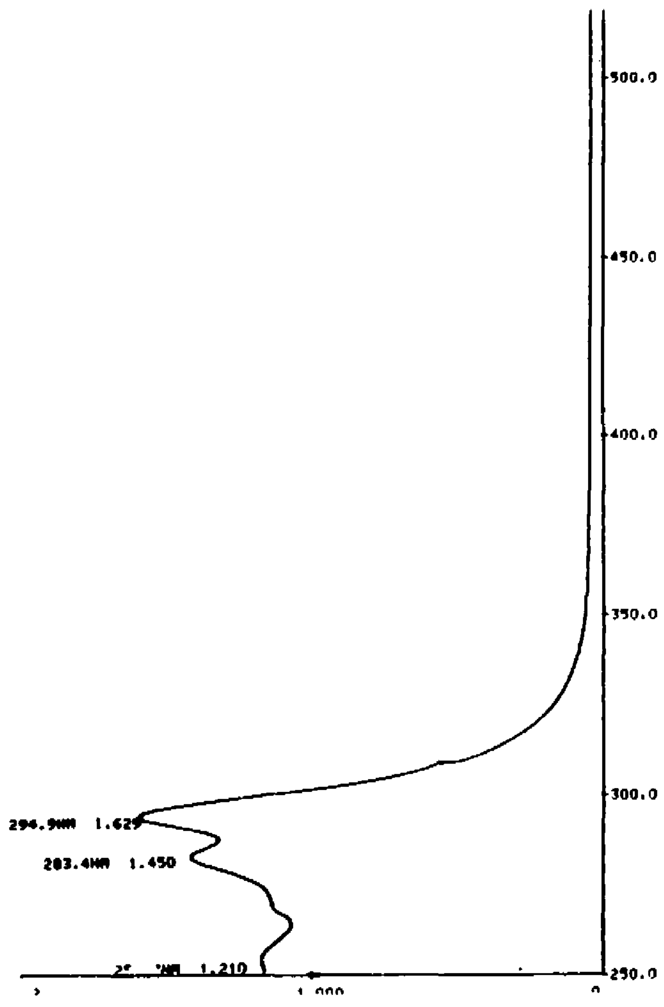
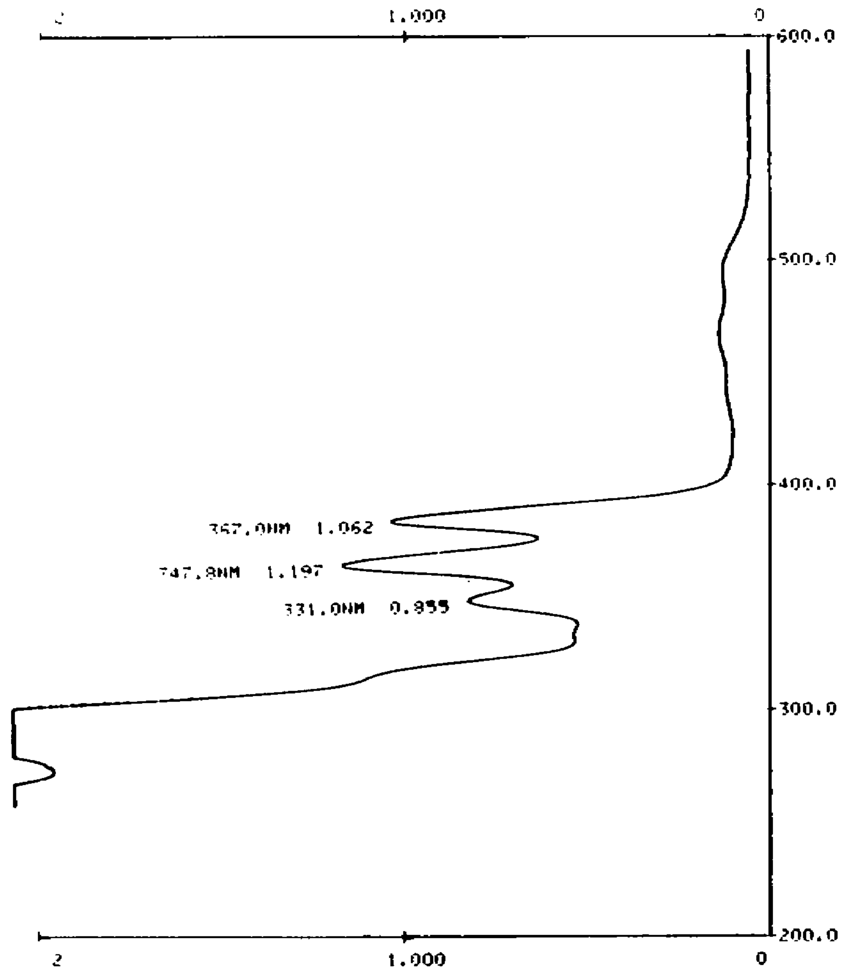
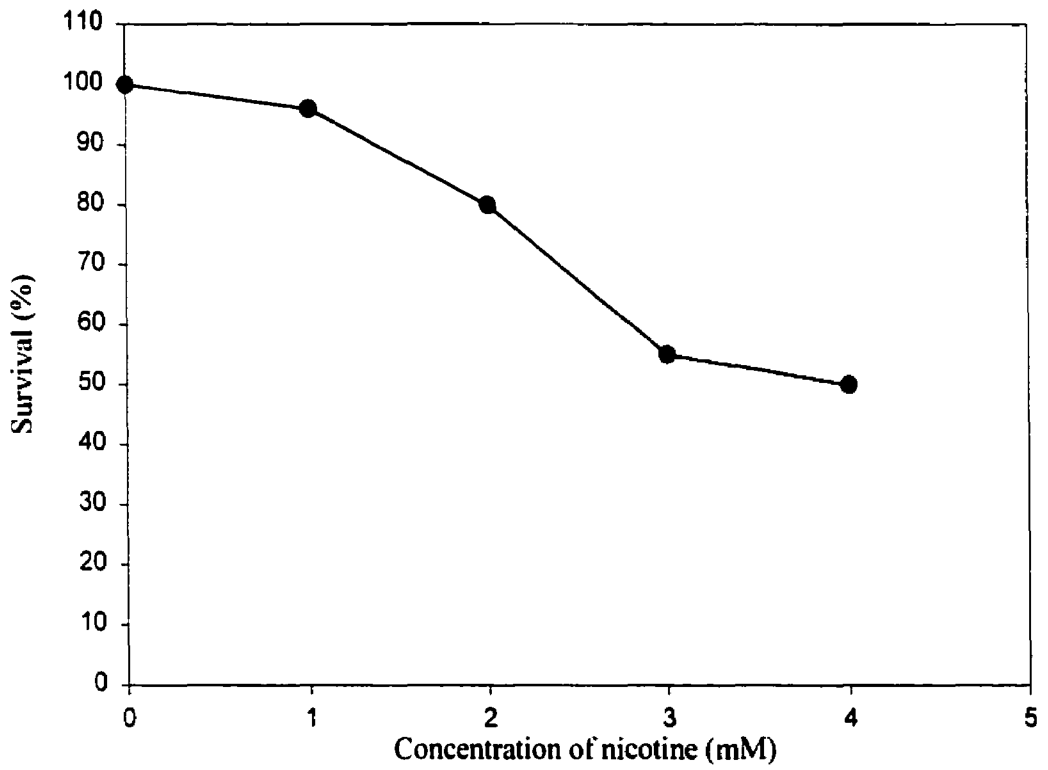

500.0
450.0
400.0
350.0
300.0
294.9NM 1.629
283.4NM 1.450
1.210
250.0
0

Fig. 6.3 Spectrophotometric spectrum of phytofluene isolated from *Rhodotorula rubra* TP1.


2
1.000
0
600.0
500.0
400.0
300.0
200.0
367.0NM 1.062
347.8NM 1.197
331.0NM 0.855
2
1.000
0



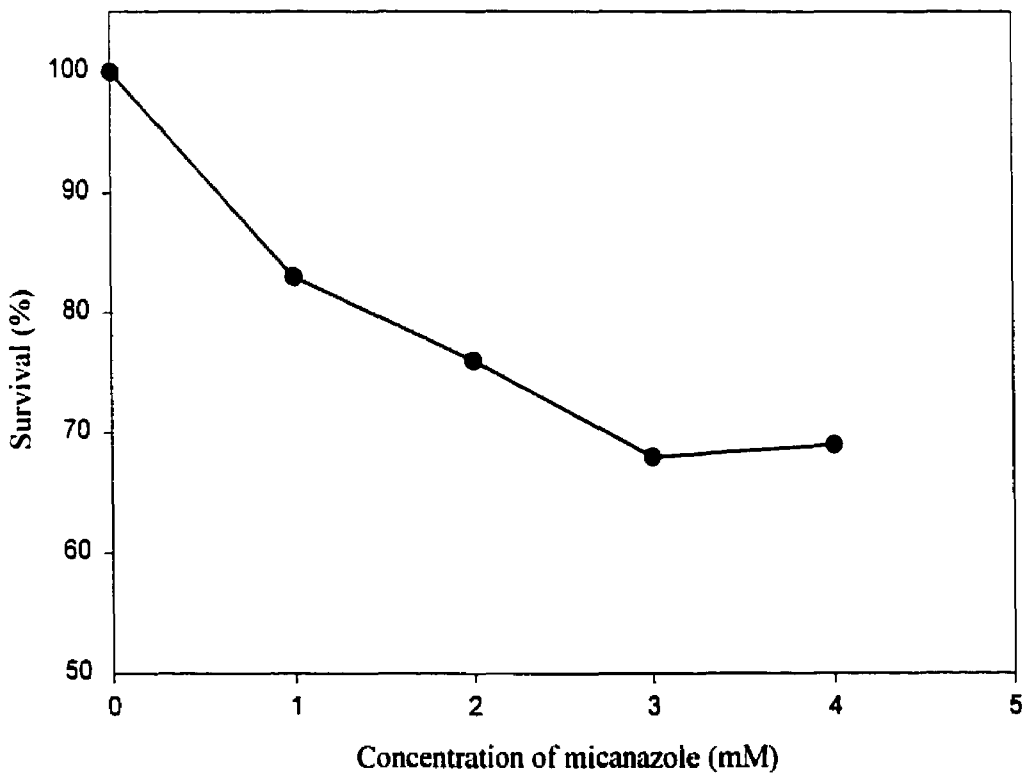
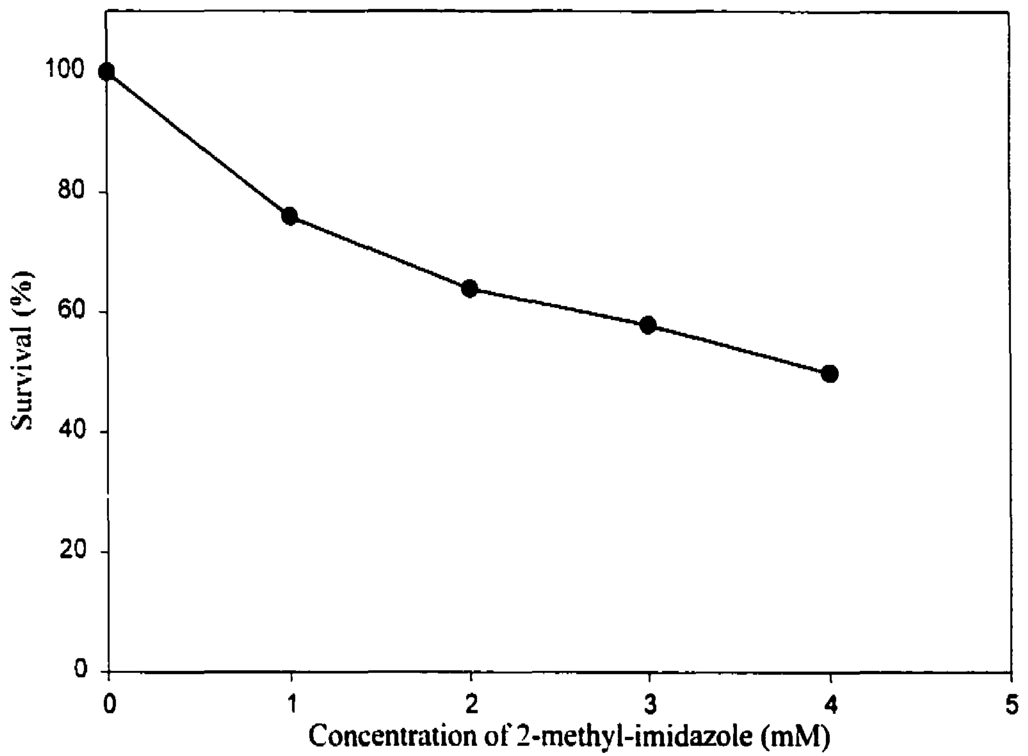


Fig. 6.4 Susceptibility of *R. rubra* TP1 parental strain to nicotene and micanazole



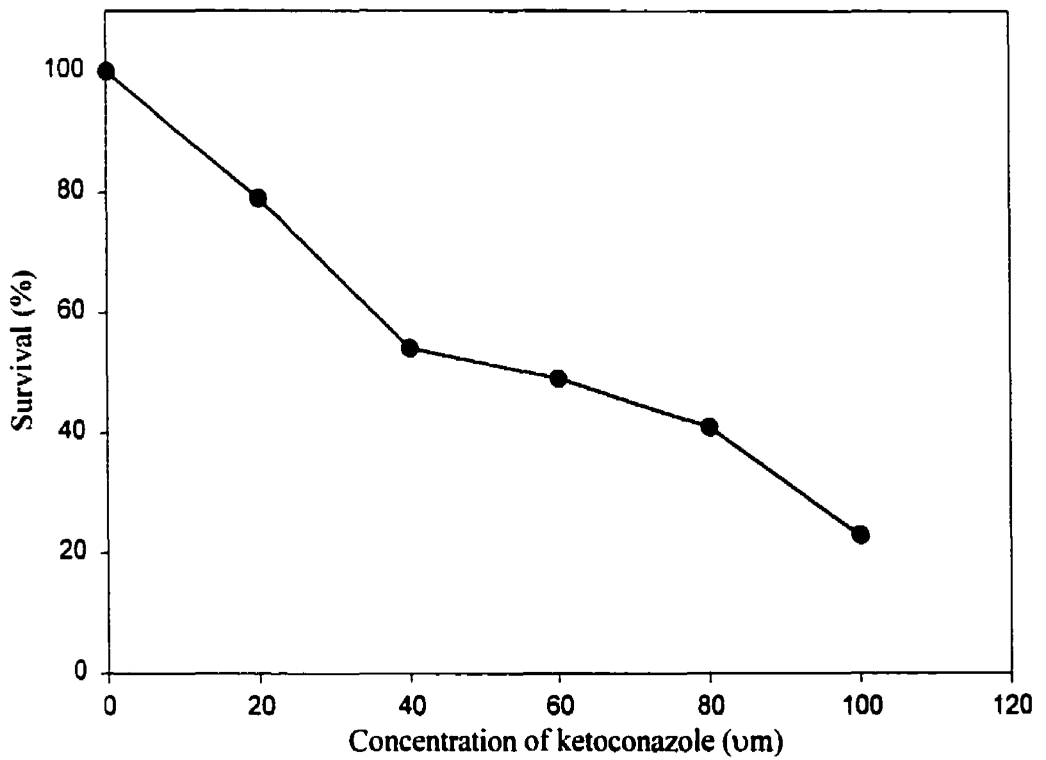
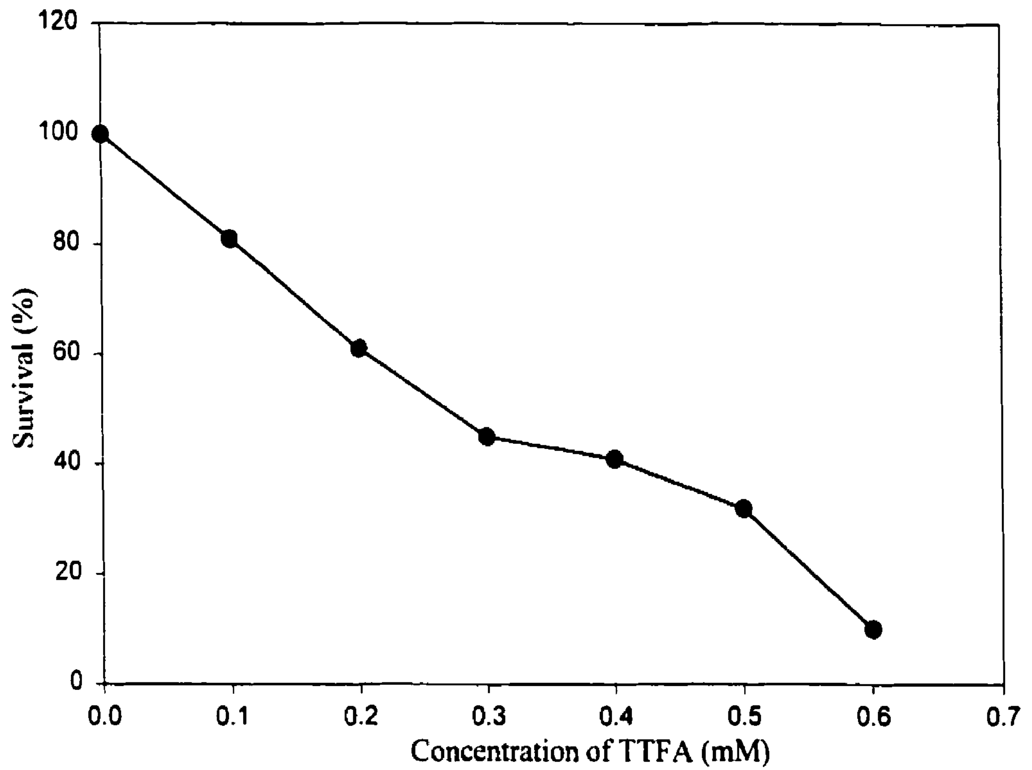


Fig. 6.5 Susceptibility of *R. rubra* TP1 parental strain to 2-methyl-imidazole and ketoconazole



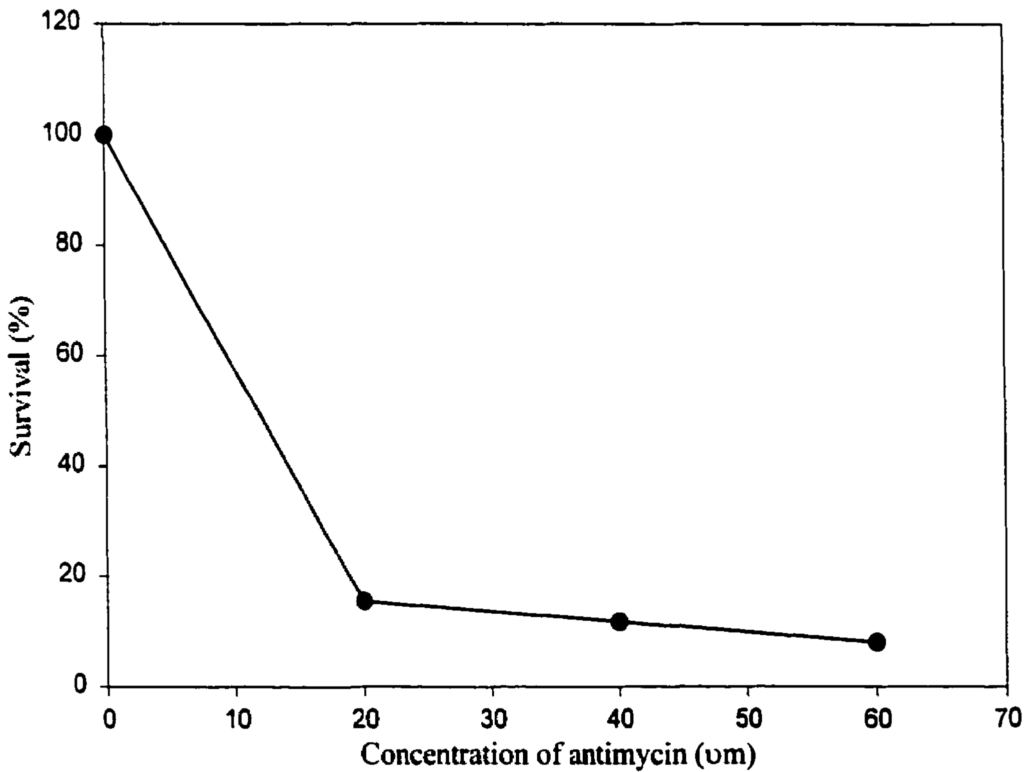


Fig. 6.6 Susceptibility of *R. rubra* TP1 parental strain toTTFA and antimycin

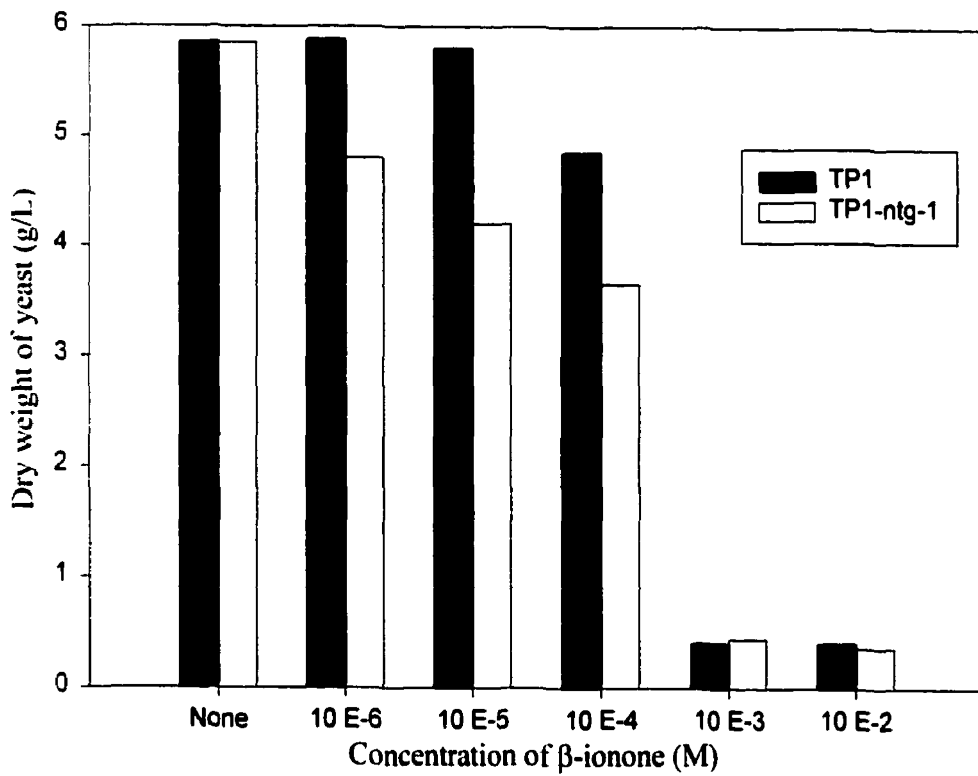
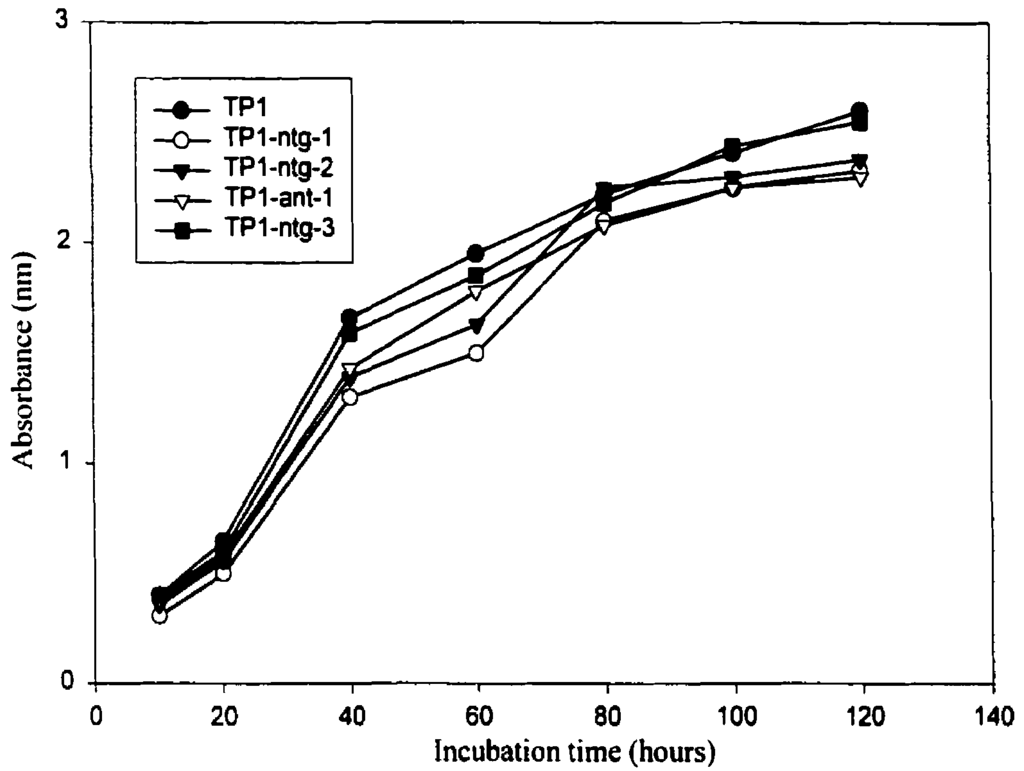


Fig. 6.7 The effects of various concentrations of β-ionone on the growth of *R. rubra* TP1 and its mutant TP1-ntg-1



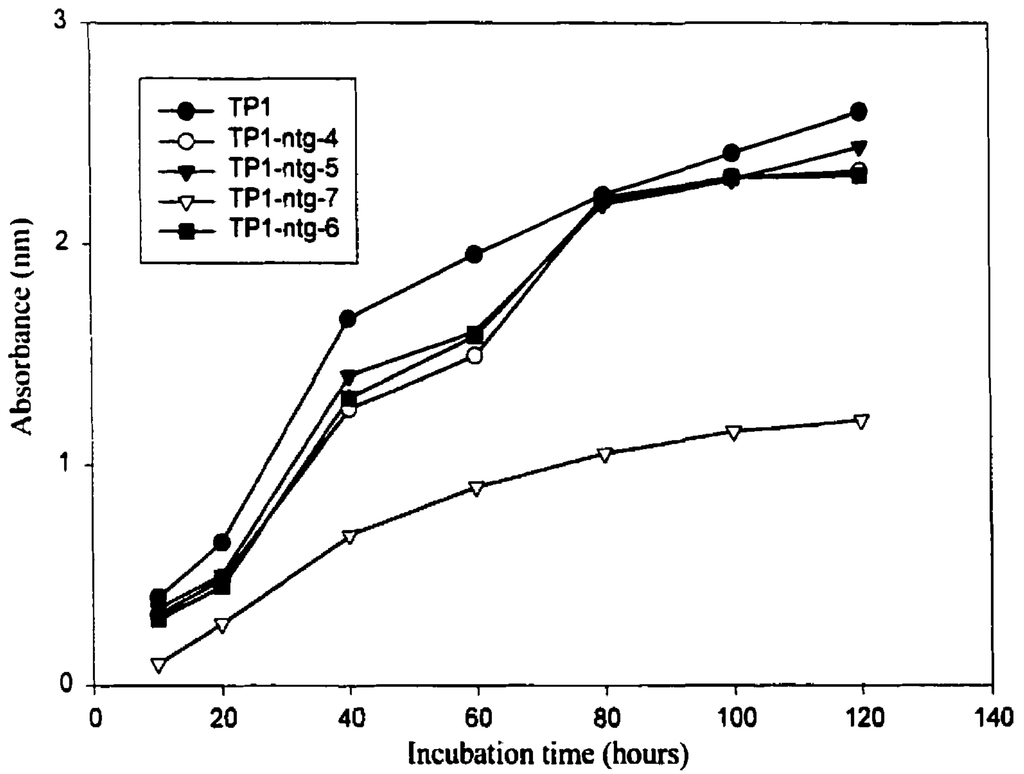


Fig. 6.8 The growth rate of *R. rubra* and its mutants in YM broth

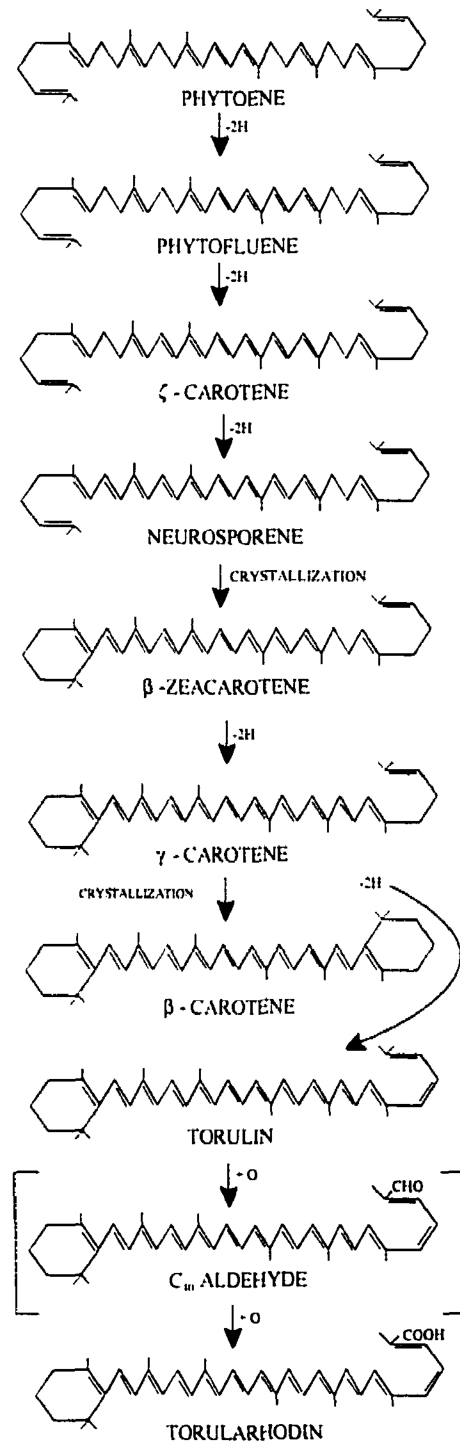


Fig. 6.9. The pathway for the production of torularhodin in *Rhodotorula rubra* (Adapted from Simpson *et al.*, 1971).

CHAPTER 7

EXAMINATION OF *R. rubra* TP1 CELL WALLS FOR THE PRESENCE OF GLUCANS

7.1 Introduction

Glucans belong to a class of drugs known as Biological Response Modifiers (BRMs). Glucans, especially those with β-1,3 linkages are known to have antitumour properties and also stimulates non-specific immune systems in animals (Azuma, 1987). They are reported to boost the immune systems of fish if administered as intraperitoneal injections, by bath or included in their diets (Jeney and Anderson, 1993). Fish in the aquaculture industry are prone to bacteria infections due to decreased immunity resulting from dense population of the stock. Streptococcosis caused by the *Streptococcus* sp. is the most important disease and can lead to the loss of an entire fish stock (Jeney and Anderson, 1993). Antimicrobial drugs have been employed for prophylactic and therapeutic purposes, however, recent occurrence of drug-resistant strains has made treatment difficult. In addition, a number of attempts have been made to produce vaccine for fish, but no vaccine is yet available (Matsuyama *et al.* 1992). Compounds that are able to boost the immune system of fish are therefore in demand in the industry. There is also a growing interest in the potential biomedical applications of polymeric β-D-glucan BRMs (Williams *et al.* 1987).

The yeast cell wall is known to contain high amounts of the polysaccharide glucan and glucans isolated from several strains of yeast are now being evaluated around the world for their ability to protect fish stock against deadly bacterial infections. Based on the therapeutic potential of glucan, this study was undertaken to examine the cell wall of the yeast *Rhodotorula rubra* TP1 for the presence of glucans.

7.2 Materials and Methods

Chemicals: Dimethyl sulfoxide (DMSO), deuterated dimethyl sulfoxide (d_6-DMSO), glucose, laminarin, Sephadex LH-20 and methyl iodide were purchased from Sigma Chemical Company, St. Louis, MO. Petroleum ether, chloroform, acetone, methanol, n-pentane, acetic anhydride, triflouroacetic acid, acetic acid, pyridine, sulphuric acid, sodium hydroxide, sodium borohydride, sodium hydride, methanol, calcium chloride, ferric sulfate, potassium chloride, magnesium sulfate, ammonium sulfate, potassium dihydrogen sulfate and silica gel plates were obtained from Fisher Scientific Ltd., Fair Lawn, New Jersey. Yeast extract was obtained from DIFCO Laboratories, Detroit, MI.

7.2.1 Growth of organisms.

Rhodotorula rubra and *Saccharomyces cerevisiae* were grown in 2 L Erlenmeyer flasks that contained 500 mL medium consisting (w/v) of 1% glucose, 0.2% yeast extract, 0.5% ammonium sulfate, 0.5% potassium dihydrogen sulfate and 0.1% (v/v) aqueous salt solution. The salt solution consisted of 5% $MgSO_4.7H_2O$, 1% $CaCl_2.2H_2O$, 1% $FeSO_4.7H_2O$ and 1% KCl.(Manners *et al.* 1974). The organisms were cultivated at 25°C and a pH of 5.5 in a Psychrotherm Controlled Environment Incubator (New Brunswick Scientific Ltd., New Brunswick, N.J.) with shaking at 150 rpm for 48 hours. The inoculum used consisted of 50 mL of medium of same composition in a 250 mL Erlenmeyer flasks which were incubated at 25°C for 48 hours. After the growth period, the cells were centrifuged at 10,000 x g, washed three

times with deionized water and then lyophilized.

7.2.2 Isolation of cell wall polysaccharides.

7.2.2.1 Isolation of cell wall polysaccharides for nuclear magnetic resonance spectroscopy (NMR).

Four different approaches were employed to isolate the polysaccharides from the yeast cell wall:

1. Polysaccharides were prepared from the cell walls of *R. rubra* TP1 by the method of Hassid *et al.* (1941) as reported by William *et al* (1991) with a minor modification. The method is given in the flow diagram in the Fig 7.0.
2. About 400 g of freeze-dried yeast was digested with preheated dimethyl sulphoxide (DMSO) at approximately 50°C. The pigments in the cells were extracted several times with acetone until the supernatant became colourless. The residue was washed twice with methanol and then several times with water. The washed cells were then subjected to the protocol described in Fig 7.0.
3. The procedure described by Misaki *et al.* (1968) was used for glucan extraction. About 400 g of dried yeast was dispersed in 6% aqueous NaOH and the mixture was stirred overnight at room temperature. The mixture was centrifuged and the insoluble residue was heated with 3% NaOH for 3 h at 75°C, the mixture was then centrifuged and the NaOH supernatant collected

and saved. The NaOH digestion was repeated twice after which the cell-wall material was collected and washed three times with deionized water, ethanol and petroleum ether and dried *in vacuo*. The dried material was then heated with 0.5 M acetic acid for 3 h at 90° C and then repeated 3 times, dispersed in water, autoclaved and centrifuged. The resulting material was washed several times with water, ethanol and petroleum ether and then evaporated to dryness. The residue was saved and analyzed for the presence of glucans. The NaOH supernatants were pooled together, pH brought to 4.0 with acetic acid and 4 volumes of ethanol added to precipitate any dissolved polysaccharides. The resulting white flocculent precipitate was removed by centrifugation, dialysed against deionized water and the resulting gel freeze-dried. This and the cell wall material collected were dissolved separately in deuterated DMSO with the help of sonication and subjected to NMR analysis.

4. Freeze-dried yeast cells were extracted with 3% NaOH at 100°C and then treated with 0.1M acetate buffer (Bacon *et al.*, 1966). The residue was washed twice with and resuspended in deionized water. One gram of sodium borohydride was added and the mixture was stirred for 16 hours at room temperature. The wet residue was extracted three times at 75°C with 3% NaOH containing 0.2 g sodium borohydride. The mixture was centrifuged, suspended in water, neutralised with acetic acid, and washed several times with water and re-suspended in water. Small portions were freeze-dried and then subjected to NMR analysis.

For comparative purposes, Brewer's yeast was subjected to the above-mentioned treatments and the polysaccharides obtained were subjected to NMR analysis.

7.2.2.2 Isolation of cell wall polysaccharides for acetylation and methylation.

The procedure described by Elinov *et al.* (1988) was used. Two day old yeast culture were disintegrated in a French Press at a pressure of 40,000 psi. The ruptured cells were then washed several times with hot deionized water and extracted twice with acetone, methanol and then ethanol to remove any traces of acetone. The cells were then washed three more times with deionized water, heated in 3% NaOH in hot water bath 100°C for 4 hours and then centrifuged at 5000 rpm, the residues were collected and washed several times with deionized water and lyophilized before being subjected to acetylation and methylation.

7.2.2.3 Isolation of cell wall mannans.

About 20 g of freeze-dried yeast cells were suspended in about 400 mL 0.1 M sodium acetate buffer (pH 7.0) and autoclaved for 30 minutes at 121°C and 20 psi. The solution was cooled, neutralized with acetic acid and centrifuged at 7000 x g and the supernatant evaporated to about 50 mL in a rotary evaporator. Four volumes of methanol were then added and the precipitate centrifuged off at 10,000 x g for 20 minutes, washed two times with methanol and then lyophilized. The freeze-dried material was re-dissolved in deionized water by heating at 100°C and the insoluble material filtered off. The dissolved portion was divided into two and one half was

freeze-dried and then portions dissolved in deuterium oxide (D_2O) for NMR analysis. This portion was referred to as the crude mannans. The other half was purified as the insoluble copper complex using the method described by Gorin and Spencer (1970). The purified mannan was also dissolved in D_2O and subjected to NMR analysis.

7.2.3 ^{13}C- and ^{1}H- nuclear magnetic resonance (NMR) spectroscopy.

The extracted polysaccharides were subjected to ^{13}C-NMR and ^{1}H-NMR spectroscopy to determine the type of inter-chain linkages in the compound. The compounds were dissolved in deuterated DMSO (d_6-DMSO) and analysed on a Varian 300 MHz NMR Spectrometer (Varian Associates, Palo Alto, California). The equipment was run in the pulsed Fourier-transform mode at 30°C with tetramethylsaline as external standard. All samples were prepared at a concentration of 50 mg/mL d_6-DMSO except the mannans, which were dissolved in D_2O. Laminarin, which is known to have β-1,3-linked triple-helical glucopyranose structure (Saito *et al.* 1977) was dissolved in d_6.DMSO and used as a standard. For comparative purposes, cell wall preparations from *S. cerevisiae* were subjected to the same treatment and analyzed by NMR.

7.2.4 Aditol acetate derivatization of cell wall polysaccharides.

The method used for the hydrolysis, reduction and acetylation of the cell wall polysaccharides followed the procedure described by Gunner *et al.* (1961) and Sawardeker *et al.* (1965). Samples of cell wall polysaccharides (ca. 10 mg) were

hydrolyzed for 16 hours in 1% trifluoroacetic acid in a water bath at 100°C. The hydrolysate was evaporated to dryness in rotary evaporator, washed 3 times with deionized water and reduced for 1 hour with sodium borohydride. After this time the reaction was stopped by the addition of glacial acetic acid and the solution was evaporated to dryness and washed 3 times with a solution of 5 % acetic acid in methanol to facilitate the removal of borate as methyl borate. The hydrolyzed cell wall polysaccharides were acetylated by heating at 100°C in a mixture of acetic anhydride/pyridine (1:1 v/v) for 30 minutes; evaporated to dryness and co-evaporated with chloroform until no traces of pyridine could be detected. The acetylated product was filtered through a sintered glass funnel, taken up in chloroform and injected and analyzed by GC-MS.

7.2.5 Methylation and hydrolysis of cell wall polysaccharides.

The method used followed the procedure described by Hakomori (1964). In this procedure, the methylsulfinyl anion (Corey and Chaykovsky, 1962; Chaykovsky and Corey, 1962) was used to generate the polyalkoxide ion of the substrate in anhydrous DMSO prior to the addition of the methyl iodide to effect methylation.

The methylsulfinyl anion was prepared as follows. About 1.5 g of sodium hydride (55% suspension in mineral oil) was weighed into 100 mL serum vial and the suspension was allowed to settle and the liquid decanted. The sodium hydride was then washed three times with 30 mL aliquots of n-pentane then dried by flushing with dry nitrogen with the aid of a hypodermic syringe. About 15 mL of DMSO, which

had previously been distilled from calcium hydride and dried over molecular sieve (4Å) was transferred into the flask which had previously been sealed with rubber septum and flushed with nitrogen via two injection needles. The vial was placed in an ultra-sonic bath and heated at 50°C until the solution became clear and hydrogen gas evolution ceased. The resulting greenish solution was stored at 4°C and used for the permethylation reactions.

For the methylation reactions, samples (1-2 mg) of cell wall preparations were mixed with 0.4 mL DMSO and a stirrer bar in a 6 mL hypo vial capped with a teflon-lined rubber septum. The contents of the vial were flushed with nitrogen gas via two injection needles for 20 seconds, stirred at room temperature (ultrasonicated when necessary) until a clear solution was obtained. To generate the polysaccharide alkoxide, 0.4 mL methylsulfinyl carbanion was added dropwise via an injection needle using a hypodermic syringe, stirred at room temperature for an hour with a 23g needle through the liner at which time a clear solution was obtained. 1 mL of methyl iodide (CH_3I) was added in a dropwise manner using a syringe with cooling. The mixture was then stirred at room temperature for another 4 hours and the resulting solution was passed through a column of Sephadex LH-20 (approx. 5 g in chloroform) and 1 mL fractions were collected. Fractions were spotted on silica gel plates, sprayed with 5% H_2SO_4 and charred to determine fractions containing methylated polysaccharides. Fractions were then combined, evaporated to dryness and taken up in 1 mL chloroform for injection on to GC-MS.

7.2.6 GC-MS Conditions.

The instrument consisted of a HP 5970 Mass Selective Detector (MSD) coupled with a HP 5890 Gas Chromatograph (Hewlett-Packard, Palo Alto, California) and a model 300 Data System from the same manufacturer. The column was a CP-Sil-5 CB, WCOT fused silica with a length of 25 m, an inside diameter of 0.25 mm, outside diameter 0.39 mm and a film thickness of 0.12 µm (ChromPack, The Netherlands). Chromatographic conditions were as follows: initial temperature of 165°C held for 15 minutes, increased to 250°C at a rate of 5°C/minute and held for 10 minutes. The carrier gas was helium with a flow rate of 1 mL/minute and a split ratio of 50:1 was used. Injector temperature and transfer lines were both maintained at 275°C. The generated mass spectra were compared with those of standards reported by Jansson *et al.* (1976) to identify the products.

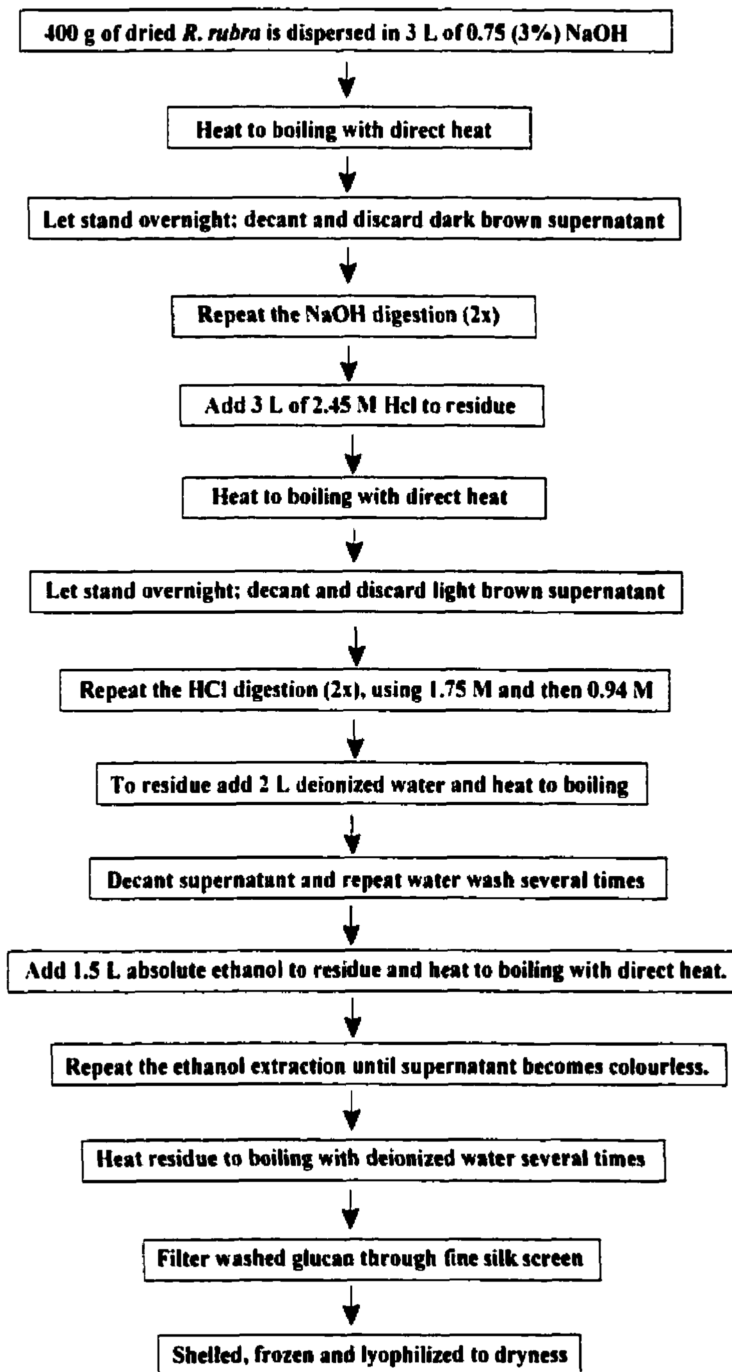


Fig. 7.0. Procedure for the extraction of glucans from yeast (Modified from Williams *et al.*, 1991).

7.3 Results.

7.3.1 NMR analyses.

To determine the type of interchain linkages present in the cell wall polysaccharides from *R. rubra* TP1, cell wall preparations were dissolved in deuterated DMSO and analyzed by both ^{13}C- and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Cell wall mannans were dissolved in D_2O. To assess the effectiveness of the method used in the preparation of the cell wall polysaccharides, cell wall polysaccharides were prepared from *S. cerevisiae* using the same protocol. Laminarin, which is known to have β-1,3-linked triple-helical glucopyranose structure (Saito *et al.* 1977) was similarly dissolved in d_6-DMSO and used as a standard. The NMR spectra of the cell wall polysaccharides analyzed by ^{13}C- and ^{1}H-NMR are depicted in Figs. 7.1 to 7.4. The ^{13}C-NMR chemical shifts measured in ppm for *R. rubra* TP1, laminarin and *S. cerevisiae* are compared with those reported by other workers in Table 7.0.

As can be seen from Fig 7.1, the cell wall polysaccharide from *R. rubra* TP1 showed only two peaks, one of which was a DMSO peak. The peaks were different from those of laminarin which is known to contain β-1,3 glycosidic linkages (Saito *et al.*, 1977; William *et al.*, 1991). The ^{13}C-NMR spectra furnished by the cell wall polysaccharides isolated from *S. cerevisiae*, on the other hand, showed excellent correspondence with those of laminarin (Fig. 7.2). This suggests that *S. cerevisiae* has the same β-1,3-linked triple helical structure possessed by laminarin as has been reported previously (Rolf *et al.* 1985, William *et al.* 1991). The ^{13}C-NMR chemical

shifts of *S. cerevisiae* depicted in Table 7.0 were in agreement with those of laminarin confirming the β-1,3-linkages. Laminarin has several other small peaks, some of which can be attributed to the occurrence of glycosyl side-chains after every 11^{th} subunit along the polymer (Rolf *et al.* 1985). It is apparent from these results that the linkages in *R. rubra* TP1 could not be identified using ^{13}C-NMR.

^{1}H-NMR spectral analyses of *R. rubra* cell wall polysaccharides and laminarin were carried out in attempt to identify the linkages present in the isolated cell wall material. Several signals were recorded for *R. rubra* TP1 but none of them were identical to those of laminarin (Fig. 7.3). The ^{1}H-NMR spectrum of *R. rubra* TP1 did not resemble that of laminarin suggesting that the cell wall polysaccharides of *R. rubra* TP1 are different from those encountered in laminarin and *S. cerevisiae*. The ^{1}H-NMR spectra of the polysaccharide isolated from the cell wall of *S. cerevisiae* and laminarin are compared in Fig. 7.4. It can be seen from the figure that the two spectra show excellent correspondence. The structure of the cell wall polysaccharides of *R. rubra* TP1 could not therefore be determined by subjecting them to NMR analysis.

Since hot alkaline extraction failed to shed light on the structure of the cell wall polysaccharides of *R. rubra*, it was reasonable to assume that the extraction procedure might have degraded the cell wall polysaccharides present and hence milder extraction techniques were employed. The ^{1}H-NMR spectra of the polysaccharides extracted by mild hydrolysis are shown in Fig. 7.5. The top spectrum represents the crude mannan and the bottom spectrum represents the other portion purified as the copper complex. It can be seen that these spectra had distinctive

finger- prints in the region of 3.7 to 3.8 ppm indicating that the cell wall is made up of mainly mannans (Gorin and Spencer, 1970).

7.3.2 Methylation analyses.

The GC-MS spectra of the fully methylated *R. rubra* TP1 cell wall polysaccharides are presented in Figs. 7.6 and 7.7. The cleavage products and structural features furnished by the methylated derivatives of the cell wall polysaccharides are presented in Table 7.1.

The fully methylated *R. rubra* TP1 cell wall polysaccharides obtained by the Hamokori (1964) procedure, on hydrolysis, furnished 1,5,6-di-O-acetyl-2,3,4-tri-O-methyl-hexitol (Fig. 7.6, top); 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl hexitol (Fig. 7.6, bottom); 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-hexitol (Fig. 7.7, top); 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl hexitol (Fig. 7.7, bottom). Unfortunately, the column could not separate 1,4,5-tri-O-acetyl 2,3,6-tri-O-methyl hexitol and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl hexitol. Fig. 7.7 (top) therefore represents either of the two structures. Since as indicated earlier, it is difficult to determine the nature of the parent monosaccharide from the mass spectra of the methylated aditol acetates, the GC analyses of the acetylated derivatives were carried out. Comparison of the retention times of these methylated derivatives with those of the standards confirmed that 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl hexitol was actually 2,3,4,6-tetra-O-methyl glucitol whereas the rest were mannosides. From the GC retention times, it can be deduced that the fully methylated cell wall polysaccharide, obtained by the Hamokori

(1964) method, on hydrolysis yielded 2,3,4,6-tetra-O-methyl glucitol; 2,4,6-tri-O-methyl- mannitol, 2,3,6-tri-O-methyl mannitol; 3,4,6-tri-O-methyl mannitol; and 2,3,4-tri-O-methyl mannitol (Table 7.1). The isolation of 2,3,4,6-tetra-O-methyl glucitol from a polysaccharide that was fully methylated suggests that the yeast cell wall may be highly branched. The linkages furnished by the various cleavage products are also depicted in Table 7.1. The sequence and the mole ratio of the various linkages shown in Table 7.1 were not determined, however, it can be inferred from these results that the cell wall contains mostly 1→3 and 1→4 linkages as indicated in Table 7.1. The mass spectra of the various monosaccharides (methylated aditol acetates) are shown in Figs. 7.8 to 7.10 and the percentage composition and the retention times of the individual monosaccharides are depicted in Table 7.2. From these results, it can been seen that the parent monosaccharides are hexitol hexacetate (i.e. glucose and mannose) and pentitol pentacetate (galactose, fucose and rhamnose). Comparison of the retention times of standard sugars run in parallel showed that the main monosaccharides present were mannose (50.53%), glucose (25.53%), galactose (12.27%), fucose (8.6%) and rhamnose (3.2%).

7.4 Discussion.

7.4.1 NMR analyses of cell wall polysaccharides.

Most fungi contain a complex mixture of polysaccharides and protein that can be found in cell membranes or wall and as exocellular components. These

polysaccharides vary widely in their sugar composition, linkage types, molecular parameters and physical properties (Bacon *et al.* 1969; Gorin, 1970). Numerous techniques including NMR have been employed in an attempt to elucidate the chemical structure of these polysaccharides (William *et al.* 1991; Gorin and Spencer, 1970; Gorin, 1980). Nuclear magnetic resonance (NMR) analysis allows the identification of the polymeric backbone and also the determination of the type of interchain linkages present in various polysaccharides (William *et al.* 1991; Ohno *et al.* 1984).

In the present study, hot alkali was used to extract polysaccharides from the cell wall of *R. rubra* TP1 and the resulting products analyzed by NMR. It was found out that NMR could not be employed to determine the chemical structure of the cell wall polysaccharides isolated from *R. rubra* TP1 with hot alkali. The failure to elucidate the chemical structure of the cell wall polysaccharides by hot alkali extraction can be attributed to several reasons. As indicated earlier, the cell wall of *Rhodotorula* species contain mainly mannan with β-(1→3) and β-(1→4)-linkages (Gorin and Spencer, 1970). These bonds are alkali-labile and suffer extensive degradation when subjected to alkali extraction (Fleet, 1987). Similarly, other bonds such as glycosyl-serine linkages, phosphodiester linkages and some peptide and disulphide bonds undergo extensive degradation when the cell walls are extracted with hot alkali (Ballou, 1976). It is therefore possible that the polypeptide and the β-(1→3) and β-(1→4) linkages present in the cell wall polysaccharides of the yeast employed in this study may have undergone extensive degradation as indicated by the

formation of viscous solutions in DMSO. According to Gorin and Spencer (1970) and Gorin (1972), regenerated polysaccharides obtained from certain types of yeast give solutions that are too viscous for NMR spectral analysis. The viscosity of the solution obtained in this study indicated that there was extensive degradation and gelatinization of the linkages present in the polysaccharide.

It can be concluded from this study that, like *Rhodotorula texensis*, *R. rubra* TP1 is not amenable to NMR analysis employing hot alkali extraction of the cell wall polysaccharides (Gorin and Spencer, 1970; Gorin, 1972).

To determine whether or not the protocol used in preparing the glucan contributed to the absence of peaks on the spectrum, glucans from *S. cerevisiae* which have been used in most studies were prepared using the same protocols used to prepare glucans from *R. rubra* TP1. Unlike *R. rubra* TP1, the *S. cerevisiae* spectrum had peaks similar to those of the standard, laminarin. Comparison of the glucan spectral peaks of *S. cerevisiae* showed excellent correspondence with that of laminarin. In addition, the ^{13}C-NMR spectra of laminarin and *S. cerevisiae* reported by Williams *et al.* (1991) were in excellent agreement with the laminarin and *S. cerevisiae* spectra reported in this study. This indicates that the protocol used in this study is applicable to *S. cerevisiae* but not suitable for *R. rubra* TP1.

The failure of the hot alkali extraction to yield any reasonable results for *R. rubra* TP1 suggests that there was either a complete destruction of the polysaccharides that occurred in the cell walls of the organism, or those polysaccharides were not amenable to hot alkali extraction. It therefore became

necessary to look at a milder extraction technique and also at the possibility that the yeast contained a different form of polysaccharide which required a different extraction protocol. Furthermore, it became necessary to employ other chemical methods that are used to elucidate the chemical structure of the cell wall polysaccharides as a complement to the NMR analysis. The decision was therefore made to use protocols that will result in extraction of mannans instead of glucans. The materials that were extracted were subjected to ^{1}H-NMR and the results indicated that the cell wall contained mainly mannans and hence shed further light on the phylogenetic affiliation of this new isolate.

Yeast cell wall mannans constitute a highly diverse series of branched homopolymers that have been investigated by ^{1}H- and ^{13}C-NMR spectroscopy (Gorin and Spencer, 1970). These polysaccharides are predominantly α-(1→2) linkages and occasionally α-(1→3) linkages. A few of the branched mannans carry residues of β-D-mannopyranose in the side chains (Gorin and Barreto-Bergter, 1982, Gorin, 1980). Yeast of the genera *Sporobolomyces* and *Rhodotorula* are unique in that their cell wall is made up of free β-D-mannan with alternate (1→3) and (1→4)-linked β-D-mannopyranose units (Gorin and Barreto-Bergter, 1982, Gorin, 1980).

In *Rhodotorula* species, the cell wall contains mainly alternate β-(1→3) and β-(1→4)-linked -β-D-mannopyranosyl units (Gorin *et al*, 1965; Gorin and Spencer, 1970). The majority of the mannose-containing polysaccharides of the cell walls from these yeasts give ^{1}H-NMR spectra containing a distinctive fingerprint region in the 3.8-4.8 ppm region arising from the H-1 signals, and this is what is normally used

in yeast identification (Gorin and Spencer, 1970). In *R. glutinis* for example, the β-(1→3)-linked units of the mannan have a chemical shift of 4.58 ppm whereas the β-(1→4)-linked units have a chemical shift of 4.69 ppm (Gorin and Spencer, 1970). The ^{1}H-NMR obtained for the polysaccharides with hot alkali from *R. rubra* TP1 had chemical shifts in the region 3.7 to 3.8 which clearly falls within the finger print region of 3.8 to 4.8 ppm reported by Gorin and Spencer (1970). It can therefore be concluded that the cell wall polysaccharides of the new isolate *R. rubra* TP1 is made up of mainly gluco-mannans.

7.4.2 Methylation analysis of the cell wall polysaccharides of *R. rubra* TP1.

As a complement to the results obtained with the NMR analyses we also methylated the isolated cell wall polysaccharides and subjected them to GC-MS analyses. Methylation analyses of polysaccharides is one of the most important and widely used method in structural determination in polysaccharide chemistry (Lindberg and Lonngren, 1978; Jansson *et al.* 1976). The method involves the methylation of all the free hydroxyl groups in the polysaccharide into methoxyl groups, which are then hydrolysed into a mixture of partially methylated sugars. The manomers formed are then analyzed by gas-liquid chromatography (GLC) (Jansson *et al.* 1976). The method provides details of the structural units present in the polymers, but gives no information on their sequence or the anomeric nature of their linkages. The mass spectra of the partially methylated aditol acetates show only minor differences making an assignment of the sugar configuration (gluco, manno

etc.) difficult. However, the mass spectra evidence together with the relative retention times of the parent monosaccharides in GLC usually leads to unequivocal identification of each component (Kamerling and Vliegenthart, 1989).

The results of the monosaccharide analysis depicted in Table 7.2 indicated that the monosaccharide content of the cell wall of *R. rubra* TP1, consisted mainly of mannose with moderate amounts of glucose and galactose and small quantities of fucose and rhamnose. These results are consistent with those reported by other workers. Sugiyama *at al.* (1985) reported that mannose and glucose were the dominant monosaccharides in *R. rubra* and other *Rhodotorula* species. They also reported the absence of xylose and the presence of rhamnose, galactose, fucose and ribose in the cell walls of these organisms. Based upon these results, they concluded that cellular carbohydrate composition is a valuable tool for the chemotaxonomy of the basidiomycetous yeasts. Similarly, Weijman and Rodrigues de Miranda (1988b) reported that the dominant monosaccharide found in several *Rhodotorula* species was mannose. The patterns of carbohydrate in the cell wall of the new isolate *R. rubra* TP1 is therefore an important evidence of the taxonomic similarity between the new isolate and the genus *Rhodotorula*.

Several cleavage products were obtained from the methylated products of *R. rubra* TP1 cell wall in an attempt to determine the cell wall structure of this yeast. The sequence of 1→3 and 1→4 linkages in the mannan were not elucidated, however, based on these results, the structure of this yeast can be constructed as follows: O-glucopyranosyl-(1→6)-O-mannopyranosyl-(1→4)-O-mannopyranosyl-O-(1→3)-O-

mannopyranosyl-O-mannose or O-glucopyranosyl-(1→6)-O-mannopyranosyl-O-(1→3)-O-mannopyranosyl-O-(1→4)-mannopyranosyl-O-mannose. These results imply that the cell wall of the yeast is made up of glucomannan with repeating units of O-D-mannopyranosyl (1→4)-O-mannopyranosyl-(1→3)-O-D-mannose or O-D-mannopyranosyl-(1→3)-O-mannopyranosyl-(1→4)-O-D-mannose. These findings are in agreement with similar structural units found in *Rhodotorula* species by other workers (Elinov *et al.* 1988; Gorin *et al.* 1965; Arai and Murao, 1978; Arai *et al.* 1978). Thus, the types of linkages between the mannoses were 1→4 and 1→3, whereas those between the mannose and glucose were 1→6. The optical rotation and whether the linkages are of α- or β-type was not determined. In most *Rhodotorula* species studied up to date, it has been reported that the linkages are mostly of β-1→3 and β-1→4 type and most of the mannans are of the D form (Gorin *et al.*, 1965; Arai and Murao, 1978; Elinov *et al.*, 1980; 1988). Since the new isolate have been identified as a strain of *R. rubra*, the linkage is, in all probability, of the β-1→3 and β-1→4 type. It was also determined in this study that some of the linkages between the mannoses were of 1→2 type. The presence of 1→2 linkage is surprising since this linkage has not been reported in mannans isolated from *Rhodotorula* species. No explanation can yet be offered for the presence of this linkage, unless the difference depends on the conditions used for culturing the yeast or the occurrence of traces of glycerol and erythritol as reported by other workers. Gorin *et al.* (1964) reported the occurrence of 1→2-linkage in the cell wall polysaccharides of *R. glutinis* and other *Rhodotorula* species but concluded upon further chemical analysis that the 1→2

linkage arose from the presence of trace amounts of erythritol in the cell walls of the yeast. They subsequently determined that the mannans in *R. glutinis* consisted of β-1→3 and β-1→4 pyranose units. Similarly, Arai and Murao (1978) detected the presence of glycerol, erythritol and O-β-D-mannopyranosyl -(1→2)-glycerol in the degradation products of oligosaccharides isolated from the cell walls of *R. glutinis* and concluded, based on the degradation products, that the oligosaccharides was O-β-D-glucopyranosyl-(1→6)-O-β-D-mannopyranosyl-(1→4)-O-β-D-mannopyranosyl-(1→3)-O-β-D-mannopyranosyl-(1→4)-D-mannose. Further support of the occurrence of 1,2 glycosidic linkages has been provided by Elinov *et al.* (1988) who reported the presence of 41-61% of 1→4 and 1→2 glycosidic bonds in the cell wall polysaccharides of *R. rubra* VKMY-341 and its mutants.

The presence of the 1→2 glycosidic linkages could also have arisen from degradation of glycogen that may have been present in the cell wall material. Yeast cell wall extracts contain large quantities of glycogen, which can be removed to a varying degree by hot acid extraction and completely by treatment with α-amylase (Manners *et al.* 1974, 1973). Since our extracts were not treated with α-amylase, it is possible that the presence of traces of glycogen may have contaminated the samples and hence contributed to the presence of 1→2 linkages. From the results presented so far, it can be inferred that the cell wall of the new isolate, *R. rubra* TP1 is made up mainly of mannans. A possible structure of the cell wall might therefore be constructed as follows: mannan cell wall consisting of linear long chain β-1→3 and

β-1→4-linked mannose chain with glucopyranosyl side chains. The structure would therefore be consistent with those determined for other *Rhodotorula* species (Arai and Murao, 1978; Gorin *et al.*, 1965) and further supports the determination that the new yeast isolate belongs to *Rhodotorula*. The occurrence of mannans with β-configuration has also been reported in *Citeromyces matritensis* (Gorin *et al.*, 1969). However unlike those in *Rhodotorula*, these mannans have α-1,6-D-mannoside chain with α-1→2 or β-1→2 mannopyranosyl side chain. The *Rhodotorula* mannans are also different from the mannans containing 1,2-α- and 1,6-α-pyranosyl links (with 1,3-α links in some cases) associated with *Saccharomyces* (Gorin *et. al.*, 1966), fungi (Hough and Perry, 1962), bacteria (Ball and Adams, 1959; Orlova, 1961) and the polysaccharides of the related genus *Cryptococcus*. In *Cr. neoformans* (Miyazaki, 1961) and *Cr. laurentii* (Ambercombie *et al.*, 1960) for example, the polysaccharides consist of glucuronic acid, mannose and xylose.

The type of linkage in the fucose, galactose and rhamnose that were detected in the cell wall of the new isolate could not be determined in this study. However, from a taxonomic point of view, the presence of these monosaccharides is very important since they confirm the phylogenetic affinity of the new isolate. *Rhodotorula* and *Sporobolomyces* are the only yeasts that are known to have galactose, fucose and rhamnose in their cell walls (Crook and Johnson, 1962). The presence of fucose is significant since it is also a characteristic of Basidiomycetes and *Rhodotorula* is known to have a basidiomycetous affiliation.

The function of polysaccharides in the cell wall of yeast and other fungi are

either unknown or at best presumed to be structural (Fleet and Phaff, 1974). In *Aspergillus nidulans*, the polysaccharide is reported to serve as storage carbohydrate (Zonnerveld, 1972), whereas in *Saccharomyces*, the cell wall is believed to be involved in maintaining the shape of the yeast cell (Lampen, 1968). The β-1,6 glucans found in certain types of yeast such as *Saccharomyces* may also play a vital role in protecting these organisms against invading pathogens as can be inferred indirectly from the ability of these glucans to boost the immune system of fish (Azuma, 1987).

Mannan is a major structural component of the cell envelope of many types of fungi and has been found to be directly involved in host-pathogen interactions between fungi and man (Phaff, 1971; Gorin and Spencer, 1970). In yeast, mannoproteins (mannans covalently linked to proteins) have been determined to play several roles. For example, they are known to be the antigenic determinants of yeast and may also function as primary receptors of some killer toxins (Schmitt and Radler, 1988). Mannans from yeasts that are linked with phosphate (phosphomannans) have been shown to have immunochemical properties. Injection of the intact cells into rabbits resulted in the formation of antibodies directed against the components of the cell wall (Raschke and Ballou, 1971; Suzuki *et al.*, 1968). Similarly, both the laboratory and industrial samples of mannans have been shown to have antiviral properties that include the inhibition of the infectiousness of the tobacco mosaic virus (Erlinov *et al.*, 1980). Also, surface exo-cellular polysaccharides of industrially

useful yeast have been studied in great detail to determine their role in various specific applications (Sandford, 1980). The glucomannan isolated from this new isolate may play a similar role. However it is not clear if they can also provide protection for fish against diseases as has been reported in the case of β-1,6 glucans of *Saccharomyces*. This function can be made the subject of future investigation.

Table 7.0. ^{13}C-NMR chemical shifts of cell wall polysaccharides from *Rhodotorula rubra* TP1, *S. cerevisiae* and laminarin in d_6-DMSO[a].

C-atom	*R. rubra* TP1	*S. cerevisiae*	*S. cerevisiae*[b]	Laminarin	Laminarin[c]
C-1	_	100.36	103.01	100.34	103.70
C-2	_	70.09	72.83	70.09	74.50
C-3	_	83.46	86.22	83.41	85.50
C-4	_	65.60	68.41	65.64	69.30
C-5	_	73.57	76.33	73.54	76.80
C-6	_	58.10	60.87	59.10	61.90

[a]Chemical shifts in ppm. [b]Chemical shifts of *S. cerevisiae* insoluble glucan reported by Williams *et al.* (1994). [c]Chemical shifts of laminarin expressed in ppm downfield from external tetramethylsaline as reported by Saito *et al.* (1977).

Table 7.1. Hydrolytic products and gas chromatography retention times of the methylated derivatives of the cell wall polysaccharides of *Rhodotorula rubra* TP1.

Cleavage product	Structural feature indicated	Retention time (minutes)
2,3,4,6-tetra-O-methyl glucitol	Glcp-(1→)	4.38
2,4,6-tri-O-methyl mannitol	Manp-(1→3)	7.59
2,3,4-tri-O-methyl mannitol	Manp-(1→6)	7.96
2,3,6-tri-O-methyl mannitol	Manp-(1→4)	7.60
3,4,6-tri-O-methyl mannitol	Manp-(1→2)	7.19

Table 7.2. Monosaccharides composition and gas chromatography retention times of cell wall polysaccharides of *Rhodotorula rubra* TP1.

Monosaccharide	% Composition	Retention time (minutes)
Rhamnose	3.2	9.13
Fucose	8.6	9.80
Mannose	50.53	21.90
Glucose	25.53	22.70
Galactose	12.73	22.90

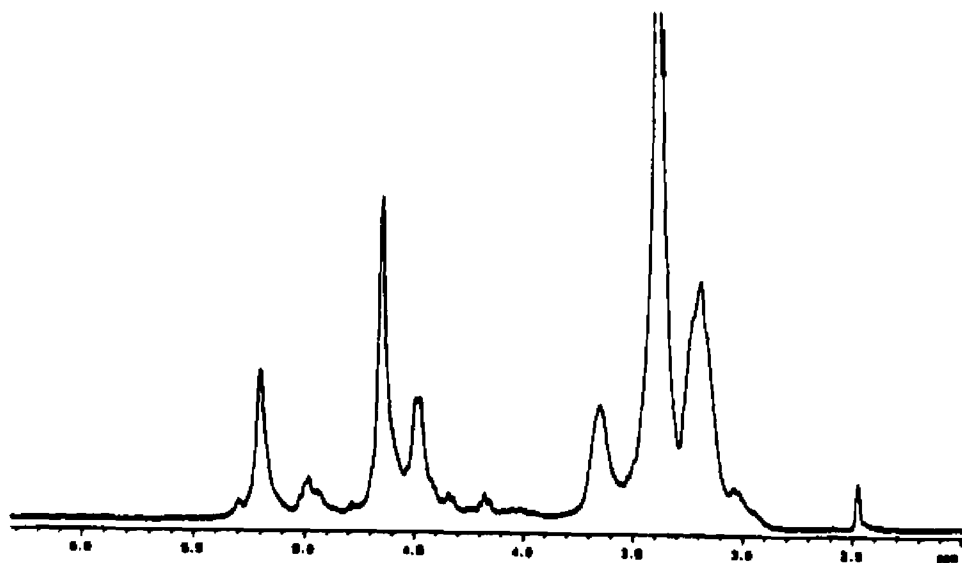
Fig. 7.1. ^{13}C-NMR spectra of the cell wall polysaccharides of *Rhodotorula rubra* TP1 (bottom) and laminarin (top). Laminarin served as the β-1,3-triple-helical polyglucose control.

200 180 160 140 120 100 80 60 40 20 0 ppm

Fig. 7.2 ^{13}C-NMR spectra of the cell wall polysaccharides of *Saccharomyces cerevisiae* (bottom) and laminarin (top). Laminarin served as the β-1,3-triple-helical polyglucose control.

110 100 70

Fig. 7.3. ^{1}H-NMR spectra of the cell wall polysaccharides of *Rhodotorula rubra* TP1 (bottom) and laminarin (top). Laminarin served as the β-1,3-triple-helical polyglucose control.



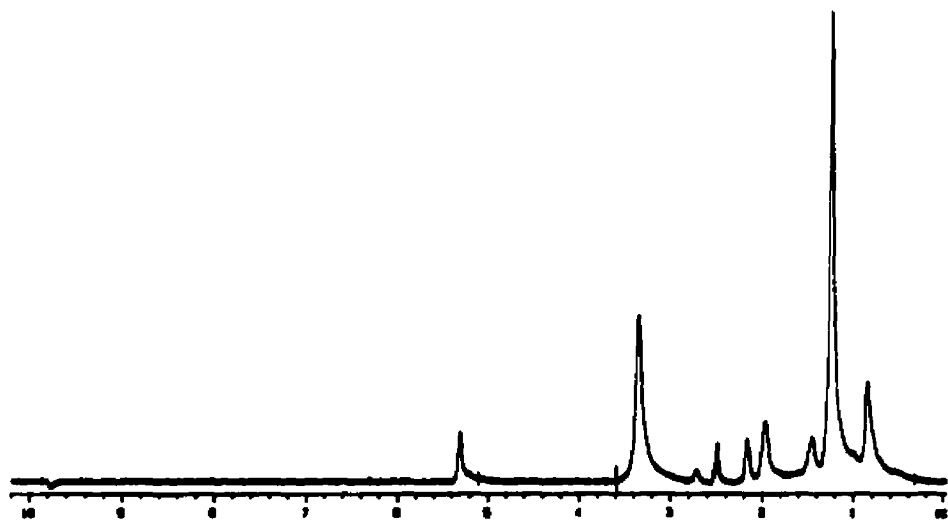
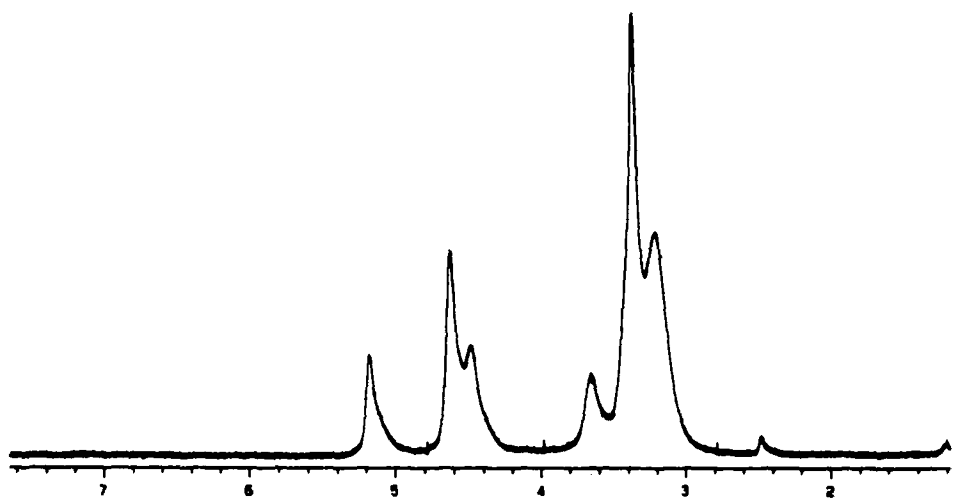


Fig. 7.4 ^{1}H-NMR spectra of the cell wall polysaccharides of *Saccharomyces cerevisiae* (bottom) and laminarin (top). Laminarin served as the β-1,3-triple-helical polyglucose control.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6
5
4
3
2

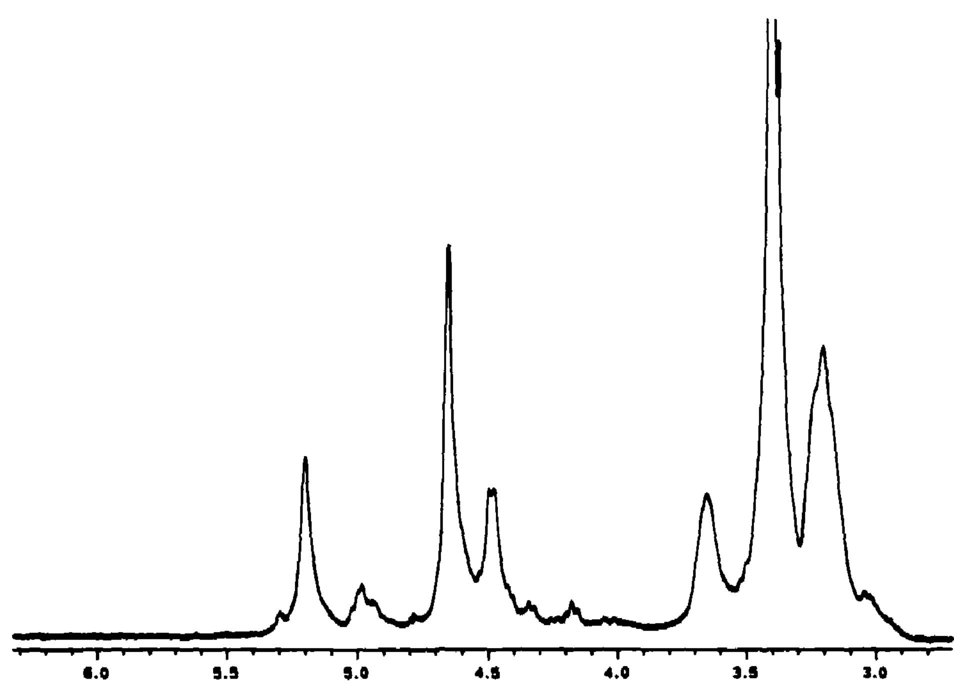
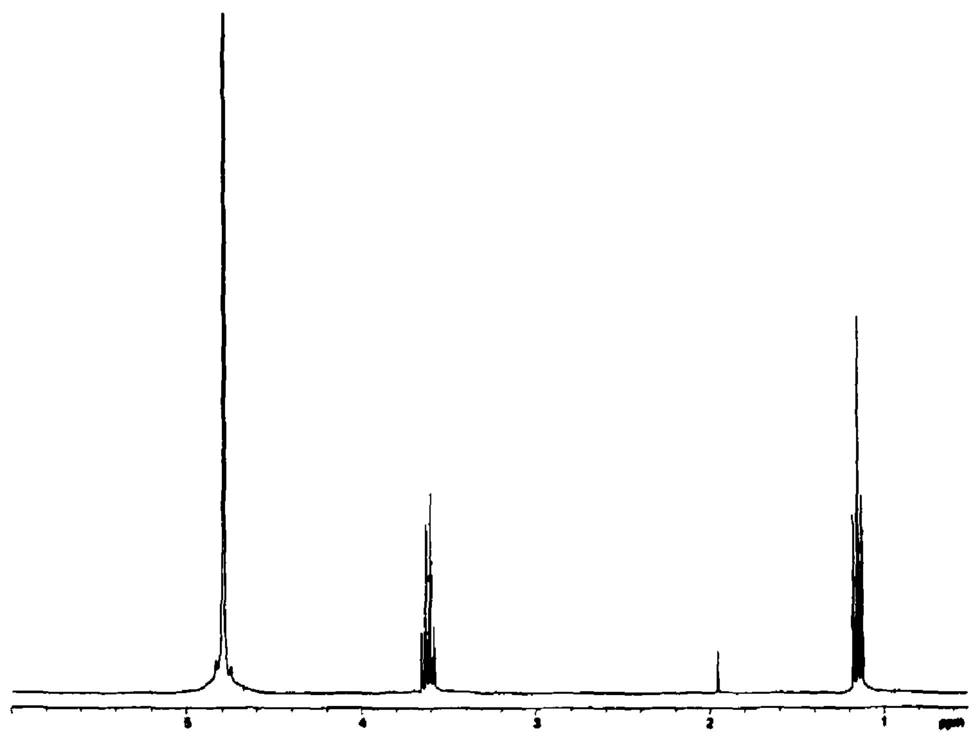

6.0
5.5
5.0
4.5
4.0
3.5
3.0

Fig. 7.5. ^{1}H-NMR spectra of mannans isolated from the cell wall of *Rhodotorula rubra*. The top spectrum is the crude mannan and the bottom spectrum is the purified copper complex.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4
3
2
1
ppm

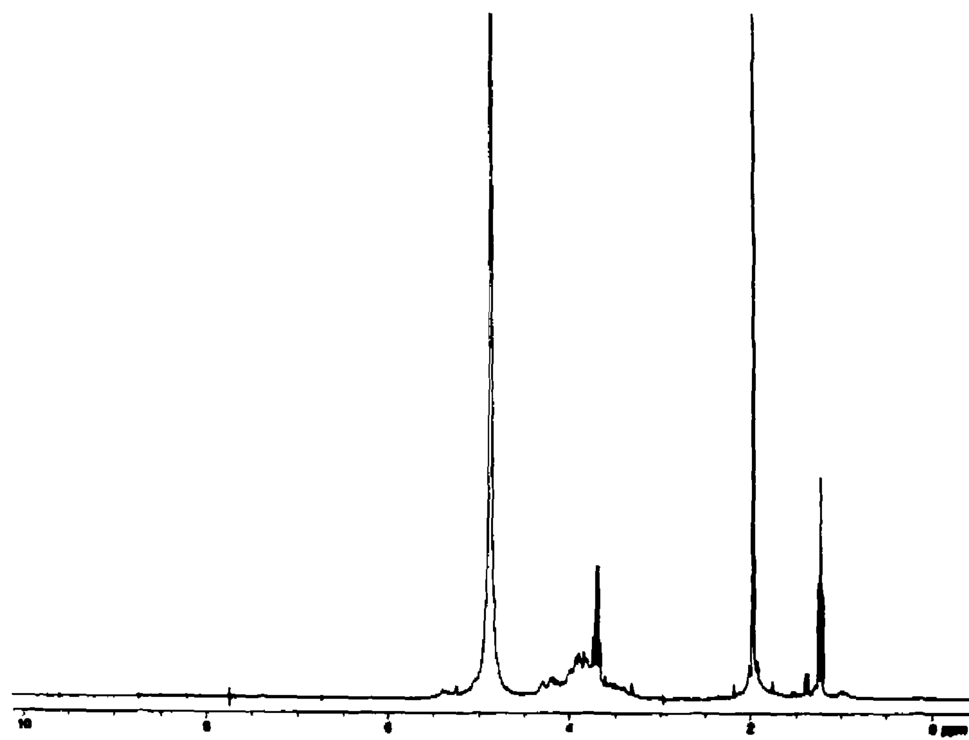
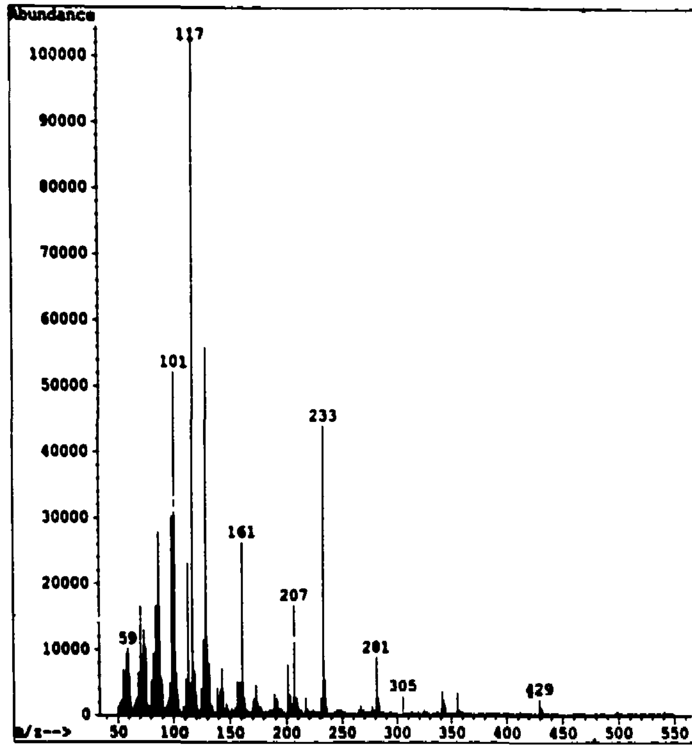

10
8
6
4
2
0 ppm

Fig. 7.6 Mass spectra of methylated aditol acetates prepared from cell wall polysaccharides of *Rhodotorula rubra* TP1. Top): 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl hexitol; bottom): 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl hexitol.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101
233
161
207
59
281
305
429
m/z-->

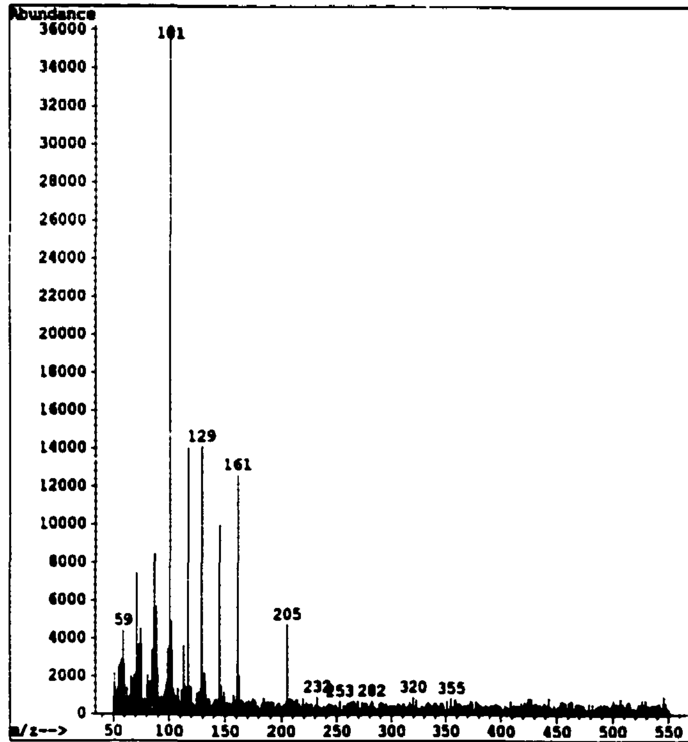
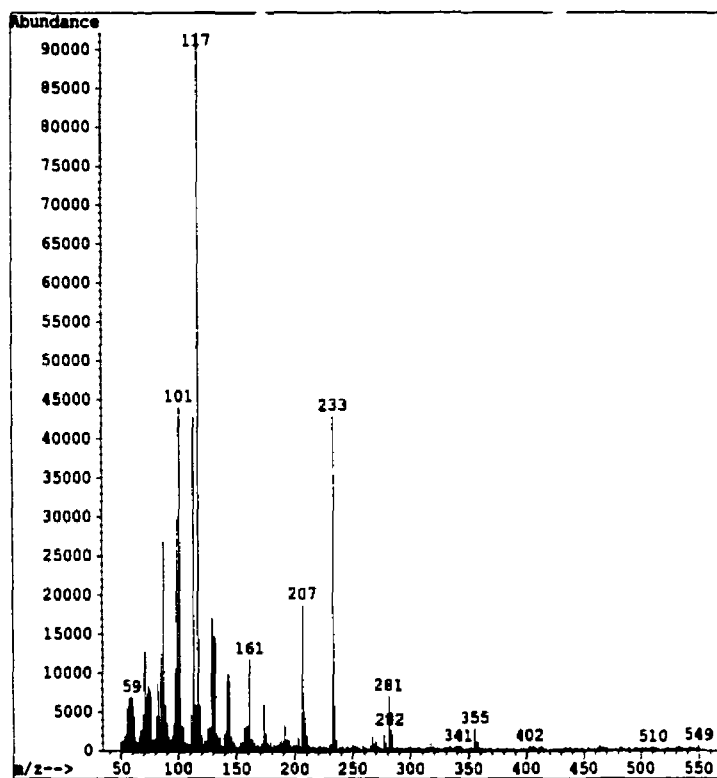
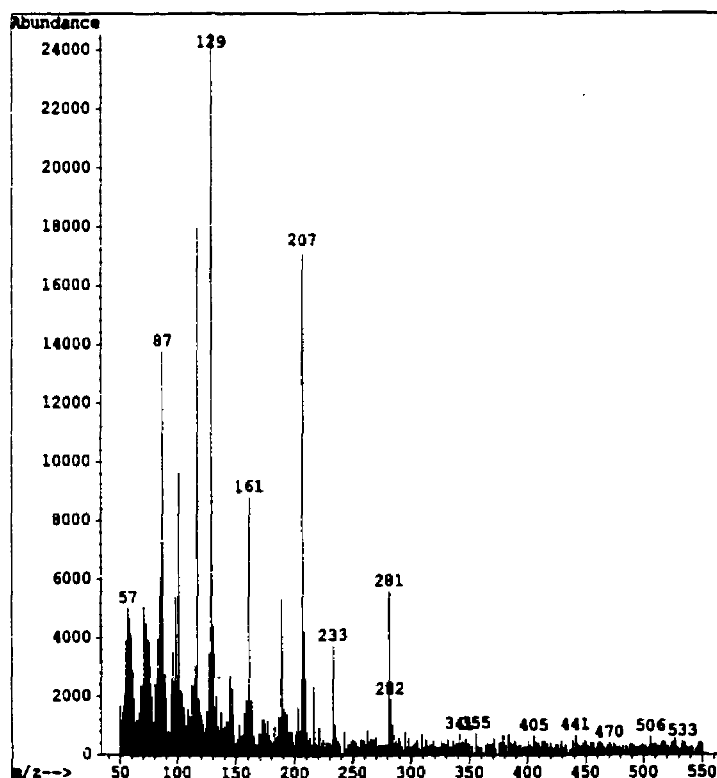

Abundance
101
129
161
205
59
320
355
m/z-->

Fig. 7.7. Mass spectra of methylated aditol acetates prepared from cell wall polysaccharides of *Rhodotorula rubra* TP1. Top): 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl hexitol and/or 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl hexitol; bottom): 1,3,5-tri-O-acetyl-3,4,6-tri-O-methyl hexitol.

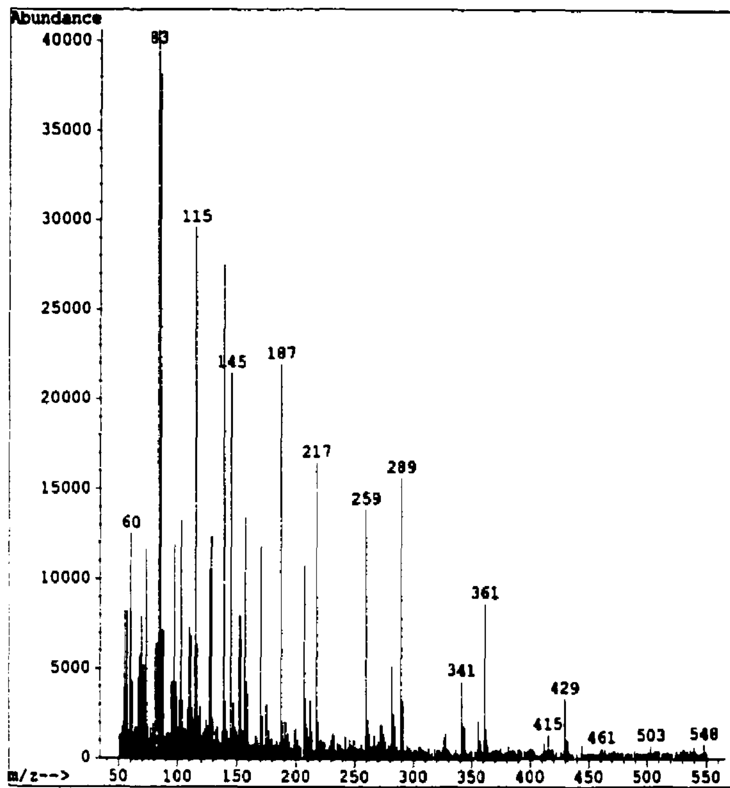


Abundance
90000
85000
80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
117
101
233
207
161
59
281
282
355
341
402
510
549
m/z-->
50 100 150 200 250 300 350 400 450 500 550

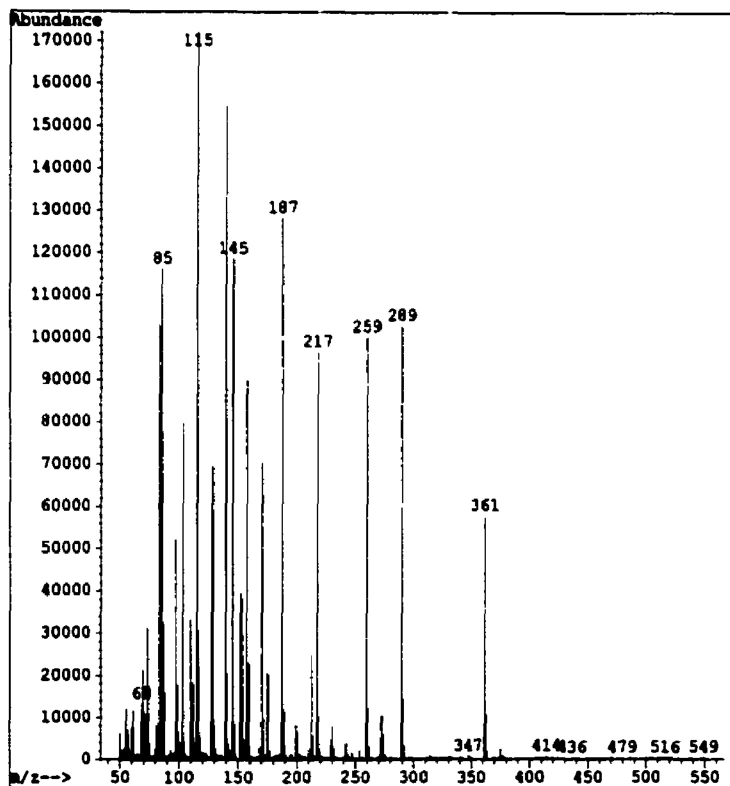


Abundance
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
129
207
87
161
281
57
233
282
405
441
470
506
533
m/z-->
50 100 150 200 250 300 350 400 450 500 550

Fig. 7.8. Mass spectra of monosaccharides isolated from the cell wall of *Rhodotorula rubra* TP1. Top): glucose, bottom): mannose.

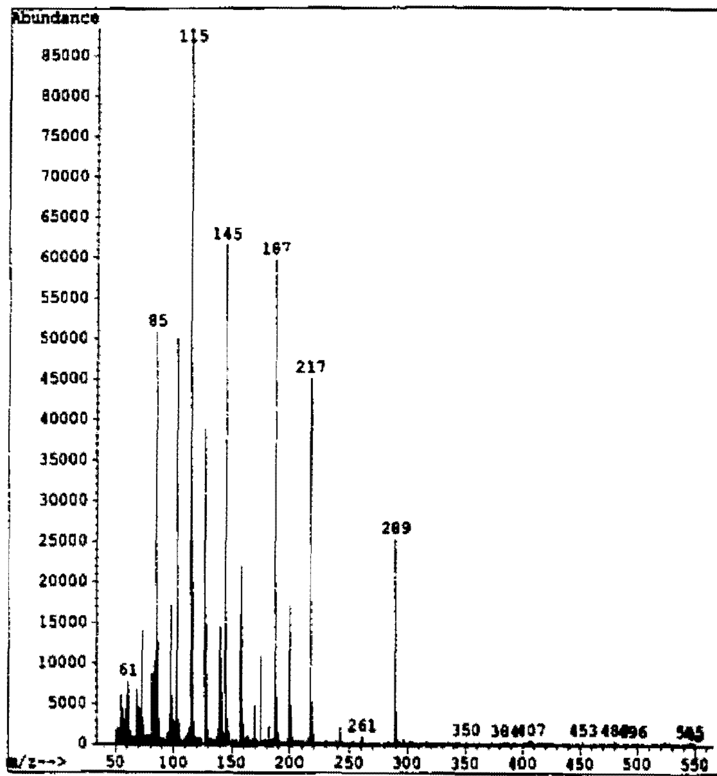


Abundance
40000
35000
30000
25000
20000
15000
10000
5000
0
m/z-->
50 100 150 200 250 300 350 400 450 500 550
83
115
145
187
217
259
289
60
361
341
429
415
461
503
548



Abundance
170000
160000
150000
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
m/z-->
50 100 150 200 250 300 350 400 450 500 550
115
187
145
85
259
289
217
361
60
347
436
479
516
549

Fig. 7.9. Mass spectra of various monosaccharides isolated from the cell wall of *Rhodotorula rubra* TP1. Top): rhamnose; bottom): fucose.


Abundance
115
145
187
85
217
289
61
261
350
384
407
453
484
496
545
m/z-->

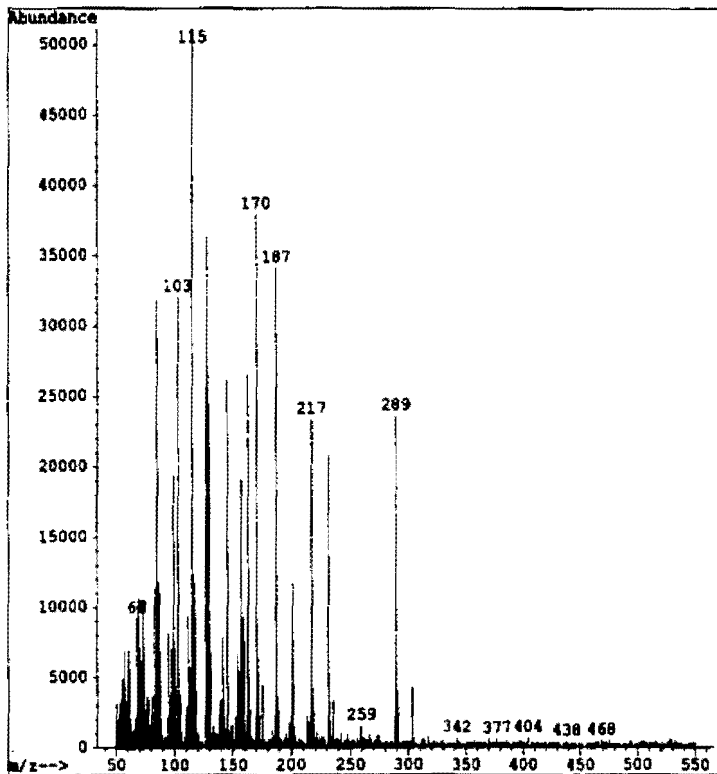
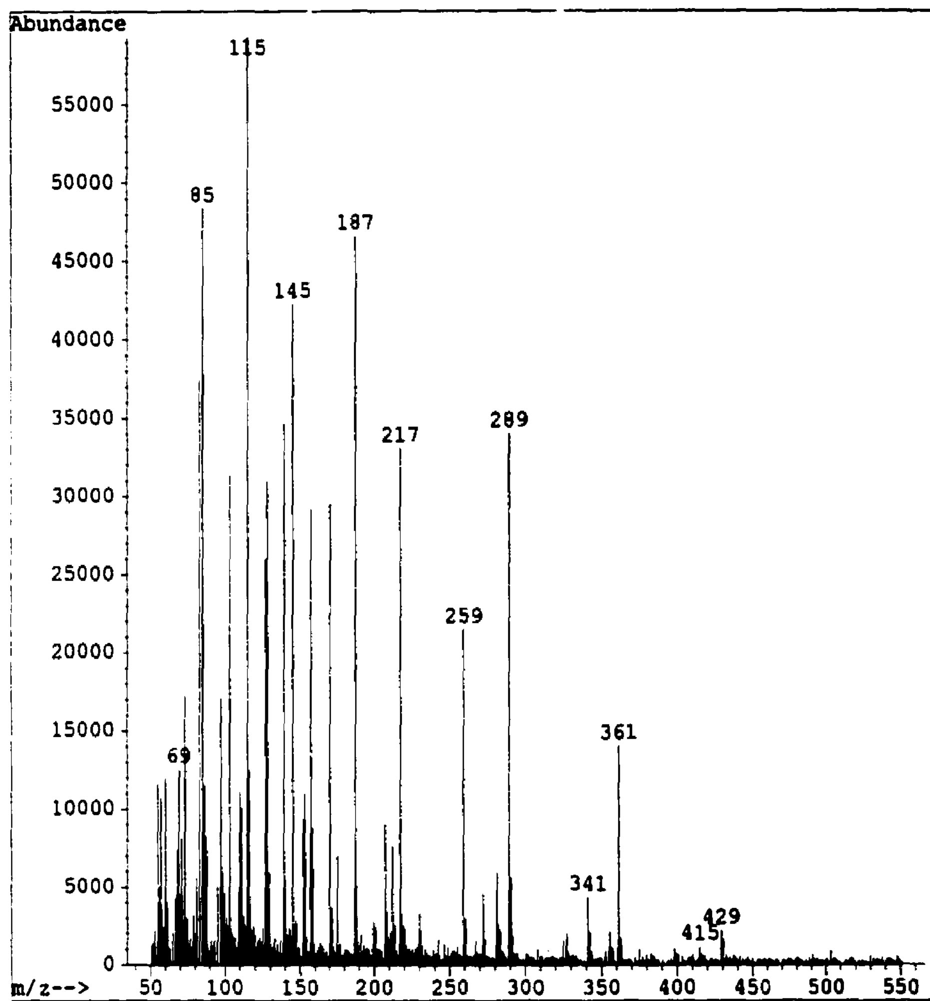

Abundance
115
170
187
103
217
289
259
342
377
404
438
468
m/z-->

Fig. 7.10. Mass spectra of galactose isolated from the cell wall of *Rhodotorula rubra* TP1.


Abundance
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
m/z-->
50
100
150
200
250
300
350
400
450
500
550
115
85
187
145
289
217
259
361
69
341
429
415

CHAPTER 8

BIOSYNTHESIS OF CAROTENOIDS FROM MEVALONIC ACID BY A PARTIALLY PURIFIED ENZYME SYSTEM FROM *Rhodotorula rubra* TP1

8.1 Introduction

In carotenoid producing organisms, the synthesis of carotenoids involves the activity of different enzyme systems that catalyze a sequence of reactions to yield the desired end product. The entire biosynthetic pathway has been demonstrated *in vitro* using cell-free extracts from various organisms such as plants (Qureshi *et al.*, 1974; Beeler and Porter, 1962; Camara *et al.*, 1982; Dogbo and Camara, 1987); fungi (Bramley and Davies, 1975; Spurgeon *et al.* 1979; Gregonis and Rilling, 1973). In yeast, Eberhardt and Rilling (1975) purified the enzyme prenyl transferase from *Saccharomyces cerevisiae* that converted dimethylallyl, geranyl and farnesyl pyrophosphates into farnesyl and geranylgeranyl pyrophosphates. However, there are no reports describing the *in vitro* conversion of labelled carotenoid precursors into carotenoids in *Rhodotorula rubra*.

There are a number of enzymes involved in the synthesis of carotenoids and among them is phytoene desaturase (PDS) which is an enzyme or a group of enzymes that catalyzes the conversion of phytoene to more saturated carotenoids. The actual number of enzymes involved in this conversion is unknown and probably varies

among different organisms (Fraser and Bramley, 1993). In higher plants, it has been reported that two enzymes are involved (Kirk and Tilney-Basset, 1978) whereas genetic studies in *Phycomyces blaskesleeanus* suggest only one is involved (Aragon *et al.* 1976).

In the present investigation, we report for the first time the isolation of an enzyme system from *R. rubra* TP1 which catalyzes the conversion of mevalonic acid (MVA) into torularhodin, torulene, β-carotene and phytoene. This demonstration is significant in that further purification and characterization of this enzyme system would aid in the isolating and cloning of the carotenogenic genes, which could have a huge economic impact on the carotenoid industry.

8.2 Materials and methods

Chemicals: (RS)-[2-^{14}C] mevalonic acid (N,N'dibenzylethylenediamine {DBED}) salt was obtained from Amersham Pharmacia Biotech; Trizma base, nicotinamide adenine dinucleotide (NAD), FAD, NADP, polyethylene glycol (PEG) 8000, bovine serum albumin (BSA), Folin and Ciocalteu's Phenol reagent, Tween 40, 60, 80, toluene and ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Company, St. Louis, Missouri; dithriothreitol (DDT), sodium dodecyl sulphate (SDS) and DEAE sepharose were from Pharmacia Biotech AB, Uppsala, Sweden; hydrochloric acid, magnesium chloride, manganese chloride, methanol, petroleum ether and acetone were purchased from Fisher Scientific Ltd., Fair Lawn, New Jersey. Thin layer plates were from EM Science, Darmstadt, Germany.

8.2.1 Preparation of crude enzyme extract

Cells were grown in YM broth for 5 days at 22°C. The cells were harvested by centrifugation at 10,000 x g for 20 minutes, the pelleted cells were washed several times with pyrogen free water and then once with ice-cold Tris-HCl buffer (pH 8.2) containing 10 mM dithiothreitol (DDT) (1:6 w/v), 10 mM EDTA (1:6 w/v). The washed cell pellets were then resuspended in the above-mentioned buffer into a slurry (25 mL buffer per 12 g wet cells) and passed through a French Press (SLM Instruments, Chicago, Illinois) at a pressure of 20,000 psi to disrupt the cells. The broken cells were then centrifuged at 40,000 x g in a Sorvall RC-5B *Plus* centrifuge (Dupont-Sorvall Instruments, Newark, DE.) for 3 hours at 4°C and the supernatant collected and stored at -85°C before being used as the cell extract. The protein concentrations of all cell extracts were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

8.2.2 Assay of carotenogenic enzymes

Incubations were done with water-soluble (2R)-[2-^{14}C] mevalonic acid (MVA, DBED salt). The incubation mixture consisted of 500 of µL of 0.4 M Tris-HCl buffer (pH 8.2), 50 µL of 5 mM DDT, 100 µL of 25 mM NAD, 100 µL of 20 mM ATP, 100 µL 20 mM NADP, 50 µL of 5 mM EDTA, 10 µL each of 0.6 M $MnCl_2$ and 0.4 M MgCl. (2R)-[2-14C] MVA (680, 000 dpm) and cell extract containing 8 mg protein (only 4 mg of PEG precipitated protein was used). The mixture was incubated at 30°C in light for 3 hours after which the reaction was stopped with the addition of 3

mL methanol and authentic β-carotene, phytoene and torularhodin were added to minimize the destruction of the pigments by light.

8.2.3 Extraction, separation and assay of carotenoids for radioactivity

The pigments were extracted three times with 6 mL aliquots of acetone and 2 mL of methanol in dim light and at 4°C. The acetone extracts were pooled together and transferred into hexane and the two phases were resolved with deionized water. The hexane phase was then evaporated to dryness with a stream of nitrogen and the residue was redissolved in 2 mL of petroleum ether. About 500 μL of this was applied to thin-layer silica gel chromatographic plates that were developed with a solvent system containing 10% toluene in petroleum ether for 1 hour. Bands were located by colour and by exposure to long UV light (366 nm) and the separated pigments were identified by co-chromatography with authentic pigments. The isolated pigmented bands were scrapped off and radio assayed by liquid scintillation counting in a Beckman Model LS-3150T Liquid Scintillation Spectrophotometer with a Beckman liquid scintillation fluid (Beckman Scientific Instruments, Irvine, California, U.S.A.). Quenching corrections were made by automatic colour quench compensation (Bramley *et al.*, 1974).

8.2.4 Solubilization of enzymes

Tweens 40, 60 and 80 were tested to determine their ability to solubilize the membrane-bound proteins. The broken cells were treated with 0.5, 1.0 and 3.0%

(w/v) of Tweens 40, 60 and 80 at a detergent:protein ratio of 2:1. The mixture was incubated with gentle stirring at 4°C for 1 hour after which it was centrifuged at 40,000 x g for 3 hours and at 4°C. The supernatant was collected and the protein content determined and assayed for carotenogenic enzyme activity as previously described (section 8.2.3).

8.2.5 Polyethylene glycol (PEG) precipitation of proteins

The carotenogenic enzymes were precipitated with 20% PEG 8000. The requisite PEG concentration was determined by obtaining an analytical precipitation curve using Tween 60 solubilized cell extract with increasing amounts of 50% (w/v) PEG solution (Ingram, 1990). The 10% PEG precipitate was redissolved in 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM DDT, 0.5% Tween 60 and 20% ethylene glycol which was then stored at -85°C. The PEG precipitated enzyme was later assayed for enzyme activity using the protocol previously described (sec 8.2.3) except that the concentration of enzyme used was 4 mg/mL.

8.3 Results.

8.3.1 Conversion of [2-^{14}C]MVA into carotenoids by crude enzyme extracts.

The biosynthesis of phytoene, β-carotene, torulene and torularhodin from mevalonic acid by cell free extracts from *Rhodotorula rubra*TP1 has been

demonstrated. Enzyme activity of the crude cell extract was estimated by the incorporation of [2-^{14}C]MVA radioactivity into phytoene, β-carotene, torulene and torularhodin. The amounts of the individual carotenoids synthesized were determined by the extraction of the incubation mixture with hexane and the chromatography of the hexane extracts on silica gel thin layer plates. The individual peaks were identified by the coincidence of radioactivity with light absorbance due to authentic carotenoids added to the hexane extracts. The results of the total radioactivity incorporated by the crude cell extract into the various carotenogenic products are presented in Table 8.0. The highest radioactivity was recorded in phytoene (7600 dpm) followed by torularhodin (6975 dpm), β- carotene (4010 dpm) and torulene (2133 dpm).

8.3.2 Solubilization of enzymes.

Varying concentrations of three detergents, Tweens 40, 60 and 80 were used to study their effects on the incorporation of [2-^{14}C]MVA into various carotenoids and their ability to solubilize membrane-bound proteins. The results are presented as percentage of the control activity (7600, 4010, 6975, 2133 dpm for phytoene, β-carotene, torularhodin and torulene, respectively) in Figs. 8.0 to 8.2. In general, cell extracts treated with all three detergents retained significant enzymatic activities. There was stimulation in phytoene and torularhodin synthesis from [2-^{14}C]MVA in all cases, however, Tween 60 at a concentration of 1% (w/v) caused the greatest stimulation in phytoene and torularhodin formation compared to the control activity

and Tweens, 40 and 60, which caused a decrease in torulene production. Tweens 40 and 60 at a concentration of 1% (w/v) maintained the original concentration level of β-carotene production whereas the other concentrations and Tween 80 were less effective in this respect. Overall, the maximum enzymatic activity was obtained with cells treated with 1% Tween 60 and this was subsequently used in the other analysis.

Protein concentrations were estimated on the S_{40} fractions before and after treatment with the various detergents at varying concentrations. The protein concentrations of the control fractions were estimated to be 16 mg/mL. From Figs. 8.0 to 8.2, it can be seen that all the detergents at varying concentrations solubilized some of the membrane proteins. The highest increase was 19.66 mg/mL produced by 1% Tween 80, and the lowest 16.19 mg/mL was furnished by 0.5% Tween 40 (Figs. 8.2 and 8.0, respectively).

8.3.3 Polyethylene glycol (PEG) precipitation of enzymes.

A 6.0 fold increase in the specific activity of the carotenoid synthesizing system was obtained by polyethylene glycol (PEG) precipitation (Table 8.1). Attempts were made to further purify the enzyme, phytoene synthetase complex by applying the PEG precipitated protein to a DEAE sepharose column (2.5 x 30 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM DDT. Proteins were washed with the same buffer and then eluted with a linear gradient of 0-1 M KCl in a Tris-HCl buffer. Fractions of 4 mL were collected. The elution profile of the phytoene synthetase complex is depicted in Fig. 8.3. However, the

activity of the enzyme was not determined at this point because specific substrate such as ^{14}C-isopentenyl pyrophosphate was not available.

8.4 Discussion.

Cell preparations capable of converting MVA or isopentinyl pyrophosphate (IPP) into colored carotenoids have been studied in numerous organisms. The most extensive studies up to date have been in tomato preparations (Camara *et al.* 1982; Dogbo *et al.* 1988; Camara, 1993). Cell free extracts have also been prepared in the fungi *Phycomyces* (Riley and Bramley, 1976; Mitzka-Schnabel and Rau, 1981, Fraser and Bramley, 1993); pea fruits (Graebe, 1968); bacteria (Bartley *et al.* 1990, Armstrong *et al.* 1989); *Neurospora* (Harding *et al.* 1978; Mitzka-Schnabel and Rau, 1976). These studies have shown that the synthesis and desaturation of phytoene to more unsaturated carotenoids require the co-ordinated activities of membrane-bound enzymes. The results from these studies also suggest that the enzyme system for the formation of phytoene may exist in different forms in different organisms. For example, in *Flavobacterium* (Brown *et al.* 1975) and *Halobacterium* (Kushwaha *et al.* 1976), the phytoene-synthesizing enzyme complex usually occurs in the 10,000 x g fraction, indicating that the enzyme in these organisms are soluble. Mitzka-Schnabel and Rau (1981), on the other hand, reported that in *Neurospora*, the enzyme that catalyzes the formation of geranylgeranyl pyrophosphate is soluble whereas the phytoene- synthesizing enzyme is a particulate one. In *Capsicum* fruits, Camara *et al.*

(1982) reported that the phytoene synthetase complex is a soluble enzyme whereas in *Phycomyces*, the enzyme has been reported to be peripheral (Sandmann and Bramley, 1985).

We report here the isolation and partial purification of the phytoene-synthesizing enzyme complex from the red yeast *Rhodotorula rubra* TP1 that converts [2-^{14}C] MVA into phytoene, β-carotene, torularhodin and torulene. Phytofluene was not detected in this study. The enzyme extract used in this study was the 40,000 x g fraction. This suggests that both the phytoene synthetase, which converts MVA into phytoene and the phytoene desaturase that converts phytoene to the more unsaturated forms of carotenoids occur in this fraction. However, whether the two enzymes occur separately or as a complex is not clear at this point. The conversion demonstrated in this study illustrates a carotenoid biosynthetic pathway identical to the one depicted in Fig. 6.9 in Chapter 6 of this thesis, and the work previously done by Simpson *et al.* (1964). The conversion of phytoene to torularhodin involves four dehydrogenation reactions and subsequent introduction of double bonds. The first dehydrogenation reaction converts phytoene to phytofluene that undergoes further dehydrogenation to yield ζ-carotene, neurosporene, β-zeacarotene, γ-carotene which may be converted to β-carotene or oxygenated to form torulene and eventually torularhodin (Simpson *et al.* 1964).

The number of enzymes involved in the four step dehydrogenation reactions is not known. It is also not known whether the dehydrogenases exists as a complex, as

individual enzymes or as a single polyfunctional protein. For some time, the general opinion was that more than one enzyme was involved with the 4 dehydrogenation reactions. This idea was deduced from studies on the cofactor requirements of these enzymes. For example in studies on the cofactor requirements for carotenoid biosynthesis in *Halobacterium cutirubrum*, Kushwaha *et al.* (1976) found that conversion of phytoene to phytofluene was dependent on NADPH, whereas the reactions from phytofluene to lycopene appeared to require FAD and Mn^{++}. These results were interpreted to mean that two enzymes with two different cofactor requirements might have been involved. Similarly, Qureshi *et al.* (1974) reported that the conversion of cis-[^{14}C] phytofluene to other carotenoids required FAD^{+}, $NADP^{+}$ and Mn^{++} for a reasonable incorporation of radioactivity into more unsaturated carotenoids. These results, like those reported by Kushwaha and co-workers (1976); suggest that there are two separate types of dehydrogenation. However, recent genetic complementation studies with *Phycomyces* mutants defective in either enzymes responsible for the desaturation of phytoene or lycopene cyclase gene have shown that, at least in this fungus, only one enzyme is involved in the dehydrogenation reactions (Credá-Olmedo, 1987). Analyses of genes from *Erwinia uredovora* (Misawa *et al.* 1990) and *Erwinia herbicola* (Schnurr *et al.* 1991) have also demonstrated that the *crtI* gene encodes for one desaturase, which is responsible for all dehydrogenation steps from phytoene to lycopene. Whether or not the presence of only one desaturase gene in an organism that form lycopene can be generalized to the yeast used in this study cannot be determined.

In order to solubilize the carotenogenic enzymes, the cell extracts were treated with Tweens 40, 60 and 80 at various concentrations for about an hour at 4°C. The Tweens were used because they have been shown to exert minimal inhibition of *in vitro* carotenogenesis in other studies (Bramley and Taylor, 1985). It was found out that Tweens 40 and 60 were the most suitable detergents for solubilizing the carotenogenic enzymes from *R. rubra* TP1. 1% Tween 60 was found to be the most suitable detergent overall resulting in a large stimulation in phytoene, torularhodin and torulene synthesis.

Detergents are routinely used in enzyme purification to solubilize membrane bound proteins. These detergents act by dislodging the tightly bound membrane protein through the dissolution of the membrane. The dissolved membranes are then replaced with aliphatic or aromatic chains which form the lipophilic part of the detergent (Rosenberg, 1996). In *Aphanocapsa*, Tween 40 is the best detergent for solubilizing carotenogenic enzymes (Bramley and Sandmann, 1987, Beyer *et al.* 1985) but sodium cholate is the detergent of choice with *Neurospora crassa* (Rau and Mitzka-Schnabel, 1985). In the C9 strain of *Phycomyces*, Bramley and Taylor (1985) reported that maximal phytoene desaturase activity was obtained with 1% (w/v) of Tween 60, whereas in daffodil chromoplasts, CHAPS ({3-[cholamidopropyl] dimethylammonio}-1-propane sulfonate) is effective in solubilizing the membrane-bound carotenogenic enzymes (Beyer *et al.* 1985). Sodium cholate and CHAPS require the addition of microsomal lipids to the incubation mixture in order to obtain

the maximum enzymatic activity. This is presumably because these detergents remove fatty acids from the microenvironment of the enzyme. Addition of the microsomal lipids therefore compensate for this removal (Fraser and Bramley, 1993). Unlike CHAPS and sodium cholate, the Tweens do not require the addition of microsomal lipids in order to effect maximum enzyme activity. This is because the fatty acid residues on the Tweens may compensate for the removal, during the solubilization process, of fatty acids from the microenvironment of the enzyme (Fraser and Bramley, 1993). The Tweens are also non-ionic detergents that in low concentrations such as used in this study, cannot dissolve the lamella structure (Helenius and Simons, 1975). Since low concentrations of detergents were used in this study, it is possible that the phytoene synthetase and desaturase enzymes encountered in this study are peripheral rather than integral membrane protein. Similar description has been provided for the phytoene desaturase and lycopene cyclase of *Phycomyces* (Fraser and Bramley, 1993; Bramley and Taylor, 1985) and *Neurospora crassa* (Mitzka-Schnabel and Rau, 1981). In *Neurospora crassa*, it has been suggested that the phytoene synthetase enzyme is loosely associated with the membrane by electrostatic forces (Mitzka-Schnabel and Rau, 1981, Spurgeon *et al.* 1979). It is therefore suggested that a more precise fractionation of the cell extracts be carried out in order to determine whether the enzymes are peripheral or membrane proteins.

A 6.0 fold increase in the specific activity of the phytoene desaturase system

was obtained with the PEG precipitation. This increase in the specific activity can be attributed to two factors: an increase in the total enzyme activity and the removal of an inhibitor of the phytoene synthesizing system or the loss of enzymes catalyzing competing reactions (Maudinas *et al.* 1977). The use of PEG offers several advantages over ammonium sulfate in fractionation of enzymes. The method is simple and products obtained are not denatured. Unlike ammonium sulfate, it is not necessary to remove PEG prior to enzyme assay since it does not inhibit enzyme activity at concentrations used (Fraser and Bramley, 1993).

This study has shown the presence of the enzymes phytoene synthetase complex in the S_{40} fraction of the cell extract of *R. rubra* TP1. This enzyme complex have also been shown to occur in the Tween 60 solubilized fraction and the PEG precipitate of the cell extract. These results indicate that all the fractions listed here contain all the enzymes of the isoprenoid pathway. It is therefore suggested that further purification be carried out to obtain a pure form of the enzyme for future studies. For example the purified enzyme can be used to raise antibodies which can be used to clone carotenogenic genes and the corresponding enzyme overexpressed for the production of antisera. The antisera can then be used for the isolation of the corresponding enzyme from other yeast. Also further characterization such as the determination of the native molecular weight, estimation if the cofactor requirements should be carried out.

Table 8.0 Products of carotenogenic enzymes in crude, Tween 60-solubilized cell extract and polyethylene glycol (PEG) precipitate of cell extracts of *R. rubra* TP1.

Preparation	Total incorporation (dpm/mg protein)[a]	% Total			
		Phytoene	Torularhodin	β-carotene	Torulene
Crude extract	2598	36.70	33.70	19.40	10.40
S40 + Tween 60	3490	43.55	25.23	18.72	12.50
PEG precipitate	15,632	46.32	22.52	17.95	13.21

[a]dpm: Disintegrations per minute.

Table 8.1 Partial purification of carotenogenic enzyme complex from *R. rubra* TP1.

Purification step	Total protein (mg)	Total activity	Specific activity[a]	Purification (-fold)
Crude extract	4000	2589	863	1
S_{40}+1% Tween 60	1700	3490	1163	1.34
10% PEG ppt.	450	15,632	5211	6.0

[a]Expressed as disintegrations per minute (dpm) (2R)-[2-^{14}C] MVA incorporated into carotenogenic products per mg protein per hour. Each assay contained 680,000 dpm (2R)-[2-^{14}C] MVA .

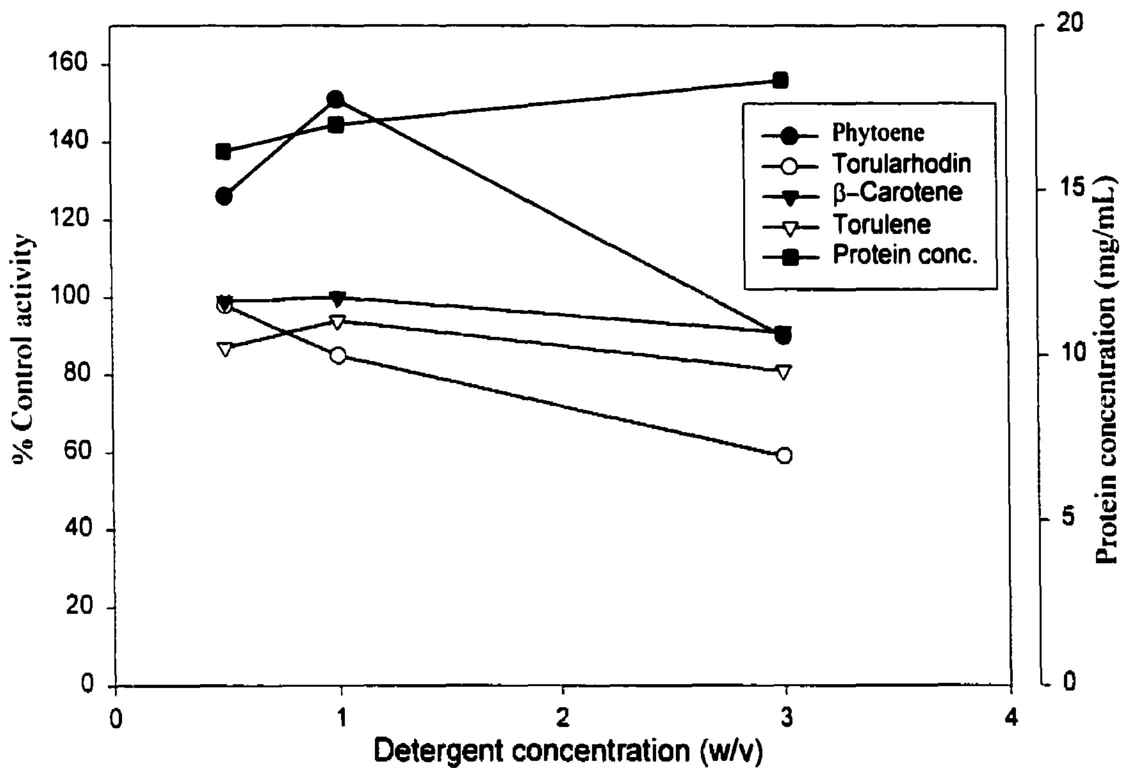


Fig. 8.0 The effects of various concentrations of Tween 40 on the enzyme activities and protein release from *R. rubra* TP1 cell extract. The carotenogenic products are expresssed as a percentage of control values (7600, 6075, 4010 and 2133 dpm for phytoene, torularhodin, β-carotene and torulene, respectively).

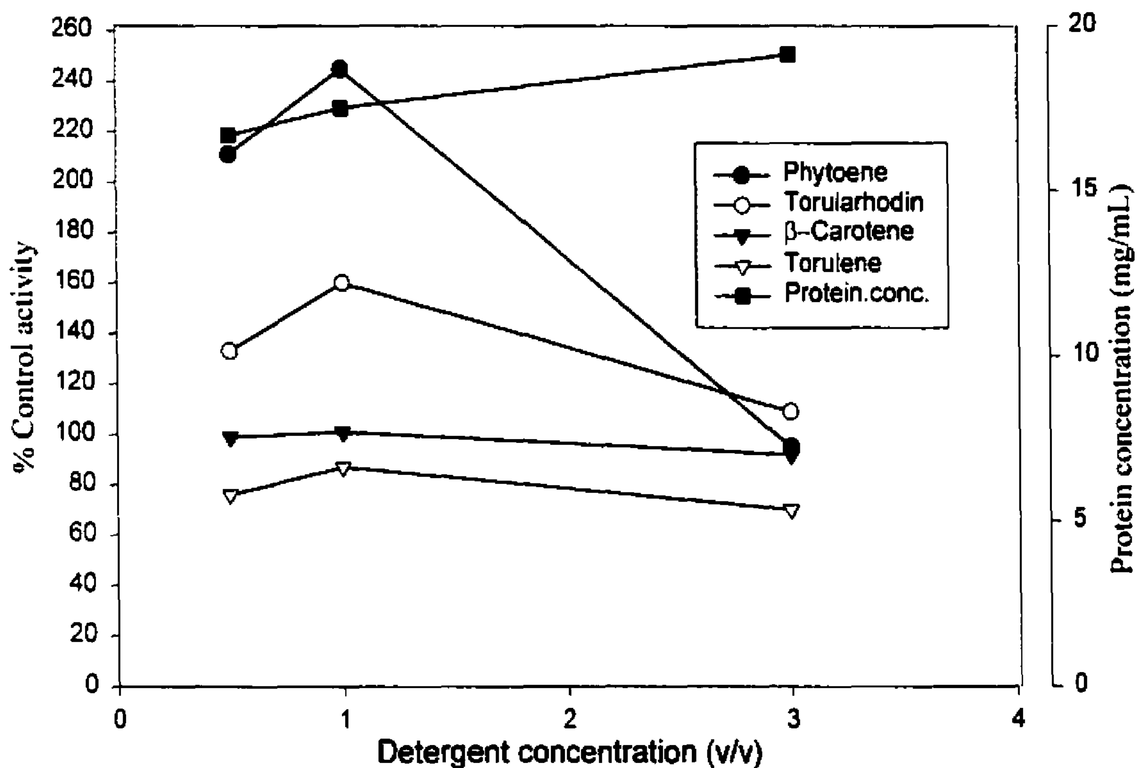


Fig. 8.1. The effects of various concentrations of Tween 60 on the enzyme activities and protein release from *R. rubra* TP1 cell extract. The carotenogenic products are expressed as a percentage of control values (7600, 4010, 6075 and 2133 dpm for phytoene, torularhodin, β-carotene and torulene, respectively)

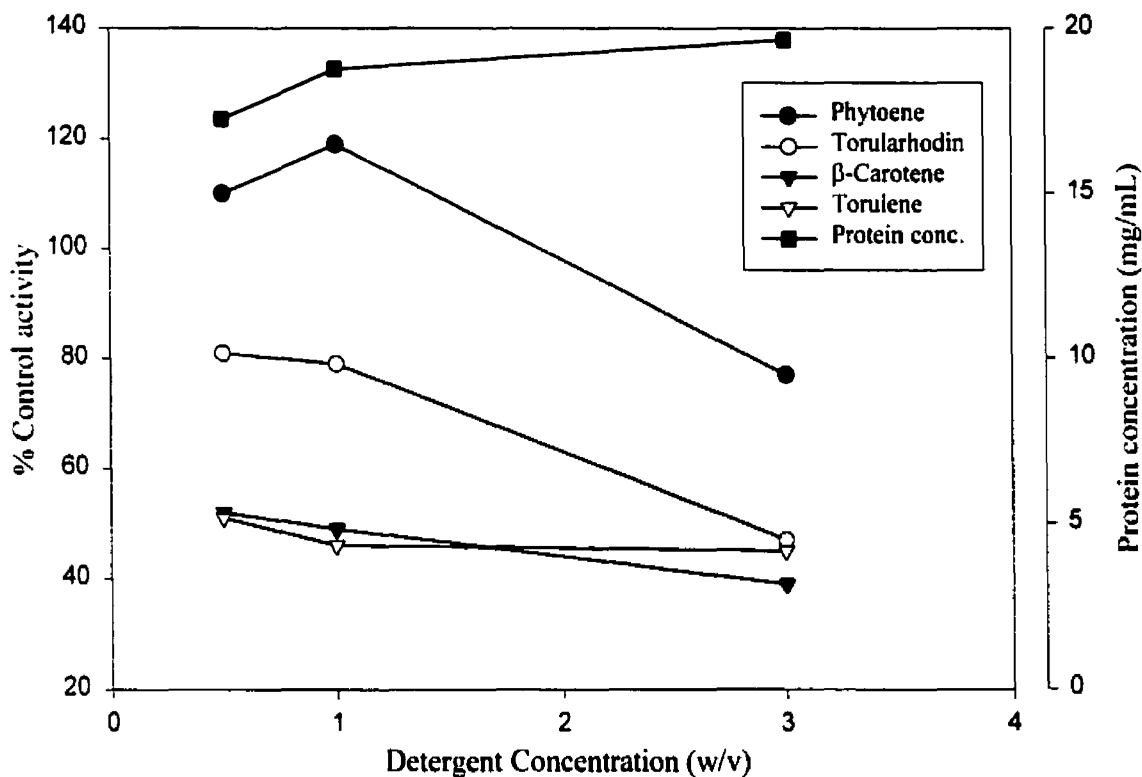


Fig. 8.2 The effects of various concentrations of Tween 80 on the enzyme activities and protein release from *R. rubra* TP1 cell extract. The carotenogenic products are expressed as a percentage of control values (7600, 6075, 4010 and 2133 dpm for phytoene, torularhodin, β-carotene and torulene, respectively).

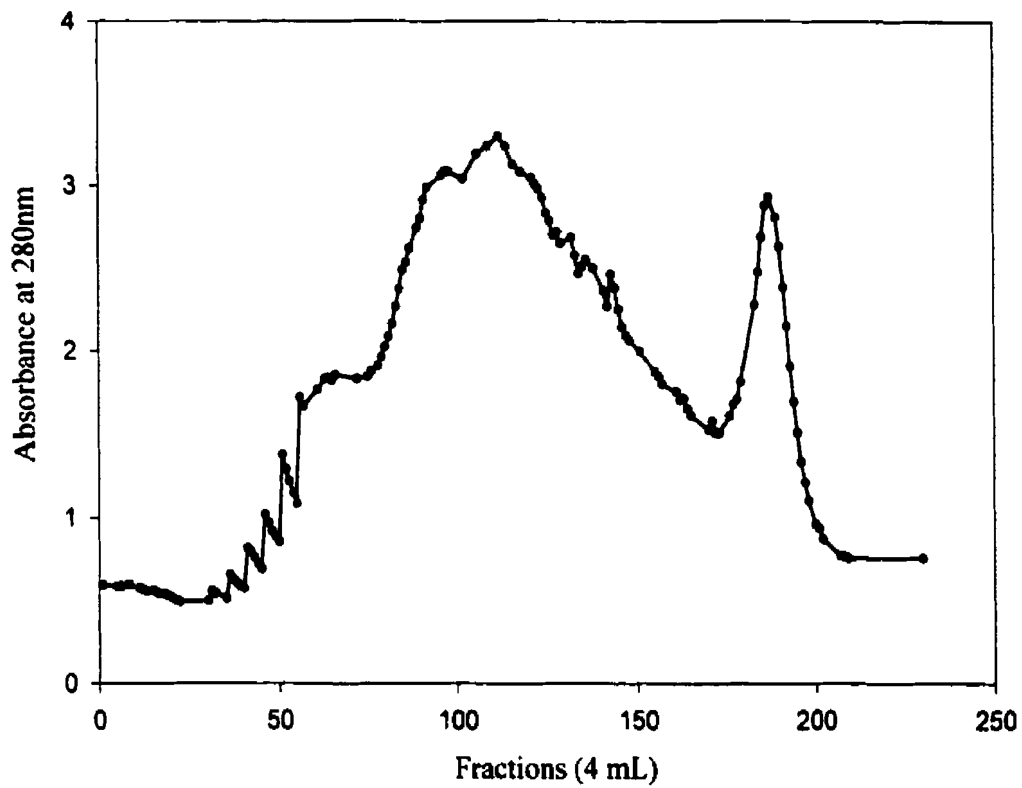


Fig. 8.3 Ion-exchange chromatography of polyethylene glycol fraction of cell extract of *R. rubra* TP1 on a DEAE-Sepharose column.

CHAPTER 9

Rhodotorula rubra TP1 AS A SOURCE OF PIGMENT AND NUTRIENTS FOR RAINBOW TROUT (*Onchorynchus mykiss*).

9.1 Introduction

Carotenoids are among the most widely distributed class of pigments in nature and have essential biological functions in animals. In salmon, the pink, orange and deep red colors of the flesh are due to the presence of carotenoids. This characteristic distinguishes them from other fish species and makes a major contribution to their elite image. The degree of flesh pigmentation is one of the most important criteria used by consumers in determining the acceptability of the product (Gentles and Haard, 1991; Johnson and An, 1991).

Salmonids, like most other animals, cannot synthesize carotenoids and they obtain their carotenoids primarily from micro-crustaceans and other invertebrates they used as food (Binkowski *et al.*, 1993). In the aquaculture industry, the pigments are supplied in their diet to impart the color to their flesh and inspire consumer acceptance and market value. Sources of carotenoids used in aquaculture include crustacean and crustacean by-products, plant and plant products and synthetic sources.

The commercial use of synthetic carotenoids is limited by their high cost, changes in regulatory climate and consumer concerns of the use of synthetics as food

additives. For this reason, lately there has been a trend towards the use of natural or biological sources of carotenoids.

This study reports a feeding trial in which *Rhodotorula rubra* TP1 was used to feed rainbow trout to determine the pigmentation of fish flesh and compared with control diets containing either no pigment or commercial pigment (canthaxanthin). The proximate analysis, pigment concentration and composition, growth and specific growth rate of fish as well as color measurements on the flesh of fish fed various diets are also reported.

9.2 Materials and Methods.

Materials: Brewer's wort was obtained from Labatt's Brewery, Montreal, Quebec; Molasses was kindly donated by Lelemand Ltd., Montreal, Quebec; yeast extract, bactopepetone and malt extract were purchased from DIFCO Laboratories, Detroit, MI. Petroleum ether, chloroform, acetone, sulfuric acid, hydrochloric acid and methanol were obtained from Fisher Scientific Ltd., Fair Lawn, NJ; MS-222 and β-carotene standard were purchased from Sigma Chemical Co., St. Louis, MO and canthaxanthin and astaxanthin standards were kind gifts from Hoffman LaRoche, Basel, Switzerland.

9.2.1 Large scale production of yeast cells.

The yeast was produced at the Agriculture Canada Food Research Center at St. Hyacinthe, Quebec using the growth conditions optimized earlier by Sangha (1994).

9.2.2. Feeding of rainbow trout (*Onchorynchus mykiss*).

The feeding trial was carried out at the Aquaculture Unit of the Fisheries and Marine Institute of Memorial University of Newfoundland, St. John's, NF. Four rectangular tanks, each with 320 L volume were set up as a flow circulatory system with a flow rate of approximately 15 L/min. The system was supplied with tap water with a regulated temperature of 10.0°C±1.0. Lighting was provided by fluorescent bulbs on a 10h light:14h dark cycle. Rainbow trout (*Onchorynchus mykiss*) obtained

from the S.C.B Fisheries Ltd., St. Albans, Newfoundland, were transferred to the tanks, each tank containing 20 rainbow trout weighing approximately 175 g per fish. Ten fish were taken as the initial sample (baseline) before the fish were distributed into the various experimental diet groups. Each group was maintained in duplicate. During acclimatization to experimental conditions (2 weeks), the fish were fed with 1% body weight using 3.0 mm non-pigmented pellets (Corey Feed Mills Ltd., Fredericton, New Brunswick). The pigmented feed was steam pelleted at the Fisheries and Marine Institute, Memorial University of Newfoundland. The composition of the feed is given in Table 9.0. Diets for the five groups were formulated as follows:

Diet 1: Diet containing 30% unbroken freeze dried *R. rubra* TP1.

Diet 2: Diet containing 5% unbroken freeze dried *R. rubra* TP1.

Diet 3: Diet containing 100 ppm pigment extract from *R. rubra* TP1

Diet 4: Diet containing 100 ppm commercial canthaxanthin (positive control).

Diet 5: Diet containing no pigment (negative control)

Fish were fed by hand with 2% body weight twice daily for 16 weeks. Weight and length of fish from each tank were measured monthly. Four fish were randomly sampled at four, eight, twelve and sixteen weeks. Prior to sampling, fish were starved for 48 hours and then euthanized with tricaine methylsulfonate (MS-222) at a concentration of 150 mg/L, the jagular vein was then cut and live-bled in ice water. The fish were immediately transferred to the laboratory where they were immediately gutted, blotted dry, filleted and colorimeter reading taken. The remainder of the fish

was stored at -85°C until used for further analysis. All samples were tested for the level, concentration and composition of pigments in the flesh and also subjected to proximate analyses.

9.2.3 Analyses of pigment in fish muscle.

9.2.3.1 Color measurements.

The Hunter color parameters were measured with a Colormet colorimeter (Instrumar Engineering Ltd., St. John's, NF.). Hunter's L*, a* and b* scale was employed (Hunter, 1975), the L* value indicating the lightness of flesh, the a* value indicating redness and the b* value indicating yellowness. The instrument was standardized with B-143 white calibration tile having Hunter L* value of 95±0.2, a* value of -1.0±0.2 and b* value 0.0. Three measurements were made on each sample by rotating the sample at 90°. There were six determinations per group.

9.2.3.2 Total carotenoid content.

Fish sample (30 g including flesh, skin and bones) were homogenized in a Warren blender with 100 mL acetone and 20 g sodium sulfate. The homogenized sample was left in the dark at 4°C for 1 hr after which the sample was centrifuged and the acetone extract decanted and saved. The residue was re-extracted two more times with 50 mL aliquots of acetone. The acetone extracts were then pooled together and then mixed with 50 mL petroleum ether and deionized water in a separatory funnel. The petroleum ether phase was removed and the aqueous phase re-extracted 2 more

times with petroleum ether. The petroleum ether extracts were pooled together and partitioned against water (3X) and then dried over anhydrous sodium sulfate and kept at -20°C overnight to precipitate all sterols present in the extract which, were then removed by centrifugation. The petroleum ether extract was evaporated to dryness with a stream of nitrogen and the residue was dissolved in an appropriate volume of petroleum ether to give an absorbance range of 0.1-0.8nm. The absorbance was recorded at 474nm in a Shimadzu Ultraviolet Visible Recording Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The total content of carotenoids was estimated using an extinction coefficient of 2100 for astaxanthin in petroleum ether according to the equation provided by An *et al.* (1989). The equation has already been described in section 6.2.1.2.

9.2.3.3 HPLC analysis of pigment.

The composition of the pigment in the flesh was determined by extracting the pigment from the fish muscle as indicated in section 9.2.3.2 and then analyzed by HPLC. Samples were dissolved in the mobile phase, 100% methanol and filtered through 0.25 μm membrane. A 10 μL sample solution was injected onto HPLC column for separation and identification of pigments. The HPLC system consisted of a HP 1050 HPLC Series (Hewlett Packard, Palo Alto, CA) coupled with a Phenomenex Partisil ODS column (100 mm x 2 mm i.d. with 5 μm packing size). The sample was eluted at a rate of 0.75 mL/min with a 15-minute linear gradient of 80 to 100% methanol in water. Separation was carried out at ambient temperature

and a HP 1050 Series variable detector was used to monitor the chromatographic effluent. The detection of carotenoids was made at 474 nm. Standards of β-carotene, canthaxanthin and astaxanthin were run in parallel and the carotenoids were identified by comparison of their retention time to those of the standards.

9.2.4 Biochemical analyses.

9.2.4.1 Total lipid and moisture contents.

Total lipid content of the fish was determined using a modified method of Bligh and Dyer (1959). The moisture content of the fish were determined by the A.O.A.C. method (Anonymous, 1990). About 3 g of the sample was dried to a constant weight at 100°C under vacuum and the moisture content determined by weight difference.

9.2.4.2 Total nitrogen and ash contents.

The total nitrogen content of the fish were determined by a modified A.O.A.C micro-Kjeldahl Method (Anonymous, 1990). The crude protein content of the sample was calculated from the percent nitrogen content using the conversion factor (N x 6.25). The A.O.A.C method (Anonymous, 1990) was used to determine the ash content of the fish.

9.2.4.3 Fatty acid analysis.

The fatty acid composition was determined by purifying the extracted lipid described previously. The extracts were stored at -85°C in chloroform to which 0.5% hydroquinone has been added. Aliquots of 1 mL of the lipid extract were placed in 6 mL conical teflon-lined, screw-capped vials and evaporated to dryness under a stream of nitrogen. The residue was transmethylated by the addition of 2 mL 6% sulfuric acid in 99.9 mol % methanol to which 15 mg hydroquinone has been added as an antioxidant. The mixture was incubated at 60°C overnight. Deionized water (1 mL) was then added to the mixture in each vial, thoroughly mixed, and then extracted three times with 1.5 mL of pesticide grade hexane. The hexane layers were removed, combined in a clean tube and washed twice with deionized water. The hexane extracts were evaporated to dryness under a stream of nitrogen. The dried samples were dissolved in Optima™ grade chloroform and analyzed by GC-MS. The GC-MS analysis was performed using a 0.25 mm x 25 m CP-Sil 5CB column (Chrompack, The Netherlands) housed in a HP 5890A gas chromatograph coupled to a HP 5970 mass selective detector (Hewlett Packard, Palo Alto, CA). Spectra were processed using Hewlett Packard ChemStation software (HPG1034C) package. One µL of sample was manually injected into the column and the following temperature program was used. An initial oven temperature of 150°C was maintained for 5 minutes then elevated at a rate of 5°C/min to 250°C and held there for 10 minutes, resulting in a total run time of 35 minutes.

9.2.5 Specific growth rate of fish.

Specific growth rate (SGR) (%) was calculated (Jobling and Wandsvik, 1983) as follows:

$$SGR = \frac{100 \times In(final\ wt) - In(initial\ wt)}{days\ of\ feeding}$$

where In = natural logarithm.

9.2.6 Statistical analysis.

The data were subjected to analysis of variance (ANOVA) and Turkey's studentized Range Test using Statistical Package for Social Sciences version 8.0 (SPSS Inc., 1998).

9.3 Results.

9.3.1 Growth of rainbow trout.

The growth data of rainbow trout during the feeding period are depicted in Tables 9.1 and 9.2. Fish in all experimental groups grew very well with a mortality of only two fish during the feeding period. At the end of the trial, rainbow trout with an initial mean weight of 180-183 g has attained a final mean weight of 397-495 g (Table 9.1). The lowest weight was recorded for fish fed diet containing 30% test yeast (diet 1) and the highest was recorded for fish fed the control diet (diet 5). The highest growth rate of 2.79 was recorded for fish in the control group (diet 5) and the

lowest, 1.94 was recorded for fish fed with diet 1 (Table 9.2). There was no difference in the length of fish recorded for fish in all groups. The specific growth rate of rainbow trout was 0.71%/day (diet 1) to 0.89% (diet 5) (Table 9.2). The maximum specific growth rate of 0.93 was recorded for fish fed with diet 5 during the 8th week of feeding but at the end of the feeding period, this value has dropped down to 0.89. Fish fed with diet 1 had the least specific growth rate. After 16 weeks of feeding, the maximum percentage weight gain of 171 was observed for the control group and 120 for fish fed diet 1. The other groups have values higher than that of fish fed with diet 1 but lower than those of fish fed with diet 5 (Table 9.2).

9.3.2 Pigmentation of fish muscle.

9.3.2.1 Measurement of different color parameters.

Tables 9.3-9.6 summarize values obtained for Hunter a* (indicating redness of flesh), b* (indicating yellowness of flesh) and L* (indicating lightness of flesh) of fish fed various diets over a 16 week period. The a* values of diet 1 and 4 fed fish showed a steady increase with increase in feeding time whereas those of diets 2 and 3 increased up to 8 weeks and declined slightly thereafter. The Hunter a* value of fish fed diet 5, on the other hand decreased steadily with an increase in feeding time. The b* values for fish fed with diets 1, 2 and 3 showed a significant increases up to week 8 ($P<0.05$) and decrease slightly from week 12 onwards. There was however, a significant decrease ($P<0.05$) in the b* value for fish fed diet 5 during the whole feeding period whereas the b* value of fish fed with diet 4 showed a steady increase

from the beginning to the end of the feeding period. The L* value showed an increase in value for fish in all groups except diet 1 which increased up to week 12 and then decreased thereafter.

The total color change between weeks 0 and 16 was highest for the fish fed diet 4 (canthaxanthin), followed by fish fed with diets 1, 2 and 3. Diet 5 fed fish on the other hand showed a substantial decrease in pigmentation during the entire period of feeding.

9.3.2.2 Total pigment concentration.

The total carotenoid content expressed as astaxanthin (μg/g flesh on dry weight basis) of the rainbow trout after 16 weeks on various diets are depicted in Tables 9.7-9.10. With the exception of fish in the negative control group (diet 5) that had a substantial decrease in pigment concentration, the total carotenoid content increased for fish fed with all other diets throughout the feeding period. At the end of the feeding period, the total carotenoid concentration had increased significantly ($P<0.05$) from an initial value of 2.02 μg/g to 4.46-12.09 μg/g tissue (4.46-12.09 mg/kg), on a dry weight basis. This exceeds the 1-3 mg/kg level reported for rainbow trout as sufficient for adequate visual color impression (Torrissen *et al.*, 1989). The highest concentration, 12.09 μg/g tissue (dry weight basis), was recorded for the fish fed diet containing canthaxanthin, (diet 4), followed by diet 1, (7.28 μg/g), and then diets 2 and 3, (4.35 and 4.46 μg/g tissue, respectively). Fish fed diets 2 and 3 seemed to have had a drop in the total carotenoid content from 5.79 and 5.83 μg/g,

respectively, at the end of the 12th week period to 4.35 and 4.46, respectively, at the end of the feeding trial

9.3.2.3 Composition of pigment in fish muscle.

Table 9.11 depicts the percentage composition of the various carotenoids obtained by HPLC analysis of the pigment extracted from fish within the various experimental groups at the end of the feeding trial. The major pigment found in fish fed the various diets was canthaxanthin which had a percentage range of 78-80%, followed by astaxanthin which had a range of 9-13% and then other pigments that could not be identified.

9.3.3 Chemical composition of fish muscle.

9.3.3.1 Total lipid, protein, moisture and ash contents of fish muscle.

The values for the total lipid content, ash and crude protein of fish in all groups are given in Table 9.12-9.15. The moisture content is given in Table 9.16. The crude protein of fish in all groups increased significantly ($P<0.05$) as fish grew but the mineral content remained relatively unchanged over the experimental period. At the end of 16 weeks of feeding, the crude protein content of fish in all groups has increased significantly ($P<0.05$) to about 8-11% with the highest increase occurring in fish in group 2. However, there were no significant ($P<0.05$) differences in the values recorded for fish from all groups.

The lipid composition of fish from all groups also increased significantly ($P<0.05$) in a pattern similar to those of the crude protein content. The highest lipid content was recorded for fish within groups 2 and 5 (5.21 and 5.26 %, respectively) and the lowest for fish in group 3 (3.76%). Fish fed with diet 1 had total lipid content of 4.23 at the end of the experiment.

9.3.3.2 Fatty acid composition.

The fatty acid compositions of the fish in all groups are depicted in Tables 9.17-9.20. It can be inferred from these tables that the level of fatty acid in the flesh of fish in all groups remained relatively constant during the first 4 weeks of feeding. After 8 weeks, however, the levels of and poly-unsaturated (PUFA) fatty acids decreased slightly for fish within groups 1, 3, 4 and 5 whereas the level of saturated fatty acid (SFA) and mono-unsaturated fatty acid (MUFA) increased slightly. Fish within group 2 had the level of SFA and PUFA remained relatively unchanged. At the end of week 16 the levels of PUFA and SFA in fish in all groups except group 1 remained relatively unchanged. Fish fed with diet 1 had a decrease in the levels of SFA and MUFA but a 6% increase in the levels of PUFA.

9.4 Discussion.

9.4.1 Growth of rainbow trout fed various diets.

The weight and length development as well as the specific growth rate of rainbow trout fed various diets are depicted in Table 9.1 and 9.2, respectively. The length development did not reveal much difference, however, the specific growth rate, the growth rate and % weight gain revealed distinct differences between groups in all these characteristics. In group 1 (fish fed with diet containing 30% *R. rubra* TP1), these three characteristics were the lowest whereas fish within group five (negative control group) have the highest values for these characteristics, followed by fish within groups 4, 3 and then 2. These results were surprising because red yeast is reported to contain more energy than Brewer's yeast due to differences in lipid content, 17 and 4.0% (w/w) total lipid for red and Brewer's yeast, respectively (Gentles and Haard, 1990; 1991). Furthermore, dietary lipids have been reported to have a sparing effect on proteins (Watanabe, 1977; Takeuchi *et al.*, 1978), allowing utilization of proteins for growth rather than for energy. It was therefore expected that fish within group 1 would do better than those within groups 4 and 5 (contained 30% Brewer's yeast). However, reports concerning which of these two yeasts (pigmented yeast and Brewer's yeast) promotes better growth in fish are mixed. Gentles and Haard (1990; 1991) fed sexually matured rainbow trout with a diet containing 15% *P. rhodozyma* or 15% Baker's yeast and reported growth rates of 6.9 and 2.4 g/week, respectively. However, because of the large variation in weight within treatments and small number of fish employed in their study, these authors

concluded that their results were not significantly different ($P< 0.05$). Laine and Gyllenberg (1969), on the other hand, fed rainbow trout with *R. sanneii* and reported that fish fed with diet containing red yeast had the lowest average weight and length whereas fish fed diet with no yeast had the highest average weight and length. They concluded that feeding fish with diet containing high proportions of yeast is not appropriate. Similarly, Haard (1992) reported that fish fed diets containing 5% intact *P. rhodozyma* or 15% milled yeast did not differ significantly in average weight gain from fish fed the control diet. However, he observed that fish reared on diets containing 10% or 15% intact yeast, and 5% or 10% milled yeast grew at a slightly lower rate than those fed control diet without added yeast. Haard (1992) therefore concluded that feeding rainbow trout a reference diet containing 5, 10 or 15% *P. rhodozyma* at 1.3 % body weight does not consistently result in weight gain when compared to the control diet with no yeast. In a fish feeding trial using diets supplemented with *P. rhodozyma*, Johnson *et al.* (1980) showed that rainbow trout fed diet supplemented with mechanically broken yeast had a greater increase in weight than fish fed diets containing intact yeast or the base ration without supplements. In this study, the lower growth rate exhibited by fish fed with diet containing *R. rubra* TP1 may be attributed to the poor feeding habits exhibited by fish fed diet supplemented with red yeast. During the feeding trial, it was observed that fish within groups 1 and 2 consumed only a fraction of their daily ration but those within group 2 consumed more than fish within group 1. The low intake of food may have contributed to the lower growth rate recorded for fish in these two groups. The

possible explanation for this observation is that the test yeast was produced in a medium containing molasses and Brewer's wort, after harvesting we could not get rid of all the media since the centrifuge used in the harvesting produced only a slurry of the cells and not pellets. As a result the cells were freeze dried in a suspension of the medium which imparted their odor to the dried product and hence to the fish diet. It may be this odor that may have affected the feeding of the fish. Hence, the low growth rate observed in fish fed with diets containing the test yeast in this study may be related to feed palatability rather than to efficiency of feed assimilation or conversions. It is therefore suggested that in any future studies efforts should me made to rid the cells off all traces of media and if possible washed with water before freeze-drying.

It was noted that in the red yeast-fed groups 1, 2 and 3 not a single fish died during the experiment. Mortality in groups 4 and 5 were one specimen each. The fish that died within group 5 may have succumbed to the MS-222 because it could not recover after it has been anesthetized.

9.4.2 Pigmentation of fish muscle.

The pigment extracted from the fish flesh was subjected to HPLC analysis and the results indicated the presence of mainly canthaxanthin and astaxanthin in the flesh of fish fed diets 1-3. The presence of these carotenoids in the fish muscle may seem remarkable since the diet contains neither of these pigments, and the general consensus is that rainbow trout cannot synthesize or transform other carotenoids into

canthaxanthin and astaxanthin (Torrissen *et al.*, 1989). It should be noted that the fish were fed on diet containing canthaxanthin at the hatchery before they were acquired for this feeding trial. Analysis of the fish at the beginning of the experiment showed only traces of astaxanthin and canthaxanthin. However, it was expected that over the period of the experiment these pigments in fish within groups 1-3 may have disappeared since their diets contain none of these pigments. Contrary to this expectation, it was observed that the level of astaxanthin and canthaxanthin increased with increase in the feeding period. This indicates that rainbow trout may have converted the pigment present in the yeast into astaxanthin and canthaxanthin. *R. rubra* TP1 contains mainly β-carotene, torulene and torularhodin and β-carotene has been postulated to be transformed into astaxanthin via canthaxanthin in fish (Johnson *et al.*, 1977; Davies *et al.*, 1965; Herring, 1968). The experimental proof for the conversion of one carotenoid to another in fish has been obtained by tracer experiments involving the various carotenoids. Hsu *et al.* (1972) fed goldfish with [^{14}C]lutein for 4 days after which the fish were returned to a regular diet. Analysis of the pigments from these fish showed that 63% of the label was in astaxanthin, 5% in α-doradexanthin, and 0.8 % β-doradexanthin. In similar experiments, Hata and Hata (1972a; 1972b) fed [^{14}C]zeaxanthin to goldfish and reported significant conversion of zeaxanthin to astaxanthin via 4-keto-zeaxanthin. In rainbow trout, Savolainen and Gyllenberg (1970) reported that when fed diets containing *Rhodotorula sanneii*, the fish flesh contained high levels of canthaxanthin and lutein. These authors proposed that lutein, β-carotene and canthaxanthin may be synthesized from other carotenoid

precursors. They did not, however, report the presence of *R. sanneii* pigments in the flesh of the rainbow trout. Schiedt *et al.* (1985) also reported that astaxanthin is reduced to zeaxanthin in rainbow trout and Atlantic salmon. They further reported the transformation of astaxanthin and zeaxanthin to vitamins A1 and A2 in vitamin A depleted rainbow trout. These studies clearly show that fish is able to transform one form of carotenoid into another. It should not be surprising therefore that canthaxanthin and astaxanthin were found in fish fed diet containing *R. rubra* TP1. In all likelihood, the fish may have converted the pigments present in the test yeast into astaxanthin and canthaxanthin, hence the high levels of these pigments in the fish muscle.

The concentration range of pigmentation in the muscle of rainbow trout fed with 30% test yeast-supplemented and canthaxanthin-supplemented diets in this study (7.28 and 12.09 mg/kg, respectively) were comparable to those reported by other workers. In rainbow trout, Seurman *et al.* (1978) reported astaxanthin concentration of the raw muscle as between 0.4 and 1.6 mg/kg after feeding them with a diet containing red crab. Similarly, Chen *et al.* (1984) reported an astaxanthin concentration of 5.2 to 9.8 mg/kg in rainbow trout that were fed diets containing astaxanthin-enriched soy-oil from crawfish. Storebakken *et al.* (1986) reported pigment concentration of 8.8-19.0 mg/kg from the flesh of rainbow trout fed diet containing synthetic canthaxanthin. The level of carotenoids in the flesh of fish which is regarded as sufficient for visual color impression in farmed salmonid is 4 mg/kg (Torrissen *et al.*, 1989) and the pigment concentration obtained with the yeast-

supplemented diets exceeded this value. Fish fed with diet supplemented with pigment extract from yeast had only about half the concentration observed in fish fed with diet supplemented with whole yeast. Even though precautions were taken to minimize the destruction of pigment by light and air during the extraction process, it is possible that destruction of pigments may have occurred during processing leading to low uptake by fish. Overall, the best pigmentation in this study was obtained from fish fed diets containing commercial canthaxanthin.

The fact that the fish were able to take up pigments and nutrients from the intact yeast may be important economically since milling of yeast can substantially increase the cost of feed production. Johnson *et al.* (1980) reported that fish fed whole and intact *P. rhodozyma* cells were unable to take up pigments from the cells. They therefore concluded that modification of the cell wall of *P. rhodozyma* was necessary before the yeast can be used as a dietary pigment source for salmonids. In this study it was not necessary to modify the cell wall since the yeast cell wall did not serve as a barrier or restricted pigment and possibly nutrient availability to fish.

9.4.3 Composition of fish muscle.

It was observed in this study that changes in the body composition of fish were primarily in their moisture, protein and lipid contents and weight gain. Tidwel and Robinette (1990) reported similar findings for channel catfish and concluded that the gain in weight by all fish may be due to the storage of additional energy as fat. pigmented and Brewer's yeasts contain 17 and 4.0% (w/w) total lipids, respectively

(Gentles and Haard, 1990; 1991). Inclusion of 30% (w/w) of these yeasts in the diets of the fish may have resulted in an increase in the overall lipid content of the fish diets which might have in turn resulted in an increase in the fat content in the muscle. This assumption is supported by studies done by Ogino *et al.* (1976) and Castledine and Buckley (1980) who reported that in rainbow trout, lipid composition of the body is directly related to dietary lipid levels. Similar observations have also been made in studies with turbot (Bromley, 1980), Arctic charr (Tabachek, 1986) and channel catfish (Garling and Wilson, 1976). In spite of the increase in the level of lipid over the experimental period, the total lipid obtained in this study for fish in all groups was lower than that reported by other workers. For example, Iwamoto *et al.* (1990) reported total lipid contents of 7.8 and 8.9 for pen-reared coho salmon strains BY 1980 and BY 1981, respectively. In wild coho salmon, on the other hand, they reported a total lipid level of only 2.2% and attributed this low lipid level to the mobilization of lipids prior to maturation. Similarly, Hörstgen-Schwark *et al.* (1986) reported low lipid levels in pen-reared rainbow trout and attributed the low level of lipid in the fish to the initiation of gonad maturation. In fish, most of the stored lipids in the somatic tissues are mobilized to the gonads during sexual maturation resulting in a decrease in the lipid content of the muscle (Dygert, 1990). This decrease in the lipid content in the muscle is normally associated with elevated levels of moisture (Love, 1988). It was observed in the present study that fish in all groups have high moisture content that continued to increase over the duration of the experiment. The high moisture levels may have been a consequence of the low lipid levels since

moisture levels has been reported to be directly related to lipid levels in the muscle, a decrease in the content of one results in an increase in the levels of the other (Reinitz, 1983).

With the exception of fish fed diet 1 that had substantial increase in the quantity of PUFA and MUFA, the fatty acid composition of the fish in all groups remained relatively unchanged over the duration of the experiment. Similar results have been reported by Yu *et al.* (1977) who indicated that the unsaturated fatty acid composition of fish remained fairly constant regardless of the dietary lipid content. Stickney and Andrews (1971) in a feeding experiment with catfish, also indicated that the fatty acid content of two groups of fish, one group fed diet containing 41% saturated fatty acid and the other 25% saturated fatty acid, remained the same after 10 weeks of feeding. Thus it seems that fish are able regulate and maintain a proper level of saturation in their body lipids (Watanabe, 1982).

In conclusion, the present study has clearly demonstrated that *R. rubra* TP1 was able to impart adequate amounts of pigmentation and coloration to the flesh of rainbow trout. However, the best pigmentation and color were obtained with fish fed with diet containing commercial canthaxanthin. Fish in all groups grew very well and had high levels of crude protein compared to values determined at the beginning of the feeding trial. Lipid levels in all fish fed various diets were generally low, however there were significant ($P<0.05$) increases in the lipid content in all groups when compared with values determined at the beginning of the feeding trial. With the exception of fish within group 1, the fatty acid composition of fish in all other

groups remained relatively unchanged. The new strain, *R. rubra* promises to be a good source of pigment for rainbow trout.

Table 9.0 Composition of feed used in feeding trial (% dry weight).

Ingredient	Diet 1 30% *R. rubra* TP1	Diet 2 5% *R. rubra* TP1	Diet 3[1] Yeast Pigment	Diet 4 Canthaxanthin	Diet 5 No Pigment
Fish Meal	33.10	33.17	33.17	33.17	33.17
Soy Protein Isolate	2.63	2.63	2.63	2.63	2.63
Corn glutein	2.95	2.95	2.95	2.95	2.95
Wheat Flour	14.00	14.00	14.00	14.00	14.00
Vitamin Mix	1.50	1.50	1.50	1.50	1.50
Mineral Mix	1.00	1.00	1.00	1.00	1.00
Guar Gum	2.00	2.00	2.00	2.00	2.00
Fish Oil	12.74	12.74	12.74	12.74	12.74
Brewers Yeast	0.00	24.95	30.00	30.00	30.00
Canthaxanthin	0.00	0.00	0.00	0.01	0.00
Rhodotorula rubra	30.10	5.05	0.01	0.00	0.00
Total	100	100	100	100	100

[1]Note: 1.9 g of pigment extract from the experimental yeast was added to diet 3 to give a pigment concentration of 100 ppm.
Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment).

Table 9.1 The growth data of rainbow trout fed diets containing pigment from various sources over 16 week period.

Feeding Period (weeks)		Diet				
	Growth Data	1	2	3	4	5
0	Body Wt.(g)[1]	179.95	174.15	178.00	185.00	182.50
	Length (cm)	25.56±1.71	25.38±2.01	25.33±2.01	25.04±0.83	25.44±1.54
4	Body Wt.(g)[1]	211.80	222.82	220.43	227.77	228.01
	Length (cm)	26.24±0.56	26.51±0.32	26.54±0.35	26.86±0.30	26.32±0.57
8	Body Wt.(g)[1]	271.22	284.79	285.30	296.74	308.61
	Length (cm)	27.45±1.57	27.60±1.89	28.00±1.60	28.33±1.34	29.10±0.9
12	Body Wt.(g)[1]	331.30	357.57	353.51	385.37	397.18
	Length (cm)	29.44±1.12	30.16±1.59	29.96±1.59	31.33±1.53	30.6±1.75
16	Body Wt.(g)[1]	396.77	433.02	454.17	471.08	495.10
	Length (cm)	31.13±2.78	32.06±1.59	32.63±1.26	32.81±1.92	33.21±1.90

[1]The fish in each tank were weighted in bulk. [2]Results are mean of 6 determinations ± standard deviation.

Table 9.2 The growth rate, specific growth rate and % weight gain of rainbow trout fed five different diets.

Feeding Period (Weeks)	Growth Data	Diet 1	2	3	4	5
4	Weight gain (%)	17.70	27.99	23.84	23.12	24.94
	SGR (% GR/day)	0.59	0.88	0.77	0.74	0.78
	Growth rate	1.14	1.74	1.52	1.53	1.63
8	Weight gain (%)	50.72	63.53	60.28	60.40	69.10
	SGR (% GR/day)	0.74	0.88	0.85	0.84	0.93
	Growth rate	1.63	1.98	1.92	1.99	2.25
12	Weight gain (%)	84.11	105.32	98.60	108.31	117.63
	SGR (% GR/day)	0.73	0.86	0.82	0.87	0.92
	Growth rate	1.80	2.18	2.09	2.38	2.56
16	Weight gain (%)	120.49	148.65	155.15	154.64	171.29
	SGR (% GR/day)	0.71	0.81	0.84	0.84	0.89
	Growth rate	1.94	2.31	2.47	2.55	2.79

Note: % Weight gain = (final weight - initial weight) ÷initial weight x 100. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment).

Table 9.3 Significant levels of differences in the lightness (Hunter L*), redness (Hunter a*) and yellowness (Hunter b*) of rainbow trout fed various diets for 4 weeks[1]

Diet	Hunter L* Value	Hunter a* Value	Hunter b* value
Baseline	38.80±2.70^{a}	6.3±2.40^{a}	13.30±2.10^{a}
1	37.90±5.10^{a}	8.20±1.70^{a}	14.20±1.20^{a}
2	42.30±4.50^{a}	8.10±3.40^{a}	14.40±1.60^{a}
3	40.80±5.10^{a}	7.30±2.30^{a}	13.80±1.50^{a}
4	37.50±5.10^{a}	10.20±2.20^{b}	14.90±1.70^{a}
5	37.90±6.70^{a}	7.00±1.30^{a}	14.6±1.60^{a}

[1]Results are mean values of 18 determinations ±standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment).

Table 9.4 Significant levels of differences in the lightness (Hunter L*), redness (Hunter a*) and yellowness (Hunter b*) of rainbow trout fed various diets for 8 weeks[1]

Diet	Hunter L* Value	Hunter a* Value	Hunter b* value
Baseline	38.80±2.70^{a}	6.3±2.40^{a}	13.30±2.10^{a}
1	43.90±6.90^{b,c}	10.54±3.28^{b}	17.72±1.90^{b}
2	46.90±4.10^{c}	10.13±2.90^{b}	18.50±1.60^{b}
3	46.50±4.5^{c}	9.54±2.92^{b}	17.60±1.50^{b}
4	42.60±6.70^{a,b}	14.63±3.24^{c}	19.00±2.10^{b}
5	47.60±3.30^{c}	9.27±3.59^{b}	17.6±2.4^{b}

[1]Results are mean values of 18 determinations ± standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment).

Table 9.5 Significant levels of differences in the lightness (Hunter L*), redness (Hunter a*) and yellowness (Hunter b*) of rainbow trout fed various diets for 12 weeks[1]

Diet	Hunter L* Value	Hunter a* Value	Hunter b* value
Baseline	38.80±2.70^{a}	6.3±2.40^{a}	13.30±2.10^{a}
1	43.98±4.67^{b}	10.71±1.69^{b}	14.46±1.29^{a}
2	44.57±4.87^{b}	8.54±3.48^{c}	13.41±1.51^{a}
3	42.69±4.78^{b}	9.49±3.19b^{b,c}	14.02±1.79^{a}
4	42.96±3.84^{b}	15.4±2.44^{d}	18.92±1.97^{b}
5	50.26±4.50^{c}	4.16±0.96^{e}	8.36±1.07^{c}

[1]Results are mean values of 18 determinations ± standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment).

Table 9.6 Significant levels of differences in the lightness (Hunter L*), redness (Hunter a*) and yellowness (Hunter b*) of rainbow trout fed various diets for 16 weeks[1]

Diet	Hunter L* Value	Hunter a* Value	Hunter b* value
Baseline	38.80±2.70^{a}	6.3±2.40^{a}	13.30±2.10^{a}
1	39.16±3.93^{a}	11.01±1.24^{b}	14.72±2.08^{b}
2	45.54±3.72^{b}	7.56±1.52^{a}	14.90±1.76^{b}
3	46.76±5.19^{b}	6.83±1.96^{a}	14.36±1.68^{b}
4	41.89±3.97^{a}	15.04±1.46^{c}	18.54±2.26^{c}
5	52.17±5.11^{c}	2.77±1.03^{d}	8.01±1.47^{d}

[1]Results are mean values of 18 determinations ±standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigment)

Table 9.7 Total carotenoid content (μg.g^{-1} tissue) of the flesh of rainbow trout after 4 weeks of feeding on experimental diets[1]

Diet	Dry Weight	Wet Weight
Baseline (At Start)	2.02±1.14^{a}	0.44±0.25^{a}
1	6.75±3.11^{b,d}	1.48±0.68^{b,d}
2	6.58±2.14^{b,d}	1.44±0.47^{b,d}
3	4.52±2.89^{a,b}	0.99±0.63^{a,b}
4	8.78±0.56^{d}	1.68±0.26^{d}
5	2.74±0.83^{a}	0.57±0.13^{a}

[1]Results are mean values of 6 determinations ± standard deviation. Values within the same column with different superscripts are significantly different (P<0.05) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.8 Total carotenoid content (μg.g^{-1} tissue) of the flesh of rainbow trout after 8 weeks of feeding on experimental diets[1]

Diet	Dry Weight	Wet Weight
Baseline (At Start)	2.02±1.14^{a}	0.44±0.25^{a}
1	5.16±2.82^{b,d}	1.79±0.46^{b,d}
2	4.42±2.27^{c,d}	1.45±0.38^{c,d}
3	4.51±2.12^{c}	0.99±0.46^{c}
4	7.69±1.20^{b}	2.03±0.17^{b}
5	1.99±0.83^{a}	0.63±0.29^{a}

[1]Results are mean values of 6 determinations ± standard deviation. Values within the same column with different superscripts are significantly different (P<0.05) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.9 Total carotenoid content ($\mu g.g^{-1}$ tissue) of the flesh of rainbow trout after 12 weeks of feeding on experimental diets[1]

Diet	Dry Weight	Wet Weight
Baseline (At Start)	2.02 ± 1.14^a	0.44 ± 0.25^a
1	6.47 ± 0.51^b	1.75 ± 0.14^b
2	5.79 ± 1.88^b	1.56 ± 0.51^b
3	5.83 ± 1.52^b	1.57 ± 0.41^b
4	10.10 ± 0.61^c	2.60 ± 0.19^c
5	0.63 ± 0.29^a	0.53 ± 0.13^a

[1]Results are mean values of 6 determinations ± standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.10 Total carotenoid content ($\mu g.g^{-1}$ tissue) of the flesh of rainbow trout after 16 weeks of feeding on experimental diets[1]

Diet	Dry Weight[2]	Wet Weight
Baseline (At Start)	2.03 ± 1.14^a	0.44 ± 0.25^a
1	7.28 ± 1.39^b	1.97 ± 0.37^b
2	4.35 ± 0.65^c	1.16 ± 0.19^c
3	4.46 ± 0.77^c	1.20 ± 0.21^c
4	12.09 ± 2.19^d	3.27 ± 0.59^d
5	1.73 ± 0.18^a	0.47 ± 0.05^a

[1]Results are mean values of 6 determinations ± standard deviation. Values within the same column with different superscripts are significantly different ($P<0.05$) from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.11 Percentage composition of carotenoid of fish muscle determined by HPLC at the end of the feeding trial.

Diet	Carotenoid composition (%)		
	Astaxanthin	Canthaxanthin	Others (unidentified)
Baseline	ND	ND	ND
1	13.36	78.63	8.0
2	11.90	77.90	10.20
3	11.20	79.10	9.66
4	9.83	80.10	10.07
5	ND	ND	ND

ND = Not determined.

Table 9.12 The chemical composition of fish fed with 5 different diets for 4 weeks[1].

Diet	Chemical composition (%)		
	Protein[1] (Nx6.25)	Lipid	Ash
Baseline	36.31±1.38 ^{a,b}	2.62±0.15 ^{b}	4.21±0.30 ^{a}
1	38.47±1.72 ^{a}	3.64±0.75 ^{a}	5.11±0.87 ^{a}
2	38.08±1.82 ^{a}	3.73±0.98 ^{a}	4.97±1.03 ^{a}
3	38.33±1.62 ^{a}	3.50±0.37 ^{a}	5.21±0.88 ^{a}
4	36.84±2.90 ^{a}	4.50±0.87 ^{a}	4.89±1.19 ^{a}
5	35.82±1.69 ^{b}	2.20±1.00 ^{b}	4.67±0.90 ^{a}

[1]Results are mean values of 6 determination ± standard deviation. Means within the same column with different superscripts are significantly ($P<0.05$) different from one another.
Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.13 The chemical composition of fish fed with 5 different diets for 8 weeks[1]

Diet	Chemical composition (%)		
	Protein[1] (Nx6.25)	Lipid	Ash
Baseline	36.31±1.38[b]	2.62±0.15[b]	4.21±0.30[a]
1	40.26±3.95[a]	4.01±1.00[a]	5.10±1.36[a]
2	38.93±1.41[c]	4.63±1.34[a]	4.73±1.22[a]
3	42.65±4.85[a]	3.82±0.74[a]	4.76±0.55[a]
4	42.54±6.49[a]	4.02±0.74[a]	4.41±0.58[a]
5	41.75±2.30[a]	4.52±0.75[a]	4.73±1.34[a]

[1]Results are mean values of 6 determination ± standard deviation. Means within the same column with different superscripts are significantly (P<0.05) different from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.14 The chemical composition of fish fed with 5 different diets for 12 weeks[1]

Diet	Chemical composition (%)		
	Protein(Nx6.25)	Lipid	Ash
Baseline	36.31±1.38[b]	2.62±1.09[b]	4.21±0.30[a]
1	46.61±1.79[a]	4.23±0.59[a]	4.69±1.09[a]
2	45.15±1.41[a]	4.50±1.09[a]	4.45±1.49[a]
3	46.77±1.63[a]	4.84±1.05[a]	4.42±0.71[a]
4	45.68±0.58[a]	4.61±1.00[a]	4.47±0.49[a]
5	42.86±1.95[c]	4.53±1.07[a]	4.88±0.98[a]

[1]Results are mean values of 6 determination ± standard deviation. Means within the same column with different superscripts are significantly (P<0.05) different from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.15 The chemical composition of fish fed with 5 different diets for 16 weeks[1].

Diet	Chemical Composition (%)		
	Protein(Nx6.25)	Lipid	Ash
Baseline	36.31±1.38^{b}	2.62±1.09^{c}	4.21±0.30^{a}
1	45.92±2.70^{a}	4.23±0.59^{a,b}	4.16±0.17^{a}
2	47.70±1.55^{a}	5.21±0.70^{b}	4.11±0.30^{a}
3	45.36±3.33^{a}	3.76±0.31^{a}	4.18±0.58^{a}
4	45.96±3.41^{a}	4.42±0.74^{a,b}	4.20±1.03^{a}
5	44.90±0.73^{a}	5.26±1.09^{b}	4.40±0.46^{a}

[1]Results are mean values of 6 determination ± standard deviation. Means within the same column with different superscripts are significantly ($P<0.05$) different from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.16 The moisture content of fish fed different diets over a 16 week period[1].

Moisture content (%)				
Diet	Week 4	Week 8	Week 12	Week 16
Baseline	74.94±0.40^{b}	74.94±0.41^{b}	74.94±0.41^{a}	74.15±1.38^{a,b}
1	68.11±0.71^{a}	76.11±0.27^{a}	74.55±1.66^{a}	74.15±1.08^{a,b}
2	68.08±2.68^{a}	75.87±1.31^{a}	74.74±1.54^{a}	74.45±0.63^{a}
3	67.66±3.44^{a}	73.83±2.56^{b}	75.32±0.34^{a}	74.04±0.22^{a,b}
4	68.25±2.32^{a}	75.97±0.17^{a}	74.40±0.15^{a}	73.08±0.22^{b}
5	74.48±1.31^{b}	75.61±0.16^{a}	71.00±1.22^{b}	73.83±0.82^{b}

[1]Results are mean values of 6 determination ± standard deviation. Means within the same column with different superscripts are significantly ($P<0.05$) different from one another. Diets: 1 = 30% test yeast, 2 = 5% test yeast, 3 = 100 ppm pigment extract from test yeast, 4 = 100 ppm commercial canthaxanthin, 5 = negative control (no pigments).

Table 9.17 The fatty acid profile of fish fed with five different diets for 4 weeks[1].

Fatty Acids	Baseline	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
12:0	2.83±0.22	3.19±0.19	3.32±0.38	2.88±0.41	2.89±0.22	2.96±0.21
14:0	0.31±0.12	0.51±0.07	0.51±0.02	0.36±0.14	0.34±0.10	0.44±0.11
16:1*w*9	19.25±0.65	20.04±0.76	20.75±0.98	19.72±0.83	20.12±0.70	20.11±0.35
18:1	0.83±0.09	0.18±0.01	0.70±0.07	0.60±0.06	0.69±0.03	0.69±0.03
18:3	22.53±0.29	24.74±0.81	23.89±0.54	24.00±0.35	23.60±0.56	23.35±0.52
20:2	-	-	-	-	-	-
20:3*w*3	7.28±0.56	7.55±0.39	7.11±0.66	6.98±0.47	6.96±0.24	7.43±0.42
20:3*w*6	13.55±0.23	13.05±0.62	13.31±0.66	13.39±0.73	13.10±0.59	13.45±0.77
22:0	-	-	-	-	-	-
22:2	19.89±0.49	17.19±1.28	16.84±1.61	18.09±1.55	18.65±1.06	17.12±0.83
22:4	12.59±1.06	11.60±0.35	11.93±0.13	12.29±0.97	12.72±0.76	12.94±0.76
22:5	0.61±0.03	0.88±0.08	0.82±0.08	0.77±0.14	0.59±0.13	0.26±0.10
22:6	0.87±0.03	0.78±0.07	0.77±0.05	0.75±0.08	0.62±0.06	0.70±0.21
∑SFA	3.14	3.7	3.83	3.24	3.23	3.40
∑MUFA	20.08	20.22	21.45	20.81	20.81	20.80
∑PUFA	77.32	75.79	74.27	76.24	76.24	75.25

[1]Values are expressed as mole percentage and are the means ± SD of 6 replicates. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

Table 9.18 The fatty acid profile of fish fed with five different diets for 8 weeks[1]

Fatty Acids	Baseline	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
12:0	2.83±0.73	3.69±0.30	4.10±0.21	3.79±0.35	3.87±0.28	3.97±0.25
14:0	0.31±0.12	0.73±0.12	0.62±0.13	0.58±0.10	0.60±0.09	0.58±0.04
16:1*n*9	19.25±0.65	19.65±0.84	20.42±0.74	20.81±0.72	20.51±0.70	21.31±1.15
18:1	0.83±0.09	0.75±0.03	0.69±0.12	0.53±0.10	0.79±0.18	0.75±0.18
18:3	22.53±0.29	23.43±1.06	23.80±0.84	23.39±1.48	23.87±0.87	24.29±1.07
20:2	-	0.42±0.11	-	0.17±0.03	0.29±0.08	-
20:3*w*3	7.28±0.56	7.07±0.36	7.00±0.45	6.94±0.47	7.13±0.43	7.32±0.39
20:3*w*6	13.55±0.23	11.74±0.28	11.82±0.74	11.31±0.57	11.32±0.66	10.97±0.48
22:0	-	0.67±0.07	0.52±0.07	0.33±0.11	0.49±0.15	0.48±0.14
22:2	19.89±0.49	19.54±1.67	18.55±2.85	19.37±2.32	18.90±2.12	18.15±1.07
22:4	12.59±1.06	10.85±0.51	11.33±0.99	11.78±0.58	10.90±0.98	10.92±0.94
22:5	0.61±0.03	0.94±0.31	0.74±0.09	0.71±0.19	0.49±0.14	0.30±0.05
22:6	0.87±0.03	0.77±0.04	0.66±0.09	0.55±0.08	0.40±0.11	0.28±0.05
∑SFA	3.14	5.09	3.12	4.70	4.96	5.03
∑MUFA	20.08	20.40	21.74	21.34	21.30	22.06
∑PUFA	77.32	74.74	74.72	74.22	73.30	72.23

[1]Values are expressed as mole percentage and are the means ± SD of 6 replicates. SFA= Saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

Table 9.19 The fatty acid profile of fish fed with five different diets for 12 weeks[1].

Fatty Acids	Baseline	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
12:0	2.83±0.73	3.29±0.20	2.95±0.11	2.99±0.35	3.03±0.33	3.54±0.27
14:0	0.31±0.12	0.43±0.15	0.47±0.14	0.49±0.09	0.48±0.10	0.52±0.07
16:1*n*9	19.25±0.65	18.94±0.81	19.12±1.05	18.57±0.65	20.19±1.13	20.79±1.02
18:1	0.83±0.09	0.35±0.05	0.78±0.08	0.62±0.24	0.71±0.02	0.72±0.03
18:3	22.53±0.29	22.87±0.81	23.05±1.83	22.57±0.73	22.85±0.58	24.25±1.19
20:2	-		-	0.21±0.04	0.30±0.10	0.15±0.06
20:3*w*3	7.28±0.56	7.20±0.32	6.97±0.48	6.98±0.26	6.96±0.52	7.31±0.47
20:3*w*6	13.55±0.23	11.47±0.33	11.39±0.38	11.83±0.92	11.47±0.67	11.77±0.41
22:0	-	0.34±0.02	0.32±0.01	0.43±0.09	0.54±0.31	0.42±0.11
22:2	19.89±0.49	22.66±2.58	21.96±1.19	22.43±2.05	20.86±1.66	18.13±1.84
22:4	12.59±1.06	11.10±0.83	11.62±0.77	11.59±1.00	10.86±0.79	10.08±0.86
22:5	0.61±0.03	0.96±0.16	0.86±0.10	0.74±0.11	0.71±0.11	0.73±0.16
22:6	0.87±0.03	0.87±0.09	0.91±0.09	0.88±0.06	0.79±0.03	0.69±0.13
∑SFA	3.14	4.06	3.74	3.91	4.05	4.48
∑MUFA	20.08	19.29	19.90	19.40	20.90	21.93
∑PUFA	77.32	77.13	76.76	77.02	74.80	72.96

[1]Values are expressed as mole percentage and are the means ± SD of 6 replicates. SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

Table 9.20 The fatty acid profile of fish fed with five different diets for 16 weeks[1].

Fatty Acids	Baseline	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
12:0	2.83±0.73	1.41±0.30	2.61±0.43	2.19±0.39	2.19±0.20	3.39±0.54
14:0	0.31±0.12	0.30±0.07	0.39±0.08	0.26±0.07	0.27±0.17	0.24±0.04
16:1*n*9	19.25±0.65	13.25±0.49	18.29±1.50	18.20±1.49	18.47±1.83	19.10±1.58
18:1	0.83±0.09	0.58±0.13	0.65±0.09	0.45±0.08	0.47±0.06	0.51±0.05
18:3	22.53±0.29	24.37±0.78	24.27±1.01	24.45±1.25	22.55±0.97	25.75±1.39
20:2	-		-	-	-	
20:3*w*3	7.28±0.56	8.13±0.52	6.61±0.81	6.74±0.45	6.50±0.27	5.93±0.72
20:3*w*6	13.55±0.23	13.36±0.86	13.42±0.60	12.89±0.38	13.95±0.88	13.70±0.64
22:0	-	0.81±0.22	0.52±0.19	-	-	
22:2	19.89±0.49	22.09±1.09	18.80±2.80	20.46±2.22	19.04±1.63	17.72±2.15
22:4	12.59±1.06	13.47±1.29	13.09±0.98	12.92±1.27	13.79±0.77	13.93±1.02
22:5	0.61±0.03	1.00±0.14	0.92±0.20	0.69±0.16	0.51±0.16	0.54±0.15
22:6	0.87±0.03	0.86±0.07	0.62±0.10	0.52±0.13	0.54±0.10	0.44±0.21
∑SFA	3.14	2.52	3.52	2.45	3.08	3.63
∑MUFA	20.08	13.83	18.94	18.65	18.94	19.61
∑PUFA	77.32	83.28	77.73	78.67	77.88	78.01

Values are expressed as mole percentage and are the means ± SD of 6 replicates. SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

A carotenoid producing yeast was isolated from yogurt and was subsequently identified as *Rhodotorula rubra* TP1 (Hari *et al.*, 1992). Studies were conducted on its morphology, sexuality, growth kinetics and possible use in the aquaculture industry as feed supplement. At the time of its isolation, it was reported that the yeast produced structures that resemble ascospores; however, other biochemical and physiological analyses suggested a *Rhodotorula* affinity (Hari *et al.*, 1992) making the phylogenetic affinity of the new isolate ambiguous. To exploit the economic potential of this isolate, it was imperative that its phylogenetic affinity be clarified and also the pigments produced by the new isolate be characterized. The present study therefore employed biochemical, physiological and molecular methods to clarify the identity of the new isolate.

To confirm the production of ascospores or any structures resembling them, the organism was cultivated on various sporulation media used in a previous study by Hari *et al.* (1992). Despite repeated trials over an extended period of time, no structures resembling ascospores were observed. Growth on various carbon sources revealed that the new isolate and *R. rubra* ATCC 9449 had identical utilization pattern for most of the carbon sources. The new isolate and *R. rubra* ATCC 9449 were also found to be nitrate negative and had identical utilization patterns for raffinose, melibiose, maltose, raffinose and erythritol, carbon sources that are widely

used in the identification of *Rhodotorula rubra* (Fell *et al.*, 1984). Thus, in terms of the nutritional and biochemical characteristics, the new isolate demonstrated the best homology with *R. rubra* ATCC 9449 than any of the yeast isolates used as controls.

Analysis of the cell wall carbohydrates revealed the presence of mainly β-(1→3) and β-(1→4) mannans which have been reported to be unique to the genus *Rhodotorula* (Spencer and Gorin, 1970). The monosaccharide content of the cell wall was also found to consist of mannose, glucose, galactose, fucose and rhamnose which is consistent with the cell wall monosaccharides of other *R. rubra* strains (Sugiyama *et al.*, 1985; Weijman and Rodrigues de Miranda, 1988b).

Cellulose acetate electrophoresis was used to separate isozymes of 8 enzymes in the new isolate and eight other yeast isolates used as controls. Cellulose acetate electrophoresis of isozymes was very effective in discriminating between the new strain and the eight other yeasts isolates. Based on the comparisons of the relative electrophoretic mobilities (μ) of the isozymes, seven enzymes (isomerase dehydrogenase, 6-phosphogluconate dehydrogenase, hexokinase, malate dehydrogenase, mannose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase) yielded zymograms that were identical for both the new isolate and *R. rubra* ATCC 9449. Only one enzyme, phosphoglucomutase had banding patterns that were different for the new isolate and *R. rubra* ATCC 9449. The overall similarities between the individual isolates were calculated from the electrophoretic mobilities. It was observed that the new isolate and *R. rubra* ATCC 9449 had the highest similarity index. Thus, based on the

isozyme analysis, the new isolate and *R. rubra* ATCC 9449 could not be separated from each other. The new isolate may therefore be considered as a variant strain of *Rhodotorula rubra*. The results also suggest that cellulose acetate electrophoresis could be an important taxonomic tool for identification of isolates of yeast. Its relatively low cost and speed make it a particularly attractive procedure.

The cell physiology of the new yeast isolate was also found to be identical to that of *R. rubra* ATCC 9449. Analysis of the cellular long-chain fatty acid by gas chromatography revealed that the major fatty acids found in both isolates were 18:0, 18:1, 18:2, 18:3, 16:1 and 16:0 and both have high quantities of C18:1 and C16:1 (70 and 13%, respectively). The concentrations of these fatty acids obtained in this study are consistent with the concentrations of the same fatty acids reported for other strains of *R. rubra* by other workers. For example, Ratledge and Evans (1987) and Zuyaginsteva *et al.* (1975) reported that the concentration of C18:1 in two strains of *R. rubra* were in the range of 57 to 81%. Similarly, Blignaut *et al.* (1996) reported that *R. rubra* had a C18:1 concentration of 68.8%. Ratledge (1982) has also reported similar results for other *Rhodotorula* species.

The determination of cellular fatty acid composition of other yeast isolates by gas-liquid chromatography as means of identification had been studied by numerous workers (van der Westhuizen *et al.*, 1991; Blignaut *et al.*, 1996; Augustyn *et al.*, 1992; Miller *et al.*, 1989; Marumo and Aoki, 1990). Multivariate statistical methods have also been useful as an objective identification procedure for differentiating between cellular fatty acid compositions, aiding in the identification of numerous

clinical microorganisms (Marumo and Aoki, 1990). The fatty acid data were also subjected to stepwise discriminant analysis, 'jackknifing' and canonical variate analyses. The results of the canonical variate and the stepwise discriminant analyses together with the 'jackknifing' procedure reported in this study clearly distinguished the new isolate and *R. rubra* ATCC 9449 from the other isolates, however, the two could not be separated from each other. These two isolates clustered together in all the applications employed in the canonical variate analysis confirming the value of this phenotypic (CFA) characteristic in the identification of yeast. In addition the high concentrations of the C18:1 fatty acid found in these two isolates were a major contributing factor in the distinguishing between them and the other isolates. Fatty acid analysis as an identification tool, has been used extensively in yeast taxonomy. Moss *et al.* (1982) used the cellular fatty acid composition of 51 isolates of various species of yeast to differentiate them into four distinct groups. Similarly, Augustyn *et al.* (1991; 1992; 1989) and Gangopadhyay *et al.* (1979) used the cellular fatty acid profile in conjunction with statistical analysis to differentiate between genera, species and strains of various types of yeast. Data from these studies show that cellular fatty acids provide useful information for rapidly distinguishing between both closely and distantly related yeast isolates. Cellular fatty acid analysis is rapid, easily applied and reliable, and can complement other methods used in yeast identification.

The strongest evidence for the phylogenetic affinity of the new isolate came from analysis of the partial base sequences of portions of the 18S rDNA gene and the internal transcribed spacer (ITS). Comparison of sequences of the 18S rDNA and

the ITS revealed that the new isolate had the highest percent sequence similarity of 100 and 93 with *R. rubra* and evolutionary distance estimates of 0.000 and 0.041 for the 18S rDNA and the ITS sequences, respectively. The percent similarities between the new isolate and *R. rubra* ATCC 9449 were the highest when compared with those between the new isolate and all the other isolates used as controls. The sequence data were subjected to Maximum Parsimony and bootstrap majority consensus tree analysis of 100 replicates. The resulting phylogenetic tree grouping isolates with most similar sequences revealed that the new isolate and *R. rubra* belongs to a single clade. This is the first genetic evidence that the two belongs to the same species, therefore the new isolate should be considered a variant strain of *R. rubra*. 18S rDNA gene and the ITS sequences alone, and in combination with other ribosomal genes, are versatile molecular probes that can be used to investigate phylogenetic relationships among yeast genera and species. It has been used in the taxonomy of several genera and species of yeast including *Rhodotorula graminis* (Fell *et al.*, 1999; 1998), *Taphrina* and *Saitoella* (Nishida and Sugiyama, 1993), *Pichia* species (Yamada *et al.*, 1994c), *Rhodosporidium dacryoidum* (Yamada *et al.*, 1994a) and the genus *Yamadazyma* (Yamada *et al.*, 1995). The results of the present study show that 18S rDNA gene and ITS sequences are sufficiently variable for population studies and yet sufficiently conservative for phylogenetic analysis.

The pigment produced by the new isolate was extracted and analyzed by column and thin layer chromatography, high performance liquid chromatography and light spectroscopy. The total concentration of the pigments produced by the new

isolate was found to be 261 µ/g yeast and that was very low when compared with that found in some mutants of *P. rhodozyma* (An *et al.*, 1989, An *et al.*, 1991). The types of pigment produced were phytoene, phytofluene, β-carotene, torulene and torularhodin. The individual pigments produced by the new isolate are consistent with those produced by members of the genus *Rhodotorula* (Simpson *et al.*, 1971, 1964). Considering the fact that economic factors play an important role in any commercialization process, and considering the fact that *P. rhodozyma* is the only economically valuable carotenoid producing organism available on the market (Johnson and An., 1991; Villadsen, 1992); if the new isolate is to be commercialized, then an enhancement of the metabolic flow of carotenogenesis resulting in yields of pigment comparable to those produced by mutants of *Phaffia rhodozyma* is required.

The new isolate was subjected to various chemicals and physical processes in an attempt to isolate pigment hyper-producing mutants and, NTG was found to be the best mutagen. Several mutants with varying pigment-producing capabilities were isolated, some of them produced more than twice the amount of pigment produced by the parental strain while others were blocked in the production of certain types of pigments.

Plating of the parental strain and NTG generated mutants on media containing various concentrations of antimycin gave rise to mutants that produced higher pigment concentration than the parental strain. These mutants also produced high concentrations of the oxycarotenoids, torulene and torularhodin than the parental strain. The physiology of the antimycin isolates and the known specificity of

antimycin for cytochrome b in the respiratory chain suggests that there was a possible alteration in the cytochrome b or P-450 components involved in oxygenation and desaturation of carotenoids. The net result was an increase in the production of oxycarotenoids (An *et al.*, 1989, Waterman *et al.*, 1986). None of the other inhibitors tested except β-ionone were able to generate any pigment-overproducing derivative strains.

β-ionone was found to inhibit the production of torulene and torularhodin in the new isolate but promoted the production of β-carotene. β-ionone appeared to have blocked torulene and torularhodin production at the β-carotene level leading to the accumulation of this carotene. β-ionone, being an end ring analog of β-carotene, may compete with β-carotene for oxygenation at C-3 and or C-4 position with β-carotene. This may lead to the accumulation of β-carotene at the expense of the oxycarotenoids, torulene and torularhodin (Lewis *et al.*, 1990).

The pigment-overproducing mutants isolated in this study can be subjected to further mutagenesis to isolate mutants that produced pigments in excess of what is produced by the parental strain. Those mutants that are blocked in the production of red pigments and therefore produced only β-carotene can also be developed further to isolate strains that produce commercial quantities of β-carotene. The collection of mutants blocked in a particular step of the carotenoid biosynthetic pathway obtained in this study would be useful tool for the cloning and characterization of carotenogenic genes.

Finally, studies on the carotenogenic enzymes in the new isolate were conducted

through the solubilization and partial purification of the enzyme system. [2-^{14}C] MVA was converted to phytoene, β-carotene, torulene and torularhodin by a cell-free 40,000 x g supernatant fraction prepared from the new isolate. Solubilization of the enzyme was achieved by subjecting the cell-free extract to varying concentrations of Tweens 40, 60 and 80 and determining the effects of these surfactants on carotenogenic activity and protein release. It was observed that Tween 60 at 1% concentration (w/v) provided the maximum stimulation for the production of phytoene, β-carotene, torulene and torularhodin. Tween 60-solubilized preparation from the new isolate is therefore a potentially suitable carotenogenic enzyme system to use in attempts to purify proteins responsible for the conversion of MVA to phytoene, and the desaturation and cyclization reactions that occur in the conversion of β-carotene to torulene and torularhodin.

The partial purification of the enzyme system was achieved through the fractionation of the Tween 60-solubilized extract with 10% polyethylene glycol and centrifugation to obtain the enzyme precipitate. The purification of the enzyme with polyethylene glycol resulted in a six-fold increase in carotenoid production. Unlike ammonium sulfate precipitation, it was not necessary to remove the polyethylene glycol prior to the enzyme assay, as it does not inhibit enzyme activity at the concentration used.

Another important aspect of this study was the determination of the efficacy of the yeast to pigment the flesh of rainbow trout. It was observed that fish within all groups grew very well, however, fish fed with diet containing the test yeast had a

lower growth rate than fish in the other groups. The low growth rate observed in this group, however, is in agreement with studies done by other workers. Laine and Gyllenberg (1969) fed rainbow trout with *R. sanneii* preparations and reported a low average weight and length gain in fish. Similarly, Haard (1992) and Johnson *et al.* (1980) fed rainbow trout with intact *P. rhodozyma* cells and reported low growth rate in the fish. The cause of the lower growth rate observed in fish fed diet supplemented with test yeast not known. The test yeast supplemented diet had the smell of molasses and Brewer's wort, as a result fish fed with diet containing the test yeast consumed only a fraction of their daily ration whereas fish in the other groups consumed almost all of their daily ration. The low consumption of the daily ration may have resulted in the lower growth rate observed in fish within this group.

Commercial canthaxanthin induced better pigmentation than the test yeast as seen from the Hunter a* and b* values. Similarly, the concentration of pigment in muscle of fish fed diet containing canthaxanthin (diet 4) was greater than that of fish fed diet containing test yeast (diet 1) which was in turn greater than those of fish in the other groups (diets 2, 3 and 5). Although the pigment concentration in fish fed diets containing intact yeast and pigment extract from yeast were low in comparison with commercial canthaxanthin, the values were comparable to those reported by other workers (Seurman *et al.*, 1978; Chan *et al.*, 1984). They were also comparable to pigmentation level regarded as sufficient for visual color impression (Torrissen *et al.*, 1989). The level of pigmentation present in the flesh of fish fed with diet containing pigment extract from yeast (diet 3) was about half the amount produced by

fish fed diet containing whole yeast. This decrease in pigment may be attributed to decomposition of the carotenoids during processing of samples.

The major carotenoids detected in the flesh of fish fed diet containing test yeast and those in groups 2-4 were mainly astaxanthin and canthaxanthin. Since these pigments were not present in the test yeast, their presence in the flesh of fish fed diet containing the yeast suggests that the fish were able to convert the pigments into canthaxanthin and astaxanthin (Savolainen and Gyllenberg, 1970; Hsu *et al.*, 1977; Hata and Hata, 1972a, 1972b).

Besides the growth, pigment and color measurements, the chemical composition of fish fed with the various diets were also determined. Fish within all groups have high protein content and no significant difference ($P>0.05$) were found between the protein content of the groups. The lipid contents of the fish in the various groups were generally low even though there were significant increases in the lipid levels at the end of the feeding period. The low lipid levels in fish observed in this study is in agreement with previous findings (Dygert, 1990; Hörstgen-Schwark *et al.*, 1986; Iwamoto *et al.*, 1990) and may be attributed to the initiation of gonads for maturation. According to Dygert (1990), most of the stored lipids in the somatic tissues of fish are mobilized to the gonads during sexual maturation resulting in a decrease in the lipid content of the muscle.

The moisture content of fish within all groups were found to be very high and this may be attributed to the low lipid content since low lipid levels are normally associated with elevated moisture content in fish and vice versa (Love, 1980; Reinitz,

1983). With the exception of fish fed with diet 1, the fatty acid composition of fish in all the other groups remained relatively unchanged indicating that the diets have no effect on the fatty acid composition of fish muscle. Fish fed with diet 1 were found to have a substantial increase in the concentration of polyunsaturated fatty acids (PUFA) which was coupled with a decrease in the levels of saturated fatty acids.

Final conclusions to be drawn from this study are:

1. Attempts to induce sporulation in the new isolate were not successful as there was no production of any structures resembling ascospores that have been reported earlier (Hari *et al.*, 1992).

2. The isolate was found to resemble the genus *Rhodotorula* in many ways. The results of the cellular fatty acid analyses, isozyme analysis, carbohydrate assimilation patterns, nitrate, DBB and urease test and the carbohydrate composition of the cell wall of the new isolate were all identical to those reported for *R. rubra* by other workers. These results suggest that the new isolate is a basidiomycetous yeast and may be a variant strain of *Rhodotorula rubra*.

3. Comparison of the DNA sequences of the new isolate with those of other yeast isolates suggests that *R. rubra* TP1 and *R. rubra* ATCC 9449 are closely related and should be considered as belonging to the same species. The phylogenetic analysis further suggests that the new isolate shares a closer relationship with other *Rhodotorula* species than the other yeast isolates used in the study.

4. The cell wall of the organism was found to be composed of mainly mannans and the linkages in the carbohydrate moiety were determined to be mainly β-

(1→3) and β-(1→4) mannopyranosyl units.

5. The total carotenoid concentration of the organism was found to be 261 μg/g yeast and the individual carotenoids were identified to be torulene, torularhodin, β-carotene, phytoene and phytofluene.

6. NTG was found to be the best mutagen for isolating mutants in the new isolate. Mutagenesis resulted in the isolation of different mutants, some of which produced more than twice the amount of pigment produced by the parental strain. Other mutants were blocked in the production of red pigment and produced only β-carotene. These mutants can be used for the cloning and characterization of the carotenoid producing genes. Furthermore, mutants with increased pigment production can be used as supplement in fish diets to determine their ability to pigment the flesh of fish.

7. The solubilization and polyethylene glycol fractionation of cell extract of the new isolate resulted in the production of an enzyme system that converted MVA into phytoene, β-carotene, torulene and torularhodin. Since new carotenoids can be obtained by combination of different genes from different organisms which can benefit the aquaculture and food industries, further purification and characterization of the enzyme system and possible cloning of the carotenogenic genes could be contemplated.

8. The new isolate, *Rhodotorula rubra* TP1 was found to induce adequate levels of pigmentation in the flesh of rainbow trout. However, commercial canthaxanthin induced better pigmentation in fish than the test yeast. All the test

diets used in the study were found to support good growth of fish.

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APPENDIX: SEQUENCE ALIGNMENTS

A. Aligned sequences of portions of 18S rDNA of *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* amplified with primer NS7mun

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                10        20        30        40        50
TP1     ......CGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAG   44
Rr      TTCCTCCGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAG   50
Phaffia ...........TTAGATGTTCTGGGCCGCACGCGCGCTACACTGACAGAG   39

                60        70        80        90       100
TP1     CCAGCGAGTCTACCACCTTTGCCGGAAGGCATGGGTAATCTTGTGAAACT   94
Rr      CCAGCGAGTCTACCACCTTTGCCGGAAGGCATGGGTAATCTTGTGAAACT  100
Phaffia CCAGCGAGTCTACCACCTTTGCCGGAAGGCATGGGTAATCTTGTGAAACT   89

               110       120       130       140       150
TP1     CTGTCGTGATGGGGATAGAACATTGCAATTATTGTTCTTCAACGAGGAAT  144
Rr      CTGTCGTGATGGGGATAGAACATTGCAATTATTGTTCTTCAACGAGGAAT  150
Phaffia CTGTCGTGATGGGGATAGAACATTGCAATTATTGTTCTTCAACGAGGAAT  139

               160       170       180       190       200
TP1     ACCTAGTAAGCGTGATTCATCAGATCGCGTTGATTACGTCCCTGCCCTTT  194
Rr      ACCTAGTAAGCGTGATTCATCAGATCGCGTTGATTACGTCCCTGCCCTTT  200
Phaffia ACCTAGTAAGCGTGATTCATCAGATCGCGTTGATTACGTCCCTGCCCTTT  189

               210       220       230       240       250
TP1     GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCC  244
Rr      GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGGCTCC  250
Phaffia GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCC  239

               260       270       280       290       300
TP1     GGATTGGCTATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTT  294
Rr      GGATTGGCTATTGGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTT  300
Phaffia GGATTGGCTATTGGGAGCTCGCGAGAGCACCCGACTGTCGAGAAGTTGTA  289

               310       320       330       340       350
TP1     CGAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGG.TTTCCGT.A  342
Rr      CGAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGG.TTTCCGT.A  348
Phaffia CGAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGGTTTCCGTTA  339

               360       370       380       390       400
TP1     GGTGAAC..GGGGAAAACCTT...GGGTTACGAATTTT.ACTTCCCCTAA  386
Rr      GGTGAACCTGGGGAAACCCCTCTAGGTTAATGGATTTA.ACTT.......  390
Phaffia GGTGAACCTGGGGAAAAACCTTT..GTTTACGAATTTACAATTCCCCTTA  387

TP1     AATT                                                390
Rr      ....
Phaffia AAT.                                                390
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B. Aligned sequences of portions of 18S rDNA of *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* amplified with primer NS12mun

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           10        20        30        40        50
TP  NNNTNGCGGACGATGGAATACAAATGCCCCCAACTATTCCTATTAATCAT   50
Rr  GAGAGGATAAATGCCCCCAACTATCCCTATTAATCATTACGGCGATCTCA   50
Pr  GTGTCTGGANCTGGTGAGTNTCNCCGTGTTGAGTCAAATTAAGTCGCAGG   50

           60        70        80        90       100
TP  TACGGCGATCTCAGAAACCAACAAAATGGGAACGCGCGTCCTATTTTATT  100
Rr  GAAACCAACAAAATGGGAACGCGCGTCCTATTTTATNATNCCATGCTAAT  100
Pr  CTCCACAACTGGTGGTGCCCTTCCGTCAATTCCTTTAAGTTTCAGTACTT  100

          110       120       130       140       150
TP  ATNCCATGCTAATGTATTCGGGNAAAGGCCTGCTTNGAACACTCTAATTT  150
Rr  GTATTCGGGCAAAGGCCTGCTTTGAACACTCTAATTCTTCCAANGTAANA  150
Pr  GTGACCATACTCCCCCCCGAATCTCATTTAAAGATTTCTCTTCGGGTGCC  150

          160       170       180       190       200
TP  TTTCAAAGTAAAAGTCCTGGTTNGCGACGACACCCAGTAAAGGACATCGC  200
Rr  GTCCTGGTTTGGGACGACACCCAGTAAAGGACATCGCCGATCACCAGGAG  200
Pr  GATACAGGCATTAATAATCCTGTTCCGATCCCCAATTGGTATAGTCTACA  200

          210       220       230       240       250
TP  CGTTCACCAGGAGGTAAGGCTCCGTCAAACAAGTACACACCAAGAAGGCG  250
Rr  GTAAGGCTCCGNCAAACAAGTACACACCAAGAAGGCGGACCGGCTGACAG  250
Pr  GAAGAGACTACAACGGTATCTAATCGTTTTCGATCCCCCTTCCTTCGTCC  250

          260       270       280       290       300
TP  GACCGGNTGACAGAGCCCGAAGTTCGACTACGAGCTTTTTAACGGNAACA  300
Rr  AGCCCGAAGTTCGACTACGAGCTTTTTAACGGCAACAATNTTAATATACG  300
Pr  TTGATCAATGAAAACATCCTTGGCAAATGTTTACGGAGGTGCTTGGTCTT  300

          310       320       330       340       350
TP  ATTTNAATATACGCTNTTNTTNTTTTTTTACCGCGGGTTGCTGGCACCA  350
Rr  CTATTGGAGCTGGAATTACCNCGGATGTTTGGTANCAGACTTGCCCTCCA  350
Pr  CCCGGCAAATCCAAGGAATTTTCAACCTCTGACGAGGGTTATATAAAATG  350

          360       370       380       390
TP  GACTTGGCCNCCAATTGGATCCNCGNTAAGGGGNTTTTNC            390
Rr  ATTGATCCTCGTTAAGGGATTAAATTGNACTCATCAAAAA            390
Pr  CCCCCCAAATCCTCCCTCTTTTAATCATTAACGGGGGGTC            390
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C. Aligned sequences of portions of 18S rDNA of *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* amplified with primer NS13mun.

```
                10        20        30        40        50
TP_   .......TAGGTGAAC..TGCGGAAGGATCA......TTAGTGAATATAG    35
Rr_   .........GGTGACC..TGCGGAAGGATCA......TTAGTGAATATAG    33
Pr_   GCGAGAGCAAGAGACCGTTGTTGAAAGTTTAATTTTGTTATTAAATTTAA    50

                60        70        80        90       100
TP_   GACGTCCA...ACTTA..............ACTTGGAGTCCGAACTCTCAC    69
Rr_   GACGTCCA...ACTTA..............ACTTGGAGTCCGAACTCTCAC    67
Pr_   TACATTCATAGACTTTGTGTTTATAAGTGAATAGGAGTTCGCTCTCTTGC   100

               110       120       130       140       150
TP_   .....TTTCTAACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAG   114
Rr_   .....TTTCTAACCCTGTGCACTTGTTGGGGATAGTAACTCTCGCAAGAG   112
Pr_   GAGAGTTACTATCCCAAACAAGTGGACAGGGTTAGAAA.....GTGAGAG   145

               160       170       180       190       200
TP_   AGCGAACTCCTATTCACTTATAAACACAAAGTC.TATGAATGTATTAAAT   163
Rr_   AGCGAACTCCTATTCACTTATAAACACAAAGTC.TATGAATGTATTAAAT   161
Pr_   TTCGGACTCCAAGT......TAAGTTGGACGTCCTAT..ATTCACTAATG   187

               210       220       230       240       250
TP_   TTCATTACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCT.CGCATC   212
Rr_   TTCATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCT.CGCATC   210
Pr_   ATCCTTCCGCAGG......TGCACCTACGGAAACCTTGTCACGACTTATC   231

               260       270       280       290       300
TP_   GATGAAGAAGGGAAAAAACCCCCTCCCCTCCCCTTCCCCCCTCCCACCCC   262
Rr_   GATGAAGAAGGGAAAAAACCCTCCCCTCTCCCCCTCCTCC.TCCTCCCCC   259
Pr_   CCCCAA......ACCCNNCCCCCTTCTTCCCCCCTAC.CCTTCCCCTCCC   274

               310       320       330       340       350
TP_   CCCCCTCCCCTC........CCCCCCCCTCAACCCCTCTCCCTCCC....   300
Rr_   CCCCTTCTCCTCTACTTCTGCCCTACCCCTTTCTCCTCCCCCCCCC....   305
Pr_   CCCCTTCCCTTC........CTCTTTCCTTT.CCCTACTCCCTCCCTTTT   315

               360       370       380       390       400
TP_   CCTCTCCCCCCCTCCTT.TCCCCTTCCTACACCCCCATCTCTCCCCTTCT   349
Rr_   CCACCCCCCCCCCTCTTCTCCCCCTGCCCCGCCCCCTTCTC.CCGCTTC.   353
Pr_   CCTCCTCCCCCTTCCTCCCCCCCTTTC....CTCCCCTCCCCCCCCTTCC   361

               410       420       430       440       450
TP_   .TCCCATTCCCTC.CTCTCCCTACATCCTACCCCCC.CCTCCCC.CCTCT   395
Rr_   ..CCC..TCCCTCTCTCTCCCT..CTCCTCCACCTC.CCTTTCTTCCTCC   396
Pr_   CTCCCCCTCCCTC.CTCTCCCT..TCCCTACTCCCCTCCTCCCC.CTTCT   407
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                460       470       480       490       500
TP_   TTCCCTACCCCT....CTCTCTTCTCC.CTCCCCCCACTCCCTCTCCTCC  440
Rr_   CCCTCTCCACCT....CGCATTTCTGC.CGCTCCCCCCTTTCCCTCATTC  441
Pr_   CCCCCTCCCCCTACTTCCCCCTTCTTCACTCTCCTCACTTCCTCTC...C  454

                510       520       530       540       550
TP_   CCGCCCCCCTCCCTCCCCCCCTTCCCCCTCCCCCCCCCTCCCTCCCC.TC  489
Rr_   CC..CCCCCTCCTCCCCCTTCTCTCCCCTTCCCTCCCCTCCCCCCCCCTC  489
Pr_   CC..CTCCCTCCC.CTCCTCCTTTCCTCTCCCCTTCTCTCTCTCATACTC  501

                560       570       580
TP_   CCCCTTCCCCCCCCCCCCTCCCTC.CTCCCCC                    520
Rr_   CCCCCTCTCGCCTTACCATCCCTCTCTCCTC.                    520
Pr_   TAC..TCACTCTTT....TCCCT...TC....                    520
```

D. Aligned sequences of portions of 18S rDNA and internal transcribed spacer (ITS) of *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* amplified with primer ITS5mun.

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                    10        20        30        40        50
TP1      AGAGCCAAGAGATCCGCTGTTGAAAGTTTAATTTTGTTATAAAATTTACT   50
Rr       ..AGCCAAGAGATCCGTTGTTGAAAGTTTTATTTTGNTATAAAATTTAAT   48
Phaffia  ....GTAGGTGACCTGCGG....AAGGATCATT.....AGTGAATATAGG   37

                    60        70        80        90       100
TP1      ACATTCATAGACTTTGTGTTT.ATAAGTGAATAGGAGTTCGCTCTCTTGC   99
Rr       ACATTCATAGACTTTGTTTTTTATAAGTGAATAGGAGTTCGCTCTCTTGC   98
Phaffia  ACGTCCA...ACTTA..............ACTTGGAGTCCGAACTCTCAC   70

                   110       120       130       140       150
TP1      GAGAGTTACTATCCCAAACAAGTGGACAGGGTT.....AGAAAGTG....  140
Rr       GAGAGTTACTATCCCAAACAAGTGCACAGGGTT.....AGAAAGTG....  139
Phaffia  .....TTTCT.TACCCT....GTGCACTTGGTTGGGATAGGAACTCTCGC  110

                   160       170       180       190       200
TP1      .AGAGTTCGGACTCCAAGTTA.............AGT...TGGACGTCCT  173
Rr       .AGAGTTCGGACTCCAAGTTA.............AGT...TGGACGTCCT  172
Phaffia  AAGAGAGCGAACTCCTATTCACTTATTAACACAAAGTCTATGAATGTATT  160

                   210       220       230       240       250
TP1      ATATTCACT.....AATGATCCTTCCGCAGGTGCACCTACGGAAACCTTG  218
Rr       ATATTCACT.....AATGATCCTTCCGCAGGTTCACCTACGGAAACCTTG  217
Phaffia  AAATTTTATTACAAAATAAAACTT........TCAACAACGGATCTCTTG  202

                   260       270       280       290       300
TP1      ..TTACGACTTTTATCAA........AAAAACCCTCCCTCCC.TCCCCTC  257
Rr       ..TTACGACTTTTATCAA........AAAACACCNNCTTCCCCTCCCCCC  257
Phaffia  GCTCTCG.CATCGATGAAGAAGGAAAAAAAAACCCNNNNNCCCCCCCCCC  251

                   310       320       330       340       350
TP1      TTCCCGCCCCT.......CCCCTCTTCCCCCCCCTCCCC...CCCTTCTC  297
Rr       TCCCCACCCCCTTCC...CCCCTCTACCTTACCCTCCCCTTACCCTTA.C  303
Phaffia  CCCCCTCCCCTTACCTTTCCCCGATTCCCCTCCCCCCTCAC.CCCTTC.C  299

                   360       370       380       390       400
TP1      TTCTTTCTCTCTTCCCTCC..CCCTCCCTCCCCCCTGTCCTCCC.CCTGA  344
Rr       TCCTCTCA.TCCTCCCTTC..CCCT..TTCCTTCCT.TCATCCCACCTTC  347
Phaffia  CCCTCTCCCCCCCCCCCCTACCCTCCCACCCCCCT...TTCCCCCCTTC  346

                   410       420       430       440       450
TP1      CTTTTTCTCGCCCCCCTCCG...CCCCACCCCCACATCCACTCTCCCCTC  391
Rr       CTCTCTCTCTTACCCCTTCTTTCCCCCACCCGTTCCTC..CTCAACACTT  395
Phaffia  CCCTC...CTT..CCCTCCTTCCCCCCTCCTCCTCCTC....CCCCCCCC  387
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E. Aligned sequences of portions of 18S rDNA and internal transcribed spacer (ITS) of *Rhodotorula rubra* TP1, *Rhodotorula rubra* ATCC 9449 and *Phaffia rhodozyma* amplified with primer ITS10mun.

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                10        20        30        40        50
TP      TGCATTAATAGGGATAGTTGGGGGCATTTGTATTCCGTCGTCAGAGGTGA   50
Rr      .TCATTAATAGGGATAGTTGGGGGCATTTGTATTCCGTCGTCAGAGGTGA   49
Phaffia ATGATTAATAGGGATAGTTGGGGGCATNTGTATTCCGTCGTCAGAGGTGA   50

                60        70        80        90       100
TP      AATTCTTGG.ATTGCCGGAAGACAAACTACTGCGAAAGCATTTGCCAAGG   99
Rr      AATTCTTGG.ATTGCCGGAAGACAAACTACTGCGAAAGCATTTGCCAAGG   98
Phaffia AATTCTCGGCATTCTCGGAGGACAAACTACTGAGAACGCATTTGTCACAG  100

               110       120       130       140       150
TP      ATGTTTTCATTGATCAAGAACGAAGGAAGGGGGATCGAAAACGATTAGAT  149
Rr      ATGTTTTCATTGATCAAGAACGAAGGAAGGGGGATCGAAAACGATTAGAT  148
Phaffia ATGTGTGCACAGATCACGCCCGAAGGAAGAGGGATCGATTTCTCTTCGAT  150

               160       170       180       190       200
TP      ACCGTTGT.AGTCTCTTCTGTAAACT........ATGCCAATTGGGGATC  190
Rr      ACCGTTGT.AGTCTCTTCTGTAAACT........ATGCCAATTGGGGATC  189
Phaffia ACCCGAGTCAGTCACTACTGAATCCTGATACCCAATCCCCAGATGGGATC  200

               210       220       230       240       250
TP      GGTACAGGATTTTTAATGACTGTATCGGCACCCGAAGAGAAATCTTTAAA  240
Rr      GGTACAGGATTTTTAATGACTGTATCGGCACCCGAAGAGAAATCTTCACA  239
Phaffia AGTT....ATCAGAAGAGACTACATCGGTATCTAATCGGTTACGTTCCCC  246

               260       270       280       290       300
TP      TGAGGTTCGGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTG.  289
Rr      TGAGGTTCGGGGGGGAGTATGGTCGCAAGGCTGAACCTTAAAGGAATTG.  288
Phaffia CTACGTTCGGTCTGGAGCATTG....AAAAC..ATCCTTG..GCAATTTC  288

               310       320       330       340       350
TP      ..ACGGA..AG...GGC.ACCACCAGGTGTGGAGCCTGCGGCTTAATTTG  331
Rr      ..ACGGA..AG...GGC.ACCACCAGGTCTGGAGCCTGCGGCTCACTTTG  330
Phaffia TTACGTAGTAGTTTGGCTACCCCCAGTCCAAGAGTC......TCACCTT.  331

               360       370       380       390       400
TP      ACTCAACACGG.GGAAACTCACCAGGTCCAGACACAATAAGGATTGACAG  380
Rr      GCTCAACACGGTGGAAACTCACCAGGCCCAGACACAATTAGGATTGTCAG  380
Phaffia .CTGAATGAGGGGTATACAAATGGGCCCC...CCCTATACCAAT....AG  373

               410
TP      ATGAAAAAAA.......                                   390
Rr      ATTAAAAAAA.......                                   390
Phaffia ATAAAATTCGGGGGGCC                                   390
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