STUDY OF THE GROWTH PARAMETERS OF THE YEAST RHODOTORULA RUBRA AND ITS CAROTENOID PRODUCTION

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STUDY OF THE GROWTH PARAMETERS OF THE YEAST *RHODOTORULA RUBRA* AND ITS CAROTENOID PRODUCTION

by

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Abstract

A red yeast, *Rhodotorula rubra*, was grown in a batch fermenter with molasses and acid extract from peat as basic substrates in separate experiments.

Different concentrations of water-diluted peat extract were tested in shake flask experiments in an attempt to determine the optimal TCH (total carbohydrate) concentration for the growth of the yeast. The best results were obtained with a TCH concentration of 15 g/L. The nutritional requirements of *R. rubra* grown in peat extract were studied to enhance the growth of the yeast biomass. The yeast required supplements of nitrogen, potassium, and magnesium for growth. Growth was also better when a combination of organic and inorganic sources of nitrogen were added than when the inorganic nitrogen source (ammonium salts) was used alone. The addition of 4 g/L ammonium sulphate, 1.5 g/L yeast extract, 0.174 g/L potassium sulphate, and 0.5 g/L magnesium sulphate to the peat extract medium produced 4.4 g/L dry biomass, with a biomass yield of 57 %, and a biomass efficiency of 29 %.

Several operational parameters, namely, pH, agitation speed, temperature, and fermentation time, were studied to optimize the biomass production by *R. rubra*. The best results produced approximately 4.8 g/L of dry biomass with a yield of 70 % and an efficiency of 32 %. The cultivation time employed was 84 hours, with an inoculum ratio of 2 % (v/v), an incubation temperature of 22 ± 1°C, an

initial pH of 5.5, and an agitation speed of 200 rpm.

In separate experiments in a 2-Liter fermenter, the growth of *R. rubra* in the peat extract and molasses was studied to optimize the agitation and aeration rates. The optimal conditions produced 5.9 g/L of biomass with a yield of 71 % in peat extract medium, and 4.5 g/L of biomass with a yield of 33 % in molasses medium. These results were obtained with an agitation speed of 200 rpm, an aeration rate of 1.0 vvm, an inoculum ratio of 2 % (v/v), an incubation temperature of 22 ± 1°C, a pH of 5.5 ± 0.1, and a cultivation time of 72 hours. The β-carotene contents of the yeast produced from the peat extract medium and the molasses medium were 1235 μg/g of dry biomass, and 1034 μg/g of dry biomass, respectively.

The proximate compositions of the *R. rubra* biomass grown in the two different media were analyzed and compared. The yeast grown in peat extract contained approximately 48.5 % crude protein, 4.1 % ash, 27.7 % total carbohydrate, and 5.7 % total fat. The yeast grown in molasses contained 43.6 % crude protein, 3.8 % ash, 30.8 % total carbohydrate and 10.2 % total fat. The amino acids and fatty acids compositions of the yeast grown in peat extract were analyzed. The yeast protein contained 10 essential amino acids, and was high in leucine, arginine, lysine, and valine. It was, however, deficient in tryptophan when compared with the FAO standard. Eleven fatty acids were identified in the biomass.

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Finally, I dedicate this thesis to my parents, Ruicong Zhang and Zongze Lu, who encouraged me and imparted to me their creed: to do everything at my best.

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CHAPTER 1

INTRODUCTION

1.1 Background

Colorant is one of the most important factors in the food industry. Products using colorants constitute an annual market of approximately twenty-five billion U.S. dollars in the world, and many of those products would not exist without colors. The market for food colors is expected to grow in the forthcoming years, with new applications being investigated to keep pace with changes in consumer trends (Dziezak, 1987).

Carotenoids, one of the main groups of natural colorant substances in food, are responsible for the colors of many edible fruits and berries, vegetables and mushrooms, flowers, and some animals (Kläui and Raunhardt, 1976). To satisfy consumer demands for attractive-looking food products, and thus to increase market value, carotenoids are added to food products such as margarine and cheese, and also to animal feeds in the aquaculture and poultry industries to produce the pink flesh of farmed fish and the yellow yolk of eggs (Lovell, 1980). These compounds are obtained by either extraction of natural carotenoids, synthesis, or fermentation methods. Natural extracts containing carotenoids have been used for coloring foods for centuries. Carotenoids produced synthetically have been used both in human foods and animal feeds since 1954 (Kläui and

Raunhardt, 1976). However, due to concern for the unknown and possibly harmful effects of synthetic chemicals, several companies are now producing carotenoids in market - size quantities by fermentative and biotechnological means (Taylor, 1990).

1.2 Carotenoids

Most carotenoids are C_{40} terpenoids (in some bacteria, they are C_{30} or C_{50}), and are biosynthesized by the well-known isoprenoid pathway from mevalonic acid (MVA; Britton, 1990). Carotenoids are widely distributed among the plants and animals, as well as in microorganisms. They are classified as either acyclic, monocyclic or bicyclic. They have been traditionally used as color additives in food for human consumption as well as in animal feeds, in which they act as pigmenters to color either the flesh or the products of the animals (Gross, 1987). Table 1.2a lists the principal carotenoids that are legally permitted in foods and feeds by most countries, together with their common sources and main areas of application.

The medical applications of carotenoids have been reviewed by Bauernfeind (1981) and by Britton and Goodwin (1982). The primary medical uses of carotenoids are the prevention or correction of vitamin A deficiency (Gross, 1987) and of certain light-sensitivity diseases in man (Mathews-Roth, 1981). Recently, carotenoid pigments were found to have potential application in preventing or slowing the growth of induced skin tumours (Britton and Goodwin, 1982). Peto *et al.* (1981) reported that the risk of cancer in human beings may be correlated

Table 1.2a Principal carotenoids permitted as additives in foods and feeds[1].

Compound	Non-microbial sources	Microbial sources	Use
β-Carotene	Carrots, Synthesis	*Blakeslea trispora*, *Dunaliella salina*	a
Lycopene	Tomatoes	*Blakeslea trispora*, *Streptomyces chrestomyceticus* subsp. *rubescens*	a
Lutein	Alfalfa, corn, marigold flowers, green plants	*Spongiococcum excentricum*, *Chlorella pyrenoidosa*	b
Zeaxanthin	cf. lutein	*Flavobacterium* sp.	b, c
Canthaxanthin	Crustacea (e.g. *Artemia*), Bird feathers, Synthesis	*Cantharellus cinnabarinus*, *Brevibacterium KY-4313*, *Rhodococcus maris*, *(Mycobacterium brevicale)*. *Corynebacterium michiganene* (mutant)	b, c
Astaxanthin	Crustacean processing waste, Bird feathers, *Adonis annua*, flowers, Synthesis	*Mycobacterium lacticola*, *Brevibacterium 103*, *Phaffia rhodozyma*, *Peniophora* sp.	c
Rhodoxanthin	Autumn leaves, Fish (*Tilapia*)	*Protomonas extorquens*	c
Capaxanthin	*Capsicum annuum* (paprika)	--	a, b
Bixin	(*Bixa orellana*) annatto	--	a, b
Crocetin	Saffron	--	a
β-Apo-8'-carotenoic acid ethyl ether	Synthesis	--	a, b
Citranaxanthin	Synthesis	--	b

a. Food colorant b. Poultry feeds c. Fish feeds.

[1](Neils and DeLeenheer, 1991)

inversely both with the level of retinol in blood and with the dietary intake of β-carotene. Considerable attention is also being given to β-carotene as a promising chemopreventive agent (Peto *et al.*, 1981, Peto, 1983; Kvale *et al.*, 1983; Winn *et al.*, 1984; Menkes *et al.*, 1986). As a result, the carotenoid market, which generated world-wide sales of 100 million U.S. dollars in 1989, is poised to expand significantly (Table 1.2b). Demand for natural β-carotene, which currently accounts for approximately 10 % of the total β-carotene market, appears to exceed supply. Thus, new production methods will be needed to meet the demand for carotenoids anticipated for the year 2000.

1.2.1 Natural carotenoids

Carotenoids biosynthesized *de novo* from small carbon units, and the metabolic precursors of such carotenoids, are considered to be natural (Liaaen-Jensen, 1990). Natural carotenoids are widely distributed in animals, plants, and microorganisms. Commercially available natural carotenoids (Table 1.2.1) are produced through the agriculture and extraction of flowering plants, enclosed bioreactor cultures of heterotrophic organisms, and mariculture of algae (Nonomura, 1990). Carotenoids thus produced, which meet the regulations of the U.S. Food and Drug Administration (1986) for use as food additives and/or feed additives for animals, are nontoxic, natural pigments that appeal to consumers' increasingly concerned about toxicity and carcinogenicity of synthetic food additives. Consumers are willing to pay more for natural carotene, which they value for its perceived

Table 1.2b Commercial market for carotenoids[1]

Carotenoid(s)	Yearly sales ($ Million)			AAG[2] (%)
	1990	1995	2000	
Beta-Carotene	70	110	175	9 - 11 %
Other Carotenoids	50	80	135	10 - 14 %
Total	120	190	310	10 - 13 %

[1]Taylor (1990)

[2]AAG: Average Annual Growth.

Table. 1.2.1. Commercial available natural carotenoids sources[1]

Source	Carotenoids (color)
NATURAL EXTRACTS:	
Annatto	Bixin, norbixin (red-orange)
Carrot	Carotenes (orange)
Palm oil	Carotenes (orange)
Saffron	Crocin, crocetin (yellow-orange)
Tomato	Lycopene (red)
Paprika	Capsanthin, Capsorubin (red)
Marigold	Lutein, Zeaxanthin (yellow)
Alfalfa	Carotenes (orange)
Crustacean waste	Astaxanthin (pink/red)
FERMENTATION:	
Fungi	Carotenes (orange)
Yeasts	Astaxanthin, other xanthaophylls (orange to red)
Algae	Carotenes (orange), Lutein (yellow) Astaxanthin (pink/red)

[1]Taylor (1990)

safety. Recent efforts in research and development include improving carotenoid-producing systems through biotechnology, finding more efficient formulations to solubilize and stabilize carotenoids, and generating new immobilized - enzyme reactors to speed carotenoid production (Taylor, 1990).

1.2.2 Microbial carotenoids

Microbial carotenoids are considered to be natural carotenoids that compare well with other high - quality carotenoid sources, and are suitable for use as food colorants (Kläui and Bauernfeind, 1981) and as pigmenters in feedstuffs for animal husbandry and aquaculture (Johnson *et al.*, 1977; Marusich and Bauernfeind, 1981; Simpson *et al.*, 1981). Many species of microbes are known to produce β-carotenes, astaxanthin and other edible, pigmented compounds, and some appear ready for investigation and commercialization. Examples of these are *Phaffia rhodozyma*, a yeast that produces red pigment, and *Monascus purpureus*, a fungus that also produces red pigment. Also, *Candida utilis* may be used to ferment beet juice to deplete its carbohydrates and nitrites (Trivedi, 1986). Commercially, microbial carotenoids are produced by means of fermentation. Table 1.2.2 shows carotenoids produced by various classes of microorganisms. In general, microbial carotenoid sources are rich in orange and red-orange - colored carotenes (Taylor, 1990).

Table 1.2.2 Commercial carotenoids from microorganisms[1].

Source (Fermentation)	Carotenoids (color)
Fungi	Carotenes (orange)
Yeasts	Astaxanthin, other xanthophylls (orange to red)
Algae	Carotenes (orange), lutein (yellow), astaxanthin (pink/red)

[1]Taylor (1990)

1.3 Single - Cell Proteins

With the increasing growth of the world population, many difficulties are encountered in solving protein deficit problems by traditional methods. Therefore, many scientists are focusing attention on a wider use and production of unconventional proteins, including protein produced by single-cell microorganisms (Kharatyan, 1978).

The term "single-cell proteins" (SCP) refers to the proteins or protein concentrates that may be extracted from cultured microbial cells that contain from 40 to 65 % protein (Reed, 1982*a*). It has become a widely accepted term for microbial biomass proteins (MBP) used as food or feed. Among the various single-cell organisms that can be used for SCP production are yeast, nonpathogenic bacteria, algae, and mycelial fungi (Kihlberg, 1972).

1.3.1 Nutritional value of SCP

The food value of SCP is quite high, in some cases comparable to that of casein. As seen in Table 1.3.1, several microbial species have well-balanced amino acid compositions. High concentrations of essential amino acids in microbial protein make it a potential dietary protein source, especially useful as a supplement to grain-based diets, as grains are deficient in lysine, threonine and tryptophan. Animal experiments have indicated that SCP can be a valuable source of protein for human consumption.

Table 1.3.1 Essential amino acids content of selected microorganisms with comparison to egg white protein (g/16g of nitrogen) [1]

Amino acids	Source [2]					
	1	2	3	4	5	6
Lysine	7.7	7.8	5.3	8.6	4.6	6.5
Threonine	4.8	5.4	4.5	4.5	4.6	5.1
Methionine	1.7	1.6	1.8	2.7	1.4	3.2
Cystine	-	0.9	0.3	-	0.4	2.4
Tryptophan	1.0	1.3	-	1.1	1.4	1.6
Isoleucine	4.6	5.3	3.9	4.6	6.0	6.7
Leucine	7.0	7.8	7.0	8.5	8.0	8.9
Valine	5.3	5.8	5.9	7.1	6.5	7.3
Phenylalanine	4.1	4.8	4.2	4.0	5.0	5.8

[1] Kharatyan (1978)

[2] 1. *Saccharomyces cerevisiae*; 2. *Candida lipolytica* (gas oil); 3. *Pseudomonas methanolica* (methanol); 4. *Hydrogenomonas eutropha*; 5. *Spirullina maxima*; 6. Whole egg.

1.4 Yeast

More than any other group of microorganisms, yeasts have been closely associated with human food supply throughout history (Miller, 1982). As a source of carotenoids and SCP for livestock, yeast have advantages over other microorganisms. Most yeasts are nontoxic, able to ferment hydrocarbons, and are good sources of protein and B - vitamins (Casida, 1964*a*). They can grow at low pH values (4.5 - 5.5), which drastically reduces bacterial contamination of the medium and the need for strict aseptic conditions (Kharatyan, 1978). They are highly acceptable to the consumer because they have been used in foodstuffs for thousands of years. Their naturally-occurring colors make them particularly suitable for animal feeds, and in this respect they may gain greater acceptance as human foods (Casida, 1964*a*).

1.4.1 Classification of yeast

According to their fermentation capacity, yeasts can be divided into three groups. In the first are yeasts with a predominantly fermentative metabolism, such as *Candida pintolopesii*, which is an imperfect form of *Saccharomyces telluris*. This species ferments glucose and takes up hardly any non-fermentable substrates. The second includes yeasts with mixed fermentation and respiratory metabolisms. These can in turn be divided into two subgroups: glucose-sensitive yeasts (Crabtree positive), such as *Saccharomyces cerevisiae*, in which glucose inhibits the biosynthesis of respiratory enzymes; and yeasts not sensitive to

glucose (Crabtree negative) such as *Candida utilis*. The third group contains the nonfermenting yeasts, including *Rhodotorula rubra*. Some yeasts in this group can be placed in the Crabtree-negative, mixed-metabolism category (Van Dijken and Scheffers, 1986).

The production of yeast for food always uses species that are Crabtree negative. Species with very weakly fermentative metabolisms are used frequently (Boze *et al.*, 1992).

1.4.2 Classification of yeast-derived products

With increased knowledge of yeast metabolism, more products from yeast are now expected to be obtained besides SCP. Products such as a variety of enzymes (Böing, 1982), organic acids (Kapoor *et al.*, 1982), amino acids (Nakayama, 1982), vinegar (Ebner, 1982), fuel alcohol (Reed, 1982*b*), *etc.*, are successful examples of yeast metabolic products produced by batch fermentation. Yeast products and yeast-based industries can be placed into seven categories: 1) Baker's yeast and bread-type products, 2) Food and feed yeast biomass, 3) Products derived from yeast biomass, e.g., autolysates and biochemicals, 4) Beer yeast for brewing, 5) Wine yeast for winemaking, 6) Distiller's yeast for making distilled spirits, 7) Industrial grade ethanol (solvents) and fuel alcohol production (Peppler, 1983). Carotenoids produced by yeast can be categorized into the third group.

1.4.3 Carotenoid-producing yeasts

Yeast has been used as human food for a long time and its genetic definition is well known. The yeasts that form carotenoid pigments are therefore considered to be a natural source of carotenoids for animal feeds. They are included in six genera out of the 1317 known yeast strains: *Rhodotorula*, *Phaffia*, *Cryptococcus*, *Rhodosporidium*, *Sporidiobolus* and *Sporobolomyces* (Andrewes *et al.*, 1976; Johnson and Lewis, 1979; Simpson, 1972; Miller, 1982). They are thought to be an attractive source of carotenoids as well as of other valuable nutrients (Johnson *et al.*, 1980). Their yield of carotene is high, compared to such other natural sources of carotene as carrots (Nonomura, 1990). Included among the yeast carotenoids are some important carotenoids, such as astaxanthin, that are rarely found in fungi. This particular carotenoid should be added to the diets of pen-raised fish. Carotenoid-producing yeasts are more suitable as a feed additive in the diets of fish than such conventional sources of carotenoids as crustacean shells, in that they contain higher carotenoid and lower mineral concentrations, which reduces several practical problems in feed formulation (Johnson *et al.*, 1980). Yeasts are also a good source of proteins and lipids, in addition to carotenoids, in feed (Johnson *et al.*, 1980).

Some properties of yeast, however, limit its large scale commercial production and use as feed additives in animal diets. The thick cell walls of yeast hinder the extraction of their metabolic products, and render their indigestible by

fish. The high production costs of yeast could be increased by the need for special engineering considerations for extraction and purification (Nonomura, 1990). So far, the most important commercial carotenoid-producing yeast is *Phaffia rhodozyma*. Many commercial companies and research institutes worldwide are developing processes for the production of carotenoids, especially astaxanthin. These include Enzymatrix Ltd. (UK), Igene Biotechnology (MD, USA), Osaka Chemical (Japan), Philips Petroleum (OK, USA), University of Wisconsin (WI, USA), LeferGen (Hamilton, Bermuda), *etc.*. Two of these, LeferGen and Igene Biotechnology, have been reported to produce astaxanthin by yeast on a commercial scale (Taylor, 1990).

1.4.4 Yeasts as a source of protein

According to Solomons (1983), the production of microbial protein in the form of yeast for human and animal nutrition has had a surprisingly long history. The greatest advantage of yeast is its high protein content; dry yeast contains about 50 % protein, which makes it a natural protein concentrate (Peppler, 1970).

Yeast has a broad amino acid composition and can be regarded as one of the richest sources of lysine. An investigation of the nutritive value of yeast (as shown in Table 1.4.4) demonstrates that its protein can be well assimilated (Kharatyan, 1978).

Table 1.4.4 Biological value of yeast protein[1]

Yeast	Test subject	Digestibility (%)[2]	Biological value (%)[3]	Protein efficiency ratio (%)[4]
Baker's	Rat	81	59	1.4
Brewer's	Rat	80 - 90	58 - 69	1.7
Fodder	Rat	85 - 88	32 - 48	0.9 - 1.4
Fodder + 0.5 % methionine	Rat	90	88	2.0 - 2.3
Dry food	Man	70 - 90	52 - 87	-

[1] Kharatyan 1978.

[2] $$\text{Digestibility (\%)} = \frac{\text{nitrogen absorbed}}{\text{nitrogen consumed}} \times 100\ \%$$

[3] $$\text{Biological value (\%)} = \frac{\text{nitrogen retained}}{\text{nitrogen absorbed}} \times 100\ \%$$

[4] $$\text{Protein efficiency ratio (\%)} = \frac{\text{body weight gain}}{\text{protein consumed}} \times 100\ \%$$

1.5 Cultivation of yeast

1.5.1 Substrates used for the production of yeast and its derived products

Yeasts possess extensive enzyme complexes that enable them to flourish successfully on a wide variety of inexpensive carbohydrate substrates such as lactose whey, molasses, polyfructose inulin and spent sulphite liquor, and also on a wide variety of non-carbohydrate substrates such as ethanol, methanol, alkanes and lipids (Boze *et al.*, 1992; Rolz, 1984). In industry, raw materials containing carbohydrates, such as molasses, sulphite waste liquor and whey, are used as substrates. Levi *et al.* (1979) has reported further research on yeast using hydrocarbons to reduce the production costs. The use of uncommon substrates for yeast to produce metabolic products has been reviewed by Johnson (1977).

1.5.2 Cultivation of yeast in fermenters

The first modern effort to manufacture microorganisms for food on a large scale was in Germany during World War I, where *Candida utilis* yeast was produced. In the years between the two world wars, production continued and reached a level of over 15,000 tons per year. The use of hydrocarbons as a substrate for the production of foodstuffs was originally directed toward lipids and vitamins. It was not until the late 1950's that the production of protein on a commercial scale was initiated (Laskin, 1977). The standard process (Figure 1.5.2) for yeast and yeast - derived products production, as developed for large-

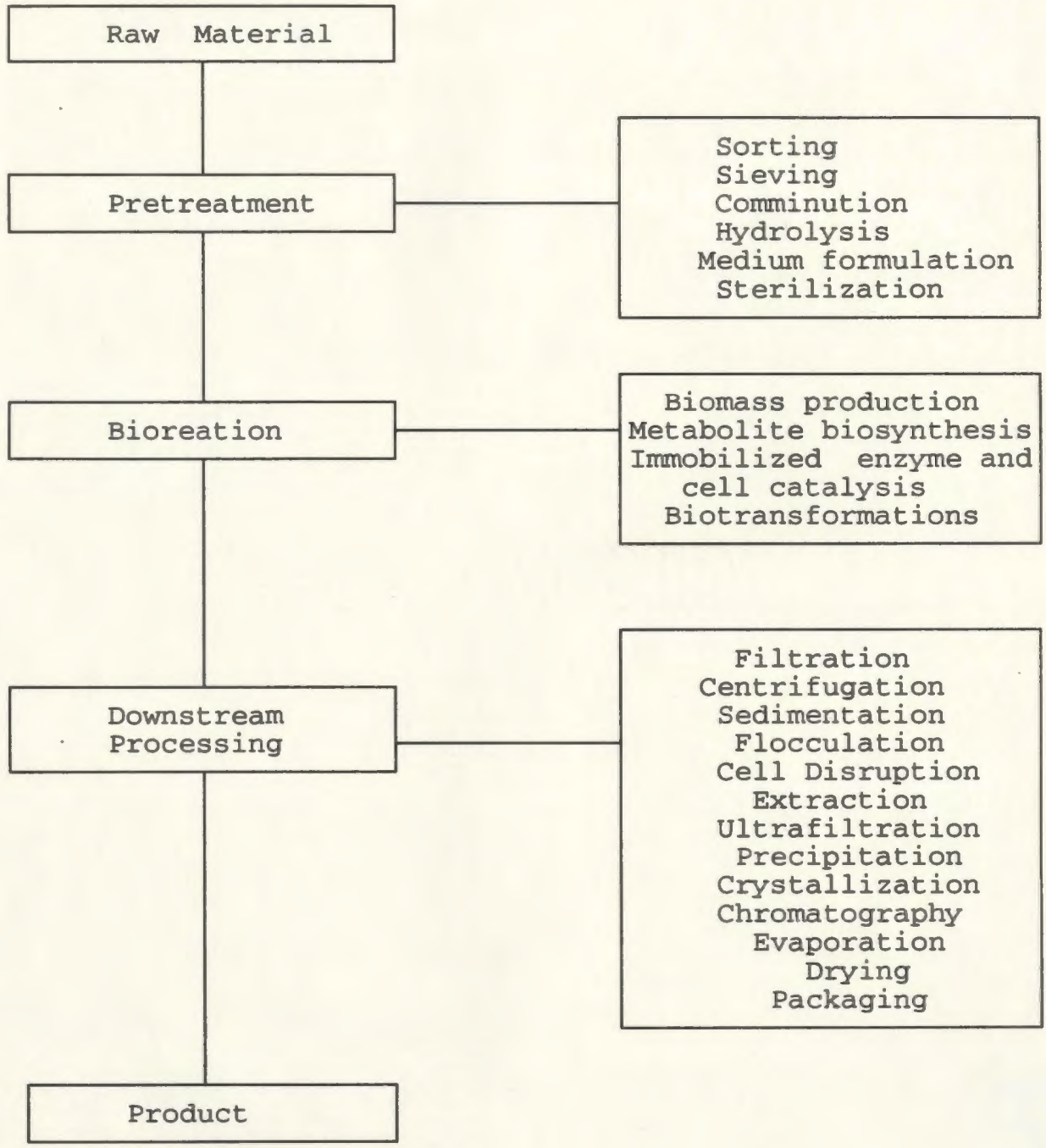


Fig. 1.5.2. The process stages for yeast and yeast - derived products production on a large scale.

scale capital-intensive plants, consists of a strictly aseptic, single-species operation carried out in synthetic, highly diluted aqueous substrates, in which biomass is continuously produced, aerobically, under mesophilic temperatures. This operation is followed by cell recovery and washing for yeast cell production, and by further extraction and purification for the derived products (Rolz, 1984; Chisti and Moo-Young, 1991). In some cases, further processing includes drying of the residues, with a nonviable, easily stored biomass or cell-derived products powder obtained as feed for livestock.

1.5.2.1 Nutritive value of yeast grown by fermenter culture

Numerous works have dealt with the nutritional qualities of yeast produced by fermenter cultivation as a protein source (Davis, 1974; Senez, 1983). The overall protein content of yeast is comparable to that of numerous other products used in animals feeds. Yeasts in animal or human diets not only constitute a protein complement, but can also provide important vitamins, lipids and sterols (Boze *et al.*, 1992), as well as carotenoids (Jacob, 1991).

1.5.2.2 Advantages of the fermenter culture process

Batch cultivation in a fermenter provides free water required by the organisms, removes the heat generated by microbial respiration, and allows control of oxygen transfer from the air supply to the bulk medium, and of the pH of the medium (Cannel and Moo-Young, 1980). These factors make this technique especially useful for the production of antibiotics, vitamins, enzymes, and

polysaccharides (Boze *et al.*, 1992).

1.5.2.3 Acceptability of the product

Commercial production of yeast metabolites is an established practice, with enzymes, such as invertase and lactase (Böing, 1982), citric acid (Kapoor *et al.*, 1982), and fuel alcohol (Reed, 1982*b*), which are well-known examples. Lactase produced from yeast has been applied in the dairy industry for the production of whole milk concentrates, whey concentrates, *etc.* (Böing, 1982).

1.5.2.4 Yeast as carotenoid source

Red - pigmented yeasts, especially those in the classes *Deuteromycetes* and *Basidiomycetes* as represented by the genera *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, and the ballisto-sporogenous genera *Sporidibolus* and *Sporobolomyces*, respectively (Phaff *et al.*, 1966; Andrewes *et al.*, 1976). As discovered more recently, the genus *Phaffia* of class *Blastomycetes* (Miller *et al.*, 1976), are able to produce carotenoid pigments. β-carotene, γ-carotene, torulene, torularhodin and astaxanthin are the principal carotenoids they produce (Simpson *et al.*, 1971; Goodwin, 1972; Miller *et al.*, 1976). The structures of the major carotenoids of yeasts are given in Figure 1.5.2.4. Commercial interest has been shown toward the β-carotene found in such yeasts, as a precursor of vitamin A, and as a yellow pigment for foods (Costa *et al.*, 1987; Neils and DeLeenheer, 1991). Astaxanthin, which is the principal carotenoid pigment produced by *Phaffia rhodozyma*, is responsible for the red and pink colors of lobsters, crabs and

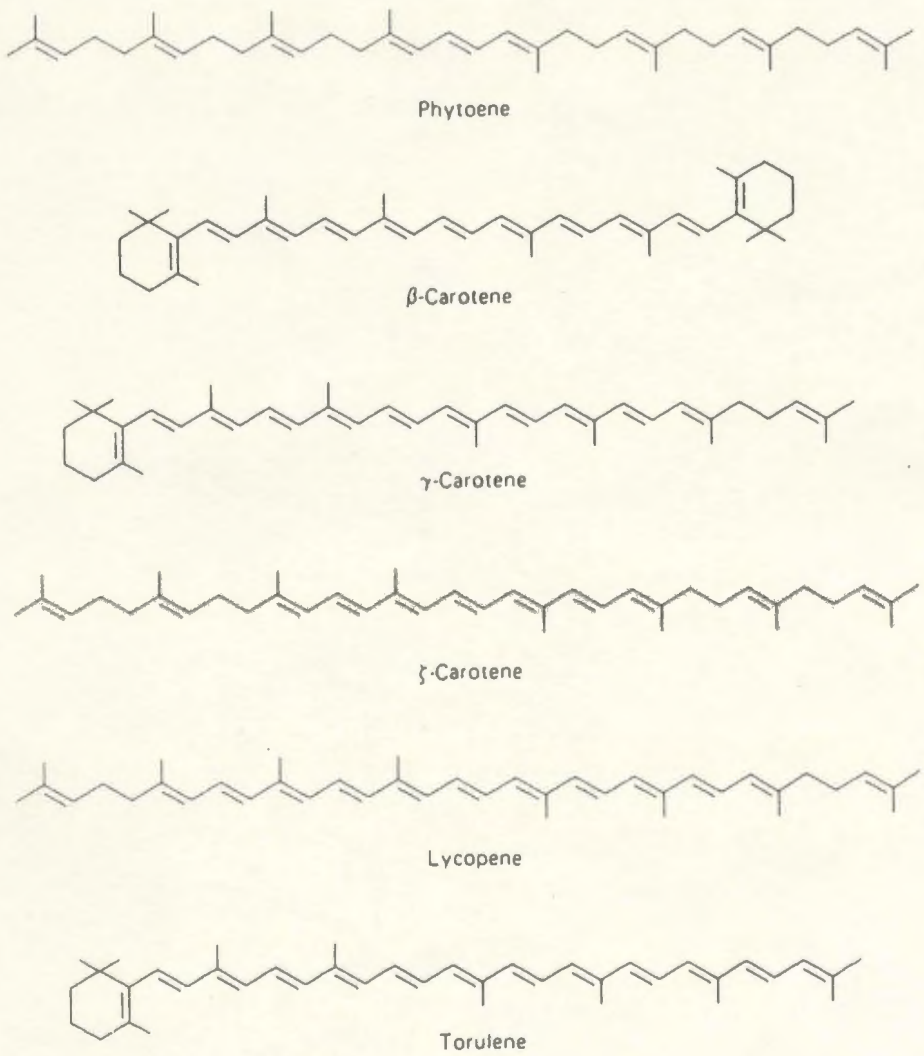


Fig. 1.5.2.4. Structures of the major carotenoids of yeast

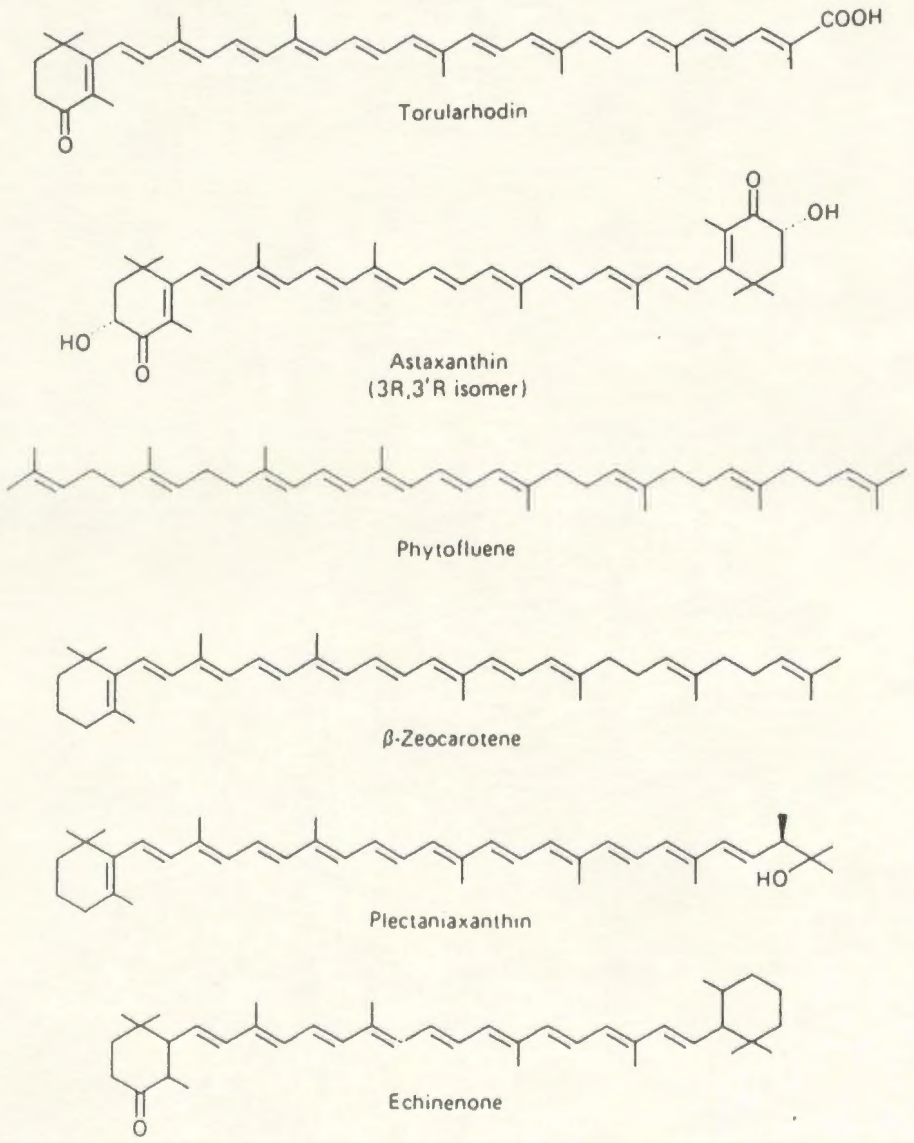


Fig. 1.5.2.4. Structures of the major carotenoids of yeast

shrimp, and the flesh color of such fish as trout and salmon (Johnson and Lewis, 1979). Considerable commercial interest has been shown in using this yeast as a pigment source for aquaculturally-produced animals (An *et al.*, 1989). Johnson *et al.* (1980) and Gentles and Haard (1990) studied the feasibility of using *Phaffia rhodozyma* biomass as a component of diets in aquaculture. However, the amounts of carotenoids produced by these yeasts are small: *Sporobolomyces* produced 250.5 μg of carotenoids per gram of dry yeast, *Rhodotorula* produced 30 to 170 μg / g (Phaff *et al.*, 1966), and *Phaffia rhodozyma* produced 295 μg / g (An *et al.*, 1989). This, combined with the difficulties of extracting the carotenoids due to the rigid yeast cell wall of glucan constituents (Phaff *et al.*, 1966), has hindered their production in commercial industry.

1.5.2.5 Enhancement of the production of yeast pigment

Efforts have been made to increase the carotenoid production of yeast by optimization of the growth medium (Simpson *et al.*, 1971; Johnson & Lewis, 1979). A growth medium contained cerelose at 20 g/L; $(NH_4)_2SO_4$ at 2 g/L; KH_2PO_4 at 1 g/L; $MgSO_4 \cdot 7H_2O$ at 0.5 g/L; $CaCl_2 \cdot 2H_2O$ at 0.1 g/L; and yeast extract at 2 g/L, has been used by Johnson and Lewis (1979) for carotenoids production. The use of alternative carbon sources like alfalfa (Okagbue and Lewis, 1984), sugar cane juice (Matelli *et al.*, 1990) and peat hydrolysate (Fuchsman, 1980) for the improvement of carotenoid production has been reported.

Inhibitors of the respiratory metabolic pathway have also been used for the

enhancement of carotenoids production by yeast. An *et al.* (1989) reported that, by growing the *Phaffia rhodozyma* strain in the presence of antimycin A, the strain contained up to 2 - 2.5 mg of astaxanthin / g of yeast. β-ionone, an inhibitor that stimulated the amount of carotenoids formed by *Phycomyces blakesleeanus* to a marked degree (Mackinney *et al.*, 1952), however, decreased the total amount of carotenoids formed by *Rhodotorula* spp. (Simpson *et al.*, 1964).

The mutation and selection methods have been used for the enhancement of carotenoid production by *Rhodotorula* spp. (Nakayama *et al.*, 1954; Villoutreix, 1960; Bonner *et al.*, 1946). Bonner *et al.* (1946) reported that one mutant of *R. rubra* produces twice as much β-carotene as the native strain. An *et al.* (1989) reported the astaxanthin production of *Phaffia rhodozyma* mutants were increased two- to five-fold compared with that of the parental, natural strain. Several patents for new mutant strains of *Phaffia rhodozyma* with increased astaxanthin production have been applied for by commercial industry (Torregross & Prevatt, 1991; Prevatt *et al.*, 1991; Evans *et al.*, 1991).

1.5.2.6 Extraction of the yeast carotenoid pigments

Treatment of the intact yeast cells for the purpose of extraction can be categorized into three methods (Fig. 1.5.2.6): physical, chemical, and biological (Crueger & Crueger, 1990). Each method has its own drawback. Chemical treatment of yeast with acids or alkalis to liberate carotenoids is unsuitable, since carotenoids are labile to them (Davies, 1976). Mechanical or physical disruption

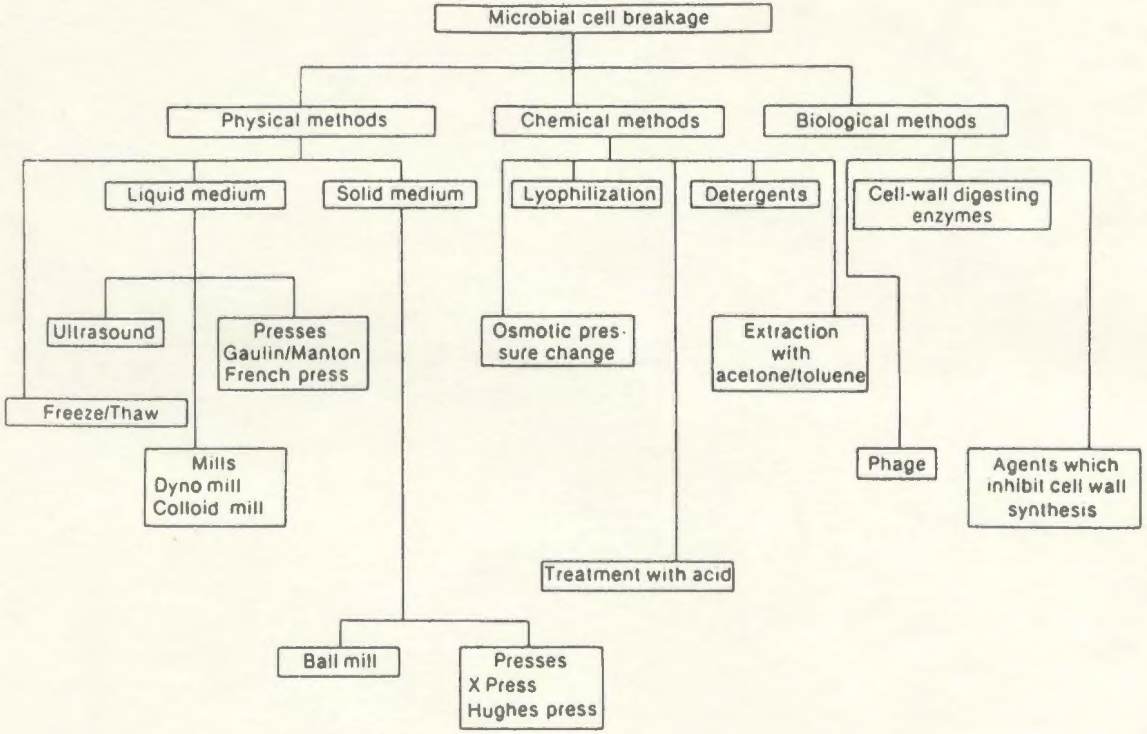


Fig. 1.5.2.6. Treatment of the intact microbial cell

of the cell walls is laborious and unsuitable for large-scale applications. Enzymatic digestion of the yeast cell walls appears to be promising, however, differences in cell wall structure among different yeast species make it difficult to choose an appropriate lytic enzyme for specific applications (Okagbue and Lewis, 1985).

Physical disruption methods such as the French Press (Costa *et al.*, 1987; Matelli *et al.*, 1990) and homogenization (Andrewes *et al.*, 1976; Haard, 1988; Johnson and Lewis, 1979) have been commonly adopted for extraction of carotenoids from yeast. Two basic kinds of enzymatic disruption methods have been reported. One uses pure commercial enzyme disruption (Gentles & Haard, 1990). The other consists of mixing yeast culture with *Bacillus circulans*, and allowing the enzymes produced by the bacilli to disrupt the yeast cell walls (Okagbue & Lewis, 1985; Johnson *et al.*, 1980). In a variation on the second method, Brady (1981) reported the relatively rapid dissolution of *Rhodotorula* spp. cell walls by an enzyme preparation made from culture filtrates of *Penicillium lilacinum*.

More recently, a new chemical disruption method developed by Sedmak *et al.* (1990) using dimethylsulfoxide (DMSO) has proven to be more efficient, and works more quickly, than all the methods previously mentioned. Experimental applications of this disruption method (Johnson and An, 1991) have been reported.

1.6 The Asporogenous yeast, *Rhodotorula rubra*

1.6.1 Biology of the *Rhodotorula* yeast

The genus *Rhodotorula* belongs to the class *Ascomycetes* in the family *Cryptococcaceae.* The genus is asporogenous (Phaff *et al.*, 1966) and includes eighteen species (Barnett *et al.*, 1983). No species has sexual reproduction or filaments; all species, as the name *Rhodotorula* suggests, are of pink to red color, with differences in kind and quantity of carotenoids produced (Liu *et al.*, 1973). Identification of species can be done through physiological tests described by Barnett *et al.* (1983).

1.6.2 Physiology of the *Rhodotorula* yeast

Species in the genus *Rhodotorula* are strictly nonfermentative (Goodwin, 1980), and strong aeration stimulates carotenogensis in them (Bekers *et al.*, 1969; Zeile, 1970). They have relatively simple growth requirements. Molasses (Zeile, 1969), whey (Zalasko *et al.*, 1973), hydrolysed peat (Zalashko *et al.*, 1974), and paraffin (Kvasnikov *et al.*, 1974; Vaskivnyuk *et al.*, 1974) are effective carbon sources for carotenogenesis and growth of *Rhodotorula* species; they can even use ethanol (Reed, 1982*a*) as the sole carbon source for growth. Media with high C:N ratios favour carotenogenesis in *Rhodotorula* spp. (Nakagawa and Tatsumi, 1960). Supplementing the substrate with fatty acids such as oleic acid (Vaskivnyuk *et al.*, 1974), vitamins such as thiamine (Lilly and Barnett, 1951), and trace elements (Goodwin and Lijinsky, 1952), stimulates the growth of

Rhodotorula spp. These yeasts can be cultivated over a wide range of temperatures (5 - 28°C), with the optimal initial pH range for carotenogenesis being 5.7 - 5.9 (Goodwin, 1980). Light stimulates carotenogenesis in *Rhodotorula* spp. (Nakayama *et al.*, 1954; Simpson *et al.*, 1964).

1.6.3 Carotenoids of *Rhodotorula* yeast

The major carotenoids in *Rhodotorula* yeast are β-carotene, γ-carotene, torulene and torularhodin (Liu *et al.*, 1973; Simpson *et al.*, 1964), the latter being found in several but not all of the red species of *Rhodotorula* (Liu *et al.*, 1973; Peterson *et al.*, 1958). Some species of *Rhodotorula* have had unique kinds of carotenoids isolated from them in limited quantities (Bonaly and Malenge, 1968; Bae *et al.*, 1971; Liu *et al.*, 1973).

Mutation has affected the production of carotenoids by yeast quantitatively and qualitatively (Bonner *et al.*, 1946; Villoutreix, 1960; Kayser and Villoutreix, 1961; Simpson *et al.*, 1964), as have changes of temperature. Nakayama *et al.* (1954) reported that, at 25°C, *Rhodotorula glutinis* produces torulin and torularhodin as the main pigments, whereas at other temperatures, less of these carotenes are produced in favour of β- and γ-carotene. The effect of temperature has also been reviewed by Goodwin (1980).

1.6.4 Nutritional value of *Rhodotorula* yeast

Rhodotorula spp. are oleaginous yeasts, containing up to 80 % of the cell dry weight as lipid (Ratledge & Evans, 1989). The amounts and types of lipids

produced by these species have been examined by Yoon and Rhee (1983), and by Yoon *et al.* (1982). The lipids consist primarily of triacylglycerols, and the central position on the glycerol molecule is found to be occupied almost exclusively by an unsaturated acid, giving the yeast oil the essential characteristics of a plant oil (Ratledge, 1981).

Rhodotorula spp. also have a relatively high crude protein content. Jacob (1991) reported a protein content of 21.6 % (dry biomass basis) for *R. gracilis*. Matelli *et al.* (1990) obtained a protein content of 38.2 % of dry biomass for *R. glutinis*, and after extracting the carotenoids, the protein content was raised to 55.7 %, which is considered a good content for a protein feed source.

1.6.4.1 Substrates for carotenoid production by *Rhodotorula* yeast

Rhodotorula spp. have for some years been used for microbial lipid production (Yoon and Rhee, 1983) and also for β-carotene production (Burden and Eveleigh, 1990), although no commercial production for either purpose has ever taken place (Ratledge, 1981; Burden and Eveleigh, 1990).

Variations in the carbon source in the culture medium affect carotenogenesis differently in different species. In *Rhodotorula sannei*, glycerol is the most effective single carbon source, although a mixture of lactose and glucose is equally good; glucose alone is non-carotenogenic, but when colorless cells produced on a glucose / gelatin medium are transferred to a suitable medium they will synthesize pigments (Fromageot and Tschang, 1938). Complex carbon

sources are also suitable for carotenogenesis in red yeasts, for example, molasses is effective for *R. gracilis* (Zeile, 1969) and *R. glutinis* (Stabnikova *et al.*, 1975); whey for *R. lactis* (Zalasko *et al.*, 1973); peat hydrolysate for *R. glutinis* (Zalashko *et al.*, 1974); and paraffin for *R. glutinis* and *R. graminis* (Kvasnikov *et al.*, 1974). Acetate also increases carotenogenesis in *R. sanniei* if the basic medium is low in nitrogen (Vuori and Gyllenberg, 1974). The stimulatory effect of leucine is apparent in *R. gracilis* and increased synthesis is also elicited by addition of glutamic acid (Vecher *et al.*, 1967). However, *R. aurantiaca* synthesizes less carotenoid if grown in the presence of sulphur-containing amino acids than in their absence (Killick, 1972). The addition of sulphur-containing amino acids also has a qualitative effect: it causes the ratio of γ-carotene: β-carotene: torulene: torularhodin to change to 1:1:8:2 from 1:2:17:30 in the standard medium (Haxo, 1950).

1.6.5 *Rhodotorula rubra*

1.6.5.1 Morphology of *Rhodotorula rubra*

R. rubra is cream, pink or red colonies with no filaments (Barnett *et al.*, 1983). Although it appears to be a physiologically stable species, morphological changes such as mutation from smooth to wrinkled colonies have been reported (Phaff and Ahearn, 1970). Mature cells grown in malt extract have a moderate to heavy pink or salmon-colored ring and usually no pellicle, but sometimes they have a partial or complete, slimy, moist film and a moderate sediment. Colonies grown

on malt agar, which have similar cell morphology, usually have a glistening, but sometimes reticulate, rugose or corrugated, surface. Their texture varies from soft to slimy, their cross section is low convex to flat, with an entire border, occasionally developing some rudimentary pseudomycelium at the thin part of the slant. Cells vary from short-ovoidal to elongate, and occur singly, in pairs, in short chains, and in clusters (Phaff and Ahearn, 1970; Fell *et al.*, 1984), and bud freely (Bessey, 1961).

1.6.5.2 Genetics of the *Rhodotorula rubra* yeast

Kreger-van Rij (1987) categorized *Rhodotorula* species into the imperfect genera, which do not usually form ascospores or basidiospores, the imperfect genera like *Rhodotorula* species are basidiomycetous. Middlehoven *et al.* (1990) described *R. mucilaginosa* (once considered the same species as *R. rubra*) as imperfect basidiomycetous. Generic differentiation of the imperfect yeasts is based on morphological and a few physiological characteristics, such as acid production and assimilation of inositol. An important characteristic of *Rhodotorula* spp. are their inability to assimilate inositol, distinguishing *Rhodotorula* from *Cryptococcus* (Kreger-van Rij, 1987).

Vegetative cells of *R. rubra* are produced by budding (Barnett *et al.*, 1983; Kreger-van Rij, 1987). The buds may have a narrow or a broad base, and they may occur multilaterally on the cell or confined to special regions, for instance on the shoulders of elongate cells or exclusively at one or both poles. Budding cells

which remain attached to each other are referred to as pseudomycelium (Kreger-van Rij, 1987). From studies by scanning electron microscope, Kurnatowska *et al.* (1977) reported that the detachment of blastospores from vegetative cells or pseudohyphae of *R. mucilaginosa* takes place by elongation of the 'bridge' joining 2 cells or by formation of a 'collar'.

1.6.5.3 Carotenoids synthesis in *Rhodotorula rubra* yeast

The entire pathway of carotenoids synthesis in *R. rubra* is described in Figure 1.6.5.3. It can be divided into three stages: (1) the synthesis of the basic C_5 terpenoid; (2) the synthesis of the C_{40} compounds from the terpene intermediates; and (3) the transformation of C_{40} chains into various carotenoids (Ratledge and Evans, 1989). Three main phases of carotenoid synthesis are observed in *R. rubra*: (1) an initial period of active synthesis leading to maximum concentration; (2) an intermediate period of persistence during which no major changes, either qualitative or quantitative, occur; and (3) a final period during which the pigments disappear (Goodwin, 1980).

1.6.5.4 Cultivation conditions for the growth and carotenogenesis of *Rhodotorula rubra*

The reported optimal initial pH for carotene synthesis of the genus *Rhodotorula* is between 4.0 - 4.7. It has been reported that during cultivation, if the pH is not controlled, it drops to pH value around 3 after 72 hrs, and then rises to a final value of 5 - 6 (Bujak and Podgorska, 1968). The pigments produced by

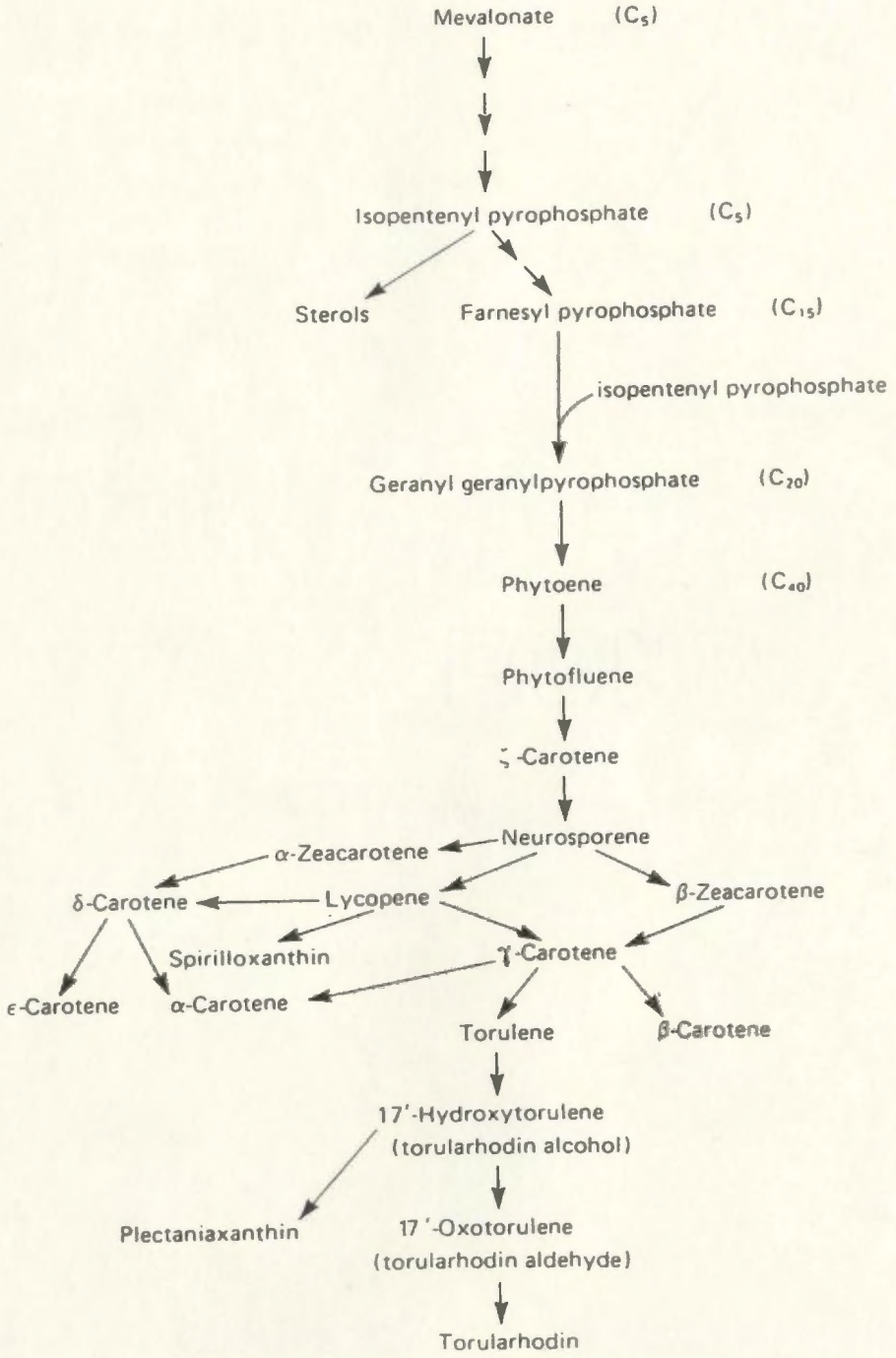


Fig. 1.6.5.3. Biosynthesize of carotenoids in *Rhodotorula rubra*. (Ratledge and Evans, 1989)

R. rubra are the same over a temperature range of 5 - 25°C (Nakayama *et al.*, 1954). *R. rubra* is a strictly non-fermentative yeast, oxygen being necessary for its growth and carotenogenesis (Goodwin, 1980). Light stimulates carotenogenesis in *R. rubra*, although it normally forms comparable amounts in the dark (Nakayama *et al.*, 1954). Other factors, such as the sources of carbon, nitrogen and energy, and especially the C:N ratio, are important for the carotenogenesis of *R. rubra*: a low carbon to nitrogen (C:N) ratio will be much less effective than a high C:N ratio (Nakayawa and Tatsumi, 1960).

1.7 Peat

Peat is the soil of peatlands, consisting largely of organic residues accumulated as a result of incomplete decomposition of the dead plant constituents under wet conditions where oxygen is limited or excluded. The vegetation constituents of the peat deposits comprise a broad spectrum of plant species ranging from trees, shrubs and herbs to sedges, grasses and mosses (LeDuy, 1979).

Peat typically accumulates at a rate of about 3 cm per 100 years in a bog whose vegetation is well-established, and where hydrological conditions are favourable. On the average, it takes 3000 or 4000 years to accumulate a meter of peat (Fuchsman, 1980).

1.7.1 Distribution of world peat resources

About 230 million hectares of peatlands are estimated to exist in the world.

These are covered with 3.0×10^{11} dry tons of organic matter, which represents 1.7 $\times$ 10^{19} kcal of potential energy for future use. Table 1.7.1 shows the world distribution of peat resources together with annual peat production figures. Over 85 % of the world's peat resource is found in Canada and in the former USSR. The contemporary world peat exploitation rate is approximately 90 million metric tons per year. Canada has 56 % of the reserves but accounts for only 0.13 % of the total world production, and exports 90 % of its product to the USA for horticulture (LeDuy, 1979).

1.7.2 Classification and chemical composition of peats

According to its geological, botanical and physicochemical characteristics, peat can be categorized mainly into two types: "low-moor" and "high-moor". The geological characteristic of greatest importance is the relationship of the water in the peat deposit to the main groundwater system. If it is continuous with the groundwater system of the adjacent mineral soils, the peatland is said to be "low-moor". If the peat water system is significantly above the groundwater system, the peatland is said to be "high-moor" (Fuchsman, 1980).

Botanical assessment of the peat consists mainly in the identification of the plants that grow most profusely in the bog. High-moor peats are characterized principally by mosses (*Sphagnum*), cottongrass (*Eriophorum*), and heath plants (various genera of the Ericaceae); low-moor peats are more likely to be characterized by frondiferous mosses (*Hypnum*), reeds (*Phragmites*), and sedges

Table 1.7.1 World peat resources [1]

Country	Extent of resources		Annual production	
	Thousand hectares	%	Thousand hectares	%
Canada	129,500	56.18	120	0.13
USSR	71,500 [2]	31.01	86,130	95.70
Finland	10,000	4.33	-	-
USA	7,500	3.24	260	0.29
Norway	3,000	1.29	70	0.08
Germany	1,618	0.68	1,005	1.12
UK	1,582	0.68	-	-
Sweden	1,500	0.64	140	0.16
Poland	1,500	0.64	45	0.05
Ireland	1,000	0.42	-	-
Others	1,784	0.89	2,230	2.47
Total	230,484	100.00	90,000	100.00

[1] LeDuy (1979)

[2] These are exploitable reserves only

(*Carex*), and by woody plants. Characteristically, high-moor plants thrive in mineral-poor waters, while low-moor plants require higher levels of inorganic solutes.

The physicochemical state of the peat pertains to the degree to which the fibrous content of the bog plants has decomposed into nonfibrous solids. Low-moor peats are most commonly 25 - 45 % decomposed, while high-moor peats are usually less than 25 % decomposed.

The main varieties of peat can also be distinguished by their acidity and by the amount of ash remaining when the peat is burned. High-moor peats are typically quite acid, having pH values of 3 - 5; in low-moor peats the pH is 4 - 7.5. The ash of high-moor peats is typically below 3 % (dry weight basis); that of low-moor peats is 3 - 14 % (Fuchsman, 1980).

Characteristically, high-moor *Sphagnum* peats are only slightly decomposed and have a high polysaccharide content, making them particularly suitable for processes such as yeast culture, which require large amounts of potentially metabolizable carbohydrates (Fuchsman, 1980).

The major chemical components of peat are listed in Table 1.7.2.

1.7.3 Hydrolysis of peat

Carbohydrates of many types exist as components in peat. They are conveniently grouped as; (1) water-soluble or easily hydrolysable, these consist of hemicelluloses, some glycosides, and possibly pectins; and (2) water insoluble

Table 1.7.2 Compositions of peat [1]

Group	Description
Bitumens	Waxes and resins soluble in ether, ethanol, benzene, etc.
Water-soluble substances	Pectins, sugars and other soluble carbohydrates, proteins, tannins
Hemicelluloses	Holocellulose fraction soluble in 5.3 N alkali at 20°C
α-Cellulose	Holocellulose fraction insoluble in 5.3 N alkali at 20°C
Humic acids	Substances soluble in alkali and precipitated by acid
Humins	Substances insoluble in alkali and in concentrated acid
Ash	Inorganic constituent

[1] LeDuy (1979)

and difficult to hydrolyse, which is limited to cellulose. Hydrolysis is the principal technique employed both in the analysis of peat carbohydrates and in the industrial preparation of the carbohydrates for further uses. Hydrolysis requires the chemical addition of a molecule of water to effect the cleavage of ether (or ester) bonds. This addition is accomplished in nature through the intermediation of enzymes. In chemical technology, acids are usually used to promote carbohydrate hydrolysis.

Typically the peat used for hydrolysis processes contains about 42 -54 % "water-soluble and easily hydrolysable substances". The difficult part to hydrolyse cellulose is present at about 24 - 27 %. As a result of hydrolysis, 90 - 95 % of the "easily hydrolysed" components and 40 - 60 % of the cellulose are recovered in the liquid phase. The composition of monosaccharides hydrolysed from the first group with dilute sulphuric acid are listed in Table 1.7.3 (Martin and Manu-Tawiah, 1989).

For the second group, except at elevated pressures and temperatures (150 - 180°C), dilute sulphuric acid is ineffective in hydrolysing cellulose. When more concentrated sulphuric acid (normally 50 - 80 % H_2SO_4) is employed, the crystal structure of cellulose is disrupted and the larger cellulose fragments dissolved. The increased concentration of the acid and elevation of the temperature results in the substantially complete conversion of cellulose to glucose.

Acid hydrolysis is not devoid of side reactions. The partial decarboxylation

Table 1.7.3 Major monosaccharide composition of the peat hydrolysate [1]

Monosaccharide	% of total reducing sugars
Arabinose	2.48 ± 1.11
Galactose	19.07 ± 1.67
Glucose	38.20 ± 1.31
Mannose	16.46 ± 1.83
Rhamnose	6.96 ± 1.62
Xylose	12.03 ± 1.15

[1] Martin and Manu-Tawiah (1989)

of galacturonic acid to give arabinose, and of glucuronic acid to give xylose, causes some uncertainty about the original chemical state of arabinose and xylose found in the hydrolysates.

Nitrogen constitutes 1 - 3 % of the dry weight of peat and, after hydrolysation, part of this exists in the form of amino acids. These play an important quantitative role in the production of high-protein yeasts.

1.7.4 Utilization of peat hydrolysate

The direct production from peat hydrolysate of individual chemical substances such as furfural, lactic and glycolic acids, polyhydric alcohols, etc., has been investigated. However, the major economic interest in peat hydrolysis centres on the use of the hydrolysate by microorganisms as fermentation substrates (LeDuy, 1979; Fuchsman, 1980; Martin, 1986; Manu-Tawiah and Martin, 1987; Martin *et al.*, 1990) to produce protein, fats, vitamins, and alcohol, *etc.* As shown in Table 1.7.4, bacteria, yeasts and fungi can use peat hydrolysate as their sole or main substrate for various fermentation processes for the production of SCP, lipids and carotenes, etc.

1.7.4.1 Cultivation of yeast in peat hydrolysates

Yeast culture has long been regarded as the best way to utilize the sugars released by peat hydrolysis. The connection between yeast and sugar has focused on fermentation, through which process quantities of alcohol, high-protein yeasts suitable for the diets of animals or humans, fat and carotene are produced.

Table 1.7.4 Production of microbial products from peat extract

Microorganisms	Purpose	References
BACTERIA		
Bacillus polymyxa	SCP	LeDuy, 1979
Clostridium acetobutylicum	SCP	LeDuy, 1979
YEAST		
Candida humicola	SCP & Lipid	LeDuy, 1979; Zalashko, 1977
Candida tropicalis	SCP & Lipid	LeDuy, 1979; Zalashko, 1977
Candida utilis	SCP	LeDuy, 1979
Candida spp.	SCP	LeDuy, 1979
Sporobolomyces pararoseus	SCP & Carotene	LeDuy, 1979; Raitsina and Evdokimova, 1977
Rhodotorula glutinis	Carotene	Raitsina and Evdokimova, 1977
Baker's yeast	SCP	LeDuy, 1979
Fodder yeast	SCP	LeDuy, 1979
FUNGI		
Oidium lactis	SCP	Kalyuzhnyi and Ivlieva, 1973
Agaricus bisporus	SCP	Martin, 1983*a,b*; 1986
Pleurotus ostreatus	SCP	Manu-Tawiah and Martin, 1987
Scytalidium acidophilum ATCC 26774	SCP	Martin *et al.* 1990

These examples were described in detail by Fuchsman (1980). The production of high levels of carotene by growing the yeasts *Sporobolomyces pararoseus T* and *Rhodotorula glutinis T-2* on peat hydrolysate has been reported (Raitsina and Evdokimova, 1977). Biomass yields, based on the dry weight of the reducing substances, of 50 % for *S. pararoseus T* and 32.2 % for *R. glutinis* have been obtained. Carotenoids contents are 250.5 μg/g of dry yeast for *S. pararoseus T* and 152.7 μg/g for *R. glutinis*. All monosaccharides present, except rhamnose, were consumed by the yeast.

1.8 Molasses

Molasses of various kinds are by-products of the sugar industry. They are the concentrated syrups or mother liquors recovered at any one of several steps in the sugar-refining process (Casida, 1964*b*). Their availability, ease of handling, and relatively low price make them the most widely-used carbon source in many industrial fermentations, and in the production of feed and fodder, where they are fortified with other ground feedstuffs for livestock and poultry (Jacob, 1991).

Major components of molasses are listed in Table 1.8, of which sucrose accounts for 33.4 % and invert sugar for 21.2 % of the amount of the cane molasses. Although molasses has been considered a potential source of both carbon and nitrogen (Jacob, 1991), the nitrogenous compounds in molasses cannot be relied upon to serve as an adequate source for microorganisms, and in practice most of the required nitrogen in molasses media is supplied by added ammonium salts, liquid

Table 1.8 Percent composition of cane and beet molasses [a]

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

[a] Imrie (1969)

ammonia, or urea (Reed, 1982*c*).

1.8.1 Classification of molasses

Cane molasses or beet molasses, or a mixture of both, is the most important raw material in the industrial production of baker's yeast. The types of molasses which have been utilized for yeast propagation are beet, high test cane, refinery cane and blackstrap. The sugar content of both cane and beet molasses is about 50 %, but cane molasses has a lower content of sucrose in proportion to the total sugar content and contains more invert sugar than does beet molasses (Olbrich, 1956). Cane molasses contains guanine, xanthine and hypoxanthine, and, in addition, the pyrimidine 5-methyl-cytosine (Binkley and Wolfrom, 1953). The biotin content of blackstrap molasses varies from 2.7 to 3.2 ppm, which is higher than that of beet molasses, which contains from 0.04 to 0.13 ppm. However, the calcium D-pantothenate content of beet molasses (50 - 110 ppm) is higher than that of cane molasses (54 ppm). The content of *myo*-inositol in blackstrap molasses is 6000 ppm (White and Munns, 1950). Blackstrap molasses contains a considerably larger number of growth factors (White and Munns, 1950), and a higher riboflavin content (Rogers and Mickelson, 1948; Binkley and Wolfrom, 1953), than does refinery cane molasses. It has been reported that both beet and blackstrap molasses additionally contain thiamine, pyridoxine and folic acid (Rogers and Mickelson, 1948; Binkley and Wolfrom, 1953).

1.8.2 Pretreatment of molasses

Generally, there are two methods for clarification of molasses: chemical methods and ion exchange resin methods. Chemical methods can be categorized into three types: 1) the addition of certain chemicals; 2) adjusting the pH; and 3) a combination of (1) and (2), followed by boiling, centrifugation to obtain clarified supernatant, and dilution to the desired concentration. Ion exchange resin methods can involve anion exchange resin, cation exchange resin, or a combination of the two. Buckley and Norton (1991), who reported treatment of molasses by both chemical and ion exchange resin methods, described these methods in detail.

1.8.3 Cultivation of yeast on molasses

Molasses has long been used as the major carbon source for yeast fermentation in industry. Its use for the production of different products is according to its quality. The highest quality molasses is usually demanded by the more fastidious industries, *i.e.*, for antibiotic production. Poor quality molasses may be used for citric acid production, and the poorest quality molasses finds use only in the production of alcohol (Ratledge, 1978). Rolz (1984), Kapoor *et al.* (1982), and Reed (1982*b*) provided details of these examples. High levels of astaxanthin (1086 µg/g) and dry biomass (14.1 g/L) production were reported by Haard (1988), who grew the yeast *Phaffia rhodozyma* on molasses, indicating that molasses is a promising material for the production of highly pigmented yeast.

1.9 Malt Wort

Wort is the extract prepared from malt, which is germinated barley, and it is used in the production of beer through yeast fermentation. Wort contains glucose, fructose, sucrose, maltose, maltotriose and maltotetraose (Harris *et al.*, 1951; Russell *et al.*,1987; Hough *et al.*, 1981), as well as small amounts of the pentoses arabinose, xylose, and galactose (Green and Stone, 1952; Montreuil *et al.*, 1961).

The nitrogenous compounds of wort vary with the quality of the barley and the treatment used (Jones and Pierce, 1963). The wort contains ammonium nitrogen in amounts of 2.18 - 2.44 mg per 100 mL wort (Ljungdahl and Sandegren, 1953). The content of α-amino nitrogen in wort varies between 99.5 and 162.2 μg/g dry grain, depending on the total nitrogen (Jones and Pierce, 1963). Okagbue and Lewis (1984) reported that wort, or wort combined with molasses, favours the growth and carotenogenesis of *P. rhodozyma*.

Table 1.9 Composition of brewer's wort [1]

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

[1] Sikyta (1983)

1.10 Objectives of this work

The main objectives of this work can be divided into five parts as follows:

1. To extract some of the organic components present in peat and use them in the production of a valuable source of carotenoids.
2. To study the operational variables and kinetic parameters in the fermenter cultivation of *R. rubra* in peat extract.
3. To obtain scientific information on the feasibility of peat extract and molasses as fermentation media, using *R. rubra* as a model.
4. To compare the nutritional value of *R. rubra* produced using peat extract as the main fermentation substrate with the nutritional value of *R. rubra* produced using molasses.
5. To provide preliminary information on potential nutrient sources for the production of red yeast as a carotenoid source and as a fodder SCP, utilizing peat, a Canadian raw material, and molasses.

1.11 Scientific information to be obtained from this work

This work seeks to determine:

1. The usefulness of the peat from some peat reserves of Newfoundland as an inexpensive substrate for producing carotenoids and feedstuffs.
2. The optimal growth and carotenogenesis conditions, kinetic parameters of growth and the physiology, including nutrient

requirements, of *R. rubra*, with peat extract as substrate.

3. The optimal growth and carotenogenesis conditions, and optimal medium composition, for the growth of *R. rubra* with molasses as substrate.
4. The β-carotene content of *R. rubra* using a modified method of carotenoids extraction.
5. The composition, including protein content, essential amino acid composition, lipid content and fatty acid composition of *R. rubra* produced with peat extract as substrate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 *Sphagnum* peat moss

A high-moor *Sphagnum* peat moss was obtained from Sundew Peat Bog, near the city of St. John's, Newfoundland, Canada. The peat was of a relatively low degree of decomposition and the humification value, in the von Post scale, corresponded to H2 (Martin and Bailey, 1984). The particle size ranged from coarse to > 60 mesh.

2.1.2 Cane molasses and Brewer's wort

The cane molasses was obtained from Lalle Nand Inc., Montreal, Quebec, Canada. The brewer's wort was kindly provided by Labatts Brewery Inc., St. John's, Newfoundland, Canada.

2.1.3 Chemicals

All the chemicals used in this work were of reagent or laboratory grade. The following were obtained from Fisher Scientific Co. Ltd. New Jersey, U.S.A.: ammonium sulphate, boric acid, chloroform, dimethylsulfoxide (DMSO), ethanol (95 %), ethyl ether (anhydrous), D-glucose (anhydrous), hydrochloric acid, hydrogen peroxide, magnesium sulphate heptahydrate, methanol, methylene red, methylene blue (water soluble), potassium phosphate dibasic, potassium phosphate monobasic, potassium sulphate, sodium hydroxide, sodium phosphate

dibasic, sodium phosphate monobasic, and sulphuric acid.

The following were obtained from Difco Laboratories, Detroit, MI, U.S.A.: yeast extract, and YM agar.

Anthrone was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ, U.S.A.

Nitrogen gas was obtained from Liquid Air Canada, St. John's, Newfoundland, Canada.

2.1.4 Culture

A new strain of *Rhodotorula rubra* yeast was isolated by Dr. T.R. Patel of the Biochemistry Department of Memorial University of Newfoundland, and its identity confirmed as indicated by Hari *et al.* (1992). It was maintained on YM agar slants, transferred every two weeks, and stored at 4°C until needed.

2.2 Methods

2.2.1 Preparation of peat extract

The peat obtained (with a moisture content of approximately 80 %), was air-dried, and the peat extract was prepared according to the method of Martin and Bailey (1985). This involved mixing peat with 1.5 % H_2SO_4 in a ratio of 100 g of dry peat to 500 g of acid solution, and autoclaving it at 15 psi (121 ± 1°C) for 2 hours. The extract was separated from the solid residues by pressing, in a Carver Laboratory Press (Model C, F.S. Carver Inc., WI, U.S.A.), followed by filtration through Whatman No. 1 filter paper. The peat extract was stored in glass

containers at 4°C until use.

2.2.2 Preparation of the semi-synthetic medium

The semi-synthetic medium was prepared by dissolving the following chemicals in distilled water, in amounts calculated to give the specified concentrations: K_2SO_4, 0.174 g/L; $MgSO_4 \cdot 7H_2O$, 0.5 g/L; $(NH_4)_2SO_4$, 4 g/L; yeast extract, 1.5 g/L. To complete its preparation, a solution of glucose was prepared and sterilized separately, and added to the above solution to give a 15 g/L total carbohydrate concentration (TCH) in the completed semi-synthetic medium.

2.2.3 Pretreatment of the molasses

The molasses was received as a very thick liquid. Its weight and volume were measured, it was diluted with distilled water, and the pH of this solution was adjusted with 10 M NaOH solution. After sterilization (by autoclaving at 15 psi., 121 ± 1°C, for 30 minutes), the solution was centrifuged and the clear supernatant was collected and stored in sterile glass container at 4°C until use.

2.2.4 Preparation of wort

The wort was sterilized (by autoclaving at 15 psi, 121 ± 1°C, for 30 mins.), followed by centrifugation to precipitate the sediment and obtain a clear wort solution. It was then stored in sterile glass container at 4°C until use.

2.2.5 Optimization of growth and carotenogenesis conditions in shake flasks

The growth and biomass production of the *R. rubra* yeast in peat extract, molasses, and semi-synthetic medium when necessary, was studied in shake flasks. The pH values of the peat extract and semi-synthetic growth media were adjusted to the required levels by the addition of 10 M NaOH, before sterilization at 121 ± 1°C for 30 minutes.

2.2.5.1 Inoculum preparation

The inocula for the shake flask experiments were prepared from fresh YM agar culture slants. One single colony of the strain was transferred aseptically to a sterile 150 mL flask containing 20 mL of sterile YM broth. After incubating the suspension at 22°C and 200 rpm for 24 hours in a Gyrotory water bath shaker (Model G 76, New Brunswick Scientific Co. Inc., Edison, NJ, U.S.A.), a sample was taken to measure the cell concentration in the suspension (in terms of the OD_{600} value) measured by the tubidimetry method from the spectrophotometer, and the rest was used to inoculate 50 mL of sterile growth media in 150 mL shake flasks at an inoculum rate of 2 % (v/v). These cultures were incubated under the same conditions as the inoculum.

2.2.5.2 Preliminary studies

Preliminary experiments were conducted to determine the appropriate TCH concentration (derived from peat extract) for the media, and to determine the

effects of yeast extract supplementation. Peat extracts, with nitrogen / vitamin supplementation (5 g/L of yeast extract), and without, were diluted with water to 12, 15, 18 g/L of total carbohydrate. After inoculating the media as described, the cultures were incubated at 22°C with an agitation speed of 200 rpm, for 96 hours (Hari *et al.* 1992).

2.2.5.3 Determination of the nutritional requirements for *R. rubra* growth and carotenogenesis

The effects of nitrogen, phosphorus, potassium, and magnesium (in concentrations specified below) on the growth and carotenogenesis of *R. rubra* in the peat extract medium were studied, as was the effect of different concentrations of wort (2, 4, 6 % v/v) as a supplement to the 10 % v/v molasses (Hari *et al.*, 1993) medium. The following growth conditions were employed in both studies: a temperature of 22°C, an initial pH of 5.5, an inoculum ratio of 2.0 % (v/v), an agitation speed of 200 rpm, and a fermentation time of 96 hours.

2.2.5.3.1 Addition of nitrogen to peat extract

The effect of different concentrations of an inorganic nitrogen source (ammonium sulphate at 3, 4, and 5 g/L), combined with different concentrations of yeast extract added (at 0.5, 1.0, and 1.5 g/L) as a source of vitamins, on the growth and the biomass production of *R. rubra* in peat extract medium were studied for the optimization of the growth of the yeast. The following growth conditions were employed in these studies: a temperature of 22°C, an initial pH

of 5.5, an inoculum ratio of 2.0 % (v/v), an agitation speed of 200 rpm, and a fermentation time of 96 hours.

2.2.5.3.2 Addition of phosphorous

The effects of the addition of different sources of phosphorus (K_2HPO_4, KH_2PO_4), at 3 mM of [P^{5+}], were studied to determine which phosphorus source was more suitable for the growth of yeast biomass in both semi-synthetic medium (15 g/L of glucose; 4 g/L of $(NH_4)_2SO_4$; 1.5 g/L of yeast extract) and peat extract medium (15 g/L TCH of peat extract; 4 g/L of $(NH_4)_2SO_4$; 1.5 g/L of yeast extract). The following growth conditions were employed in this study: a temperature of 22°C, an initial pH of 5.5, an inoculum ratio of 2.0 % (v/v), an agitation speed of 200 rpm, and a fermentation time of 96 hours.

2.2.5.3.3 Addition of potassium and magnesium

Experiments were conducted to study the effects of potassium and magnesium on the growth and β-carotene production of *R. rubra* in the peat extract medium. Potassium was supplied in the form of potassium sulphate (K_2SO_4) and magnesium was supplied in the form of magnesium sulphate ($MgSO_4 \cdot 7H_2O$). A nitrogen supplementation of 4 g/L $(NH_4)_2SO_4$ and 1.5 g/L yeast extract was also used.

Potassium was tested at concentrations of 2, 3, and 4 mM. Magnesium was tested at concentrations of 2, 4, and 6 mM. The following growth conditions were employed in these studies: a temperature of 22°C, an initial pH of 5.5, an

inoculum ratio of 2.0 % (v/v), an agitation speed of 200 rpm, and a fermentation time of 96 hours.

2.2.5.4 Studies on the operational conditions for the growth and carotenogenesis of *R. rubra*

Using peat extract, diluted to give a solution of 15 g/L total carbohydrate, and an inoculum ratio of 2.0 % (v/v), and with 1.5 g/L yeast extract, 4 g/L $(NH_4)_2SO_4$, 0.174 g/L K_2SO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ added as supplementation, the effects of temperature (18, 20, 22, 24, and 26°C), initial pH (4, 4.5, 5, 5.5, and 6), agitation speed (150, 200, 250, and 300 rpm) and fermentation time (60, 72, 84, 96, and 120 h) were investigated in three separate experiments.

2.2.6 Batch fermentation

Batch fermentation experiments were conducted to optimize the mechanical agitation speed and aeration rate, and also to study the kinetics of the growth of *R. rubra* in the fermenter. The experiments were conducted in a 2-Litre Multigen fermenter (Model F-2000, New Brunswick Scientific Co.), operated with control of pH, aeration, agitation and temperature. The fermenter was equipped with a four-blade, turbine-type impeller to mix the medium, and a built-in mechanical pump that delivered filter-sterilized air to the culture through the agitator shaft. The pH of the growth medium was adjusted to 5.5 manually every 12 h to keep it constant using a pH meter (Model DO-40) and a Model pH-40 pump module (New

Brunswick Scientific Co.) to add the acid or base.

The fermenter system was operated under aseptic conditions, at a controlled temperature of 22 ± 1°C and pH of 5.5 ± 0.1. The pH was adjusted by the addition of 0.5 M NaOH or 1.0 M H_2SO_4 solution. Microscopic observations of culture samples were used to verify the asepticity of the conditions during the experiments.

2.2.6.1 Preparation of Inocula and Growth Media

For the batch fermentation experiments, *R. rubra* was propagated in two different media for inoculation, according to the type of medium each was to be used for. Type A inocula were propagated in 150 mL shake flasks with 20 mL of growth medium A. Growth medium A consisted of peat extract diluted to 15 g/L, to which was added 4 g/L $(NH_4)_2SO_4$, 1.5 g/L yeast extract, 0.174 g/L K_2SO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$. Type B inocula were propagated in 150 mL shake flasks with 30 mL of growth medium B. Growth medium B consisted of molasses (10 % v/v), and wort (4 % v/v). The propagation conditions for both inocula were as follows: an incubation temperature of 22.0 ± 1.0°C, an initial pH of 5.5 ± 0.1, an inoculum ratio of 2.0 % (v/v) and an agitation speed of 200 rpm. A fermentation time of 24 h was used to obtain active yeast starter cultures.

The growth of one shake flask was used to inoculate 1 L (for medium A) or 1.5 L (for medium B) of sterile (autoclaved at 121 ± 1°C for 30 minutes) growth medium at an inoculum ratio of 2.0 % (v/v).

2.2.6.2 Optimization of agitation speed and aeration rate

Three levels of mechanical agitation speed (150, 200 and 250 rpm) and four rates of aeration (0.75, 1.0, 1.5 and 2.0 vvm) were tested in separate experiments to optimize the growth and carotenogenesis of the yeast in peat extract medium in batch fermentations. Four levels of mechanical agitation speed (150, 200, 250, and 300 rpm) and three rates of aeration (0.75, 1.0, and 1.5 vvm) were tested in separate experiments to optimize the growth and carotenogenesis of the yeast in molasses medium in batch fermentations. All the batch cultivation experiments were performed under the same operating conditions : an incubation temperature of 22.0 ± 1.0°C, an initial pH of 5.5 ± 0.1, an inoculum ratio of 2.0 % (v/v) for 72 h.

2.2.6.3 Determination of the growth kinetic parameters

The optimal conditions of temperature (22°C), initial pH (5.5), mechanical agitation speed (200 rpm) and aeration rate (1.0 vvm) previously determined were employed to study the kinetics of the growth of the *R. rubra* strain in peat extract medium (Medium A). The progress of the cultivation was monitored over a period of 134 h for two separate fermentations by determining the growth in terms of biomass concentration, specific growth rate, and residual TCH concentration, and the carotenogenesis in terms of β-carotene production.

2.3 Analytical methods

2.3.1 Determination of the dry biomass

The total biomass concentration (calculated as dry weight) was determined by the turbidimetry method, using a Beckman UV/VIS Spectrophotometer (Model DU-8) at 600 nm. For this, a standard curve was set up of the light absorbence values (*ca.* 0.2 - 0.9) of a series of suspensions in distilled water (8 mL) of increasing amounts (0 - 6 mg) of yeast cells, versus their corresponding dry weights (determined by oven-drying to constant weight at 108°C an equivalent amount of yeast cells). After fermentation, the yeast cells were centrifuged, washed twice with distilled water, resuspended into a volume of distilled water equivalent to the original supernatant, and the absorbence value measured at 600 nm. Using this, the dry weight of the biomass was read from the standard curve. The initial inoculum dry weight was determined by the same procedure and was subtracted from the total biomass dry weight to obtain the dry weight of the yeast produced. The biomass yield was calculated as grams of yeast dry weight produced per gram of the total carbohydrate consumed, and the efficiency was calculated as grams of yeast dry weight produced per gram of the total carbohydrate supplied.

2.3.2 Determination of the total carbohydrate (TCH) concentration

The TCH concentrations of the growth medium before and after fermentation were determined by the Anthrone reagent method (Hodge and

Hofreiter, 1962). The samples were diluted to the appropriate range (*ca.* 20 - 200 mg/L of equivalent glucose), 2 mL of this was placed in test tubes, and 4 mL of anthrone reagent was added. After heating these solutions in boiling water for 16 minutes and then cooling them, they were read against a blank solution (2 mL distilled water and 4 mL anthrone reagent) at 625 nm, using a Beckman UV/VIS Spectrophotometer (Model DU-8). The results were expressed as equivalent glucose concentrations. The TCH concentrations in the dry biomass were determined based on the same procedure. About 0.25 g of freeze-dried sample was extracted with ether for two hours (i.e. until essentially fat-free). The extracted sample was transferred to an evaporating dish and dried at 60°C to constant weight. The sample was then heated for three hours with 100 ml of water and 10 ml of concentrated hydrochloric acid in a 250 ml flask provided with a reflux condenser. The sample was cooled and filtered through Whatman No.1 filter papers. The solution was then diluted and 2 ml of this dilution was added with 4 ml anthrone reagent, and the results obtained with the same procedure mentioned above.

2.3.3 Determination of moisture

The moisture content of the raw peat was determined using a Compu-Trac moisture analyzer (Motorola Process Control Inc., Tempe, Arizona, USA). It involved drying approximately 1 g of wet matter to constant weight at 107°C. The moisture content of the yeast was determined by drying approximately 1 g of the

sample to a constant weight at 108°C under vacuum. The moisture content was calculated by weight difference.

2.3.4 Determination of total nitrogen and crude protein

The total nitrogen content of the yeast *R. rubra* was determined by a modified A.O.A.C. micro-Kjeldahl Method 47.021 - 47.023 (Anonymous, 1980*a*). This involved placing 0.1 - 0.3 g of freeze-dried sample in a Kjeldahl flask and adding 2 Special Kjeltabs (S. 3,5), 20 mL of concentrated sulphuric acid and 10 mL of 30 % hydrogen peroxide. The sample was digested, using a Kjeltec Digestion System 6 (Model 1007 Digester, Tector Inc., Boulder, Co., U.S.A.), for 3 h until the solution had become colorless or clear. The digestion was continued for another 30 minutes, and the solution was allowed to cool. About 40 mL of water was added, carefully and a little at a time, to the flask. The flask was cooled under running water and placed in an Ammonia Distillation Unit (Kjeltec system 1002). About 50 mL of 40 % NaOH solution was carefully added to the flask without agitation, and the solution was distilled for 30 - 40 minutes into a receiving flask containing 50 mL of 4 % boric acid. A few drops of a methyl red indicator was added to the distillate and the latter was titrated with a standard 0.1 M hydrochloric acid solution.

A blank determination was carried out as above, with distilled water replacing the yeast sample.

The crude protein content of the sample was calculated from the percent

nitrogen content using the conversion factor (N × 6.25).

2.3.5 Extraction and determination of carotenoids

The total carotenoids content of the yeast was extracted and determined using a modified procedure of Sedmak (1990). The method involved extracting approximately 0.1 mg of yeast cells with 0.5 mL of hot (55°C) dimethylsulfoxide (DMSO) with agitation for 20 - 30 seconds, then adding 0.1 mL of 0.01 M sodium phosphate (pH 7.0) and 1 mL of diethyl ether to separate the carotenoids. Agitation was repeated for 30 - 40 seconds to mix the aqueous and organic phases, followed by separating the two phases by centrifugation for five minutes. The organic phase was recovered and the pigmented carotenoid content of the organic phase determined spectrophotometrically from its absorbency at 448 nm. The specific absorption coefficient $A_{1cm}^{1\%} = 2659$ (Craft and Soares, 1992) was used in the calculation of β-carotene concentration, by the formula reported by Davies (1976).

2.3.6 Determination of total lipids

The total lipids content of the yeast was determined using a modified form of the procedure of Morton (1985). The method involved extracting approximately 0.5 g of freeze-dried yeast cells with 100 mL of chloroform : methanol (2:1, v/v) for 24 h, followed by micro-filtration. The filtrate was then added to 100 mL of 5 % (w/v) Na_2SO_4 solution in a separatory funnel, which was shaken vigorously and left overnight for the phases to separate. The volume of the chloroform layer was

recorded and the methanol (upper) layer was removed. The chloroform (lower) layer, which contained the lipids, was transferred to a flask, evaporated to dryness under a stream of nitrogen gas, and weighed.

2.3.7 Determination of amino acids

Freeze-dried samples were hydrolysed with 6 N HCl under vacuum for 24 h at 110°C. The samples were reconstituted with 0.6 M lithium citrate buffer and analyzed with a Beckman 121 MB amino acid analyzer using a single column method. The concentrations of tryptophan and cystine were determined according to the methods of Penke *et al.* (1974), and Blackburn (1968), respectively.

2.3.8 Determination of fatty acids

The modified procedure of Morton (1985) previously described was used to extract the total lipids from the sample. The fatty acids composition was determined using a modified form of the procedure of Thompson (1969). It involved evaporating 0.5 - 2.0 mL aliquots of the lipid extract to dryness under a stream of nitrogen gas. The residues were transmethylated by adding 2 mL of 6 % H_2SO_4 in 99.9 mol % methanol and 15 mg hydroquinone, and placing the mixture in an oven at 55 - 60°C overnight. After that, 1 mL of deionized water was added and the sample was extracted 3 times with 1.0 mL portions of high-quality hexane. The hexane extract was collected and washed twice with 1.5 mL portions of deionized water and the hexane was then evaporated under a stream of nitrogen gas. The lipid was dissolved in about 15 - 40 µL of CS_2 and about 4 µL

were analyzed with a Perkin Elmer gas chromatograph (Model 8310) with a flame ionization detector and a Perkin Elmer graphic printer (Model GP-100). The column was packed with SP 2330 resin (Supelco) and operated isothermally at 170°C and with a sensitivity of 3×10^{-10} amp/mv.

Fatty acid esters were identified qualitatively by comparing their retention times to those of fatty acid ester standards.

2.4 Statistical analysis

All the data reported for the shake flask experiments represent mean values of three flasks. All the data for the batch cultivation experiments in the fermenter represent mean values of two fermentations. The data for proximate analysis of samples represent mean values of at least three determinations of three replicate samples.

Comparison between means was made using the T statistics test (Miller and Miller, 1988).

CHAPTER 3

RESULTS AND DISCUSSION

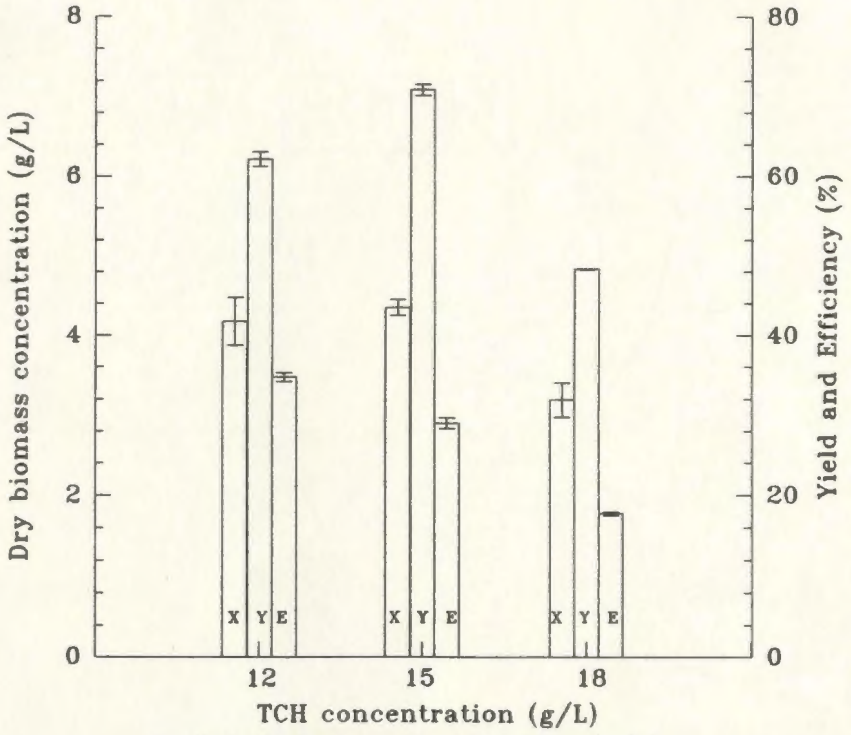
3.1 Shake flask experiments.

3.1.1 Optimization of medium compositions for the growth of *R. rubra* in shake flask experiments.

Preliminary experiments were conducted to determine the optimum carbon substrate concentration (total carbohydrate concentration of diluted peat extract), and the amounts of the nitrogen, phosphorous, potassium, and magnesium sources required to be added for the growth of *Rhodotorula rubra* in shake flask culture.

3.1.1.1 Substrate concentration

When *R. rubra* was inoculated in both peat extracts obtained from the hydrolysis process and in diluted peat extract solutions, practically poor growth was observed. The yeast was, therefore, tested for growth in peat extract media of various total carbohydrate (TCH) concentrations with 5 g/L yeast extract supplementation. The results of this experiment are presented in Figure 3.1.1.1. It was observed that a maximal growth, in terms of the dry biomass concentration and yield, was obtained at 15 g/L TCH, and this concentration was used for all subsequent experiments. A higher initial TCH concentration (less dilution of the peat extract) hampered the yeast growth, probably due to the higher



X– Dry biomass concentration (g/L)
Y– Yield (%)
E– Efficiency (%)

Fig. 3.1.1.1. Effect of TCH concentration of peat extract on the growth of *R. rubra* in shake flask.

concentrations of inhibitory substances (Martin and Bailey, 1984). According to Prescott and Dunn (1949), in an aerated growth medium, a decrease of the level of sugar results in an increase in the activities of the enzymes of the tricarboxylic acid cycle (TCA) and of the enzymes involved in the glyoxylate cycle and the electron-transport system, so that increasing TCH concentrations will not result in better growth indefinitely.

The reduced growth obtained at lower initial TCH concentrations was probably due to a lack of nutrients.

3.1.1.2 Utilization of carbon sources

Carbohydrates are usually the best sources of carbon and energy for microbial biomass production. All yeasts are able to utilize D-glucose, D-fructose, and D-mannose, although a particular yeast may utilize other carbon sources more efficiently (Miller, 1982). Fell *et al.* (1984) reported that *R. rubra* readily assimilated sucrose, trehalose, raffinose, and D-xylose. Hari *et al.* (1993) reported that the new strain of *R. rubra* readily assimilated glucose, adonitol (ribitol), galactose, maltose, sucrose, trehalose and melezitose; but less readily assimilated L-arabinose, xylose, sorbitol (glucitol), raffinose, and glycerol; and that it did not assimilate inositol, cellobiose or lactose, nor complex carbohydrates like 2-keto-D-gluconate, methyl-D-glucoside and N-acetyl-D-glucosamine.

The glucose in the semi-synthetic medium provided the same TCH concentration (15 g/L) as did the peat extract diluted to a concentration of 15 g/L

Table 3.1.1.2 Effect of carbon source on the on the growth and β-carotene synthesis of *Rhodotorula rubra* [1]

Carbon source	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)
Peat extract	4.35 ± 0.10^a	70.92 ± 0.69^a	29.02 ± 0.66^a
Glucose	2.58 ± 0.01^b	24.60 ± 0.02^b	17.22 ± 0.02^b

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium or glucose medium with $15 g \cdot L^{-1}$ TCH and $5 g \cdot L^{-1}$ yeast extract, at a temperature of 22°C, a fermentation time of 4.0 d, an agitation speed of 200 rpm, and a pH of 5.5.

with water, and both media were supplemented with 5 g/L yeast extract. A higher dry biomass concentration, yield and efficiency, were obtained in the peat extract medium than in the semi-synthetic medium (Table 3.1.1.2). An explanation of this difference in growth could be that yeasts possess extensive enzyme complexes that enable them to utilize a wide variety of carbohydrates (Boze *et al.*, 1992). The peat extract contained a mixture of different carbohydrates (Table 1.7.3), which were utilized more efficiently and stimulated better yeast growth than glucose as the sole carbon and energy source. In addition, many of the inhibitory substances that exist in peat extract, may have been at least partly removed in its pretreatment by sedimentation during the pH adjustment and subsequent separation through centrifugation.

3.1.1.3 Nitrogen sources

No growth of the yeast *R. rubra* occurred in the peat extract medium without nitrogen supplementation. Therefore, organic and inorganic nitrogen sources were used to supplement the peat extract. Studies were conducted using a semi-synthetic medium supplemented with various concentrations of both an inorganic and an organic nitrogen source. The effect of the nitrogen supplementation on the growth of the yeast *R. rubra* depended on the nitrogen sources and concentrations used. Fell *et al.* (1984) reported that nitrate salts were not assimilated by this species. Hence, nitrate salts were not considered as nitrogen supplements for the growth of *R. rubra*. In this work, maximal growth was

obtained in the peat extract medium supplemented with 1.5 g/L yeast extract and 4.0 g/L ammonium sulphate as the nitrogen sources (Table 3.1.1.3a). That this supplementation stimulated the growth of the yeast indicated that the peat extract was deficient in nitrogen and thiamine for good growth of *R. rubra*. It has also been observed in this work that the yeast cells produced less carotenoids when peat extract supplemented by a higher concentration of yeast extract (5 g/L), although the dry biomass concentration and yield were higher than those obtained with 4 g/L of ammonium sulphate and 1.5 g/L of yeast extract supplementation (Table 3.1.1.3b).

Yeast extract increased the pH of the medium, while ammonium sulphate decreased it, and when the two nitrogen sources were added as supplements together, the pH of the medium did not change significantly (around ± 0.1 - 0.3).

It was observed that the growth of *R. rubra* was very poor (indeed, it was not even detectable) when the semi-synthetic medium was supplemented with ammonium sulphate alone, compared to the growth when it was supplemented with yeast extract together with ammonium sulphate. A possible explanation is that the effective components of yeast extract, which may include any or all of thiamine, riboflavin, nicotinic acid, vitamin A and the vitamin B complex (Goodwin and Lijinsky, 1952), supplied additional nutrients and growth factors for the growth of the yeast. This explanation is in agreement with Fell *et al.* (1984), who

Table 3.1.1.3a Effect of concentration of $(NH_4)_2SO_4$ and yeast extract on the growth of *Rhodotorula rubra* [1]

$(NH_4)_2SO_4$ added ($g \cdot L^{-1}$)	Yeast extract added ($g \cdot L^{-1}$)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)
0	0.5	0.87 ± 0.4^{ac}	10.09 ± 0.7^{a}	5.81 ± 0.1^{a}
4	0.5	1.18 ± 0.2^{a}	12.66 ± 0.7^{b}	7.86 ± 0.5^{b}
5	0.5	1.10 ± 0.1^{a}	12.02 ± 0.2^{b}	7.35 ± 0.8^{b}
0	1.0	1.49 ± 0.9^{abc}	20.31 ± 0.8^{c}	9.92 ± 0.2^{c}
3	1.0	2.43 ± 0.7^{ab}	23.85 ± 0.6^{d}	16.16 ± 0.9^{d}
4	1.0	2.38 ± 0.2^{b}	24.29 ± 0.9^{d}	15.89 ± 0.9^{d}
5	1.0	2.39 ± 0.2^{b}	23.57 ± 0.9^{d}	15.93 ± 0.2^{d}
0	1.5	1.73 ± 0.1^{c}	19.69 ± 0.7^{c}	11.50 ± 0.1^{e}
3	1.5	2.43 ± 0.1^{b}	28.66 ± 0.7^{e}	16.21 ± 0.3^{d}
4	1.5	2.65 ± 0.4^{bc}	30.89 ± 0.9^{e}	17.65 ± 0.9^{d}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium with $15 g \cdot L^{-1}$ TCH, at an incubation time of 4.0 d, a temperature of 22°C, an agitation rate of 200 rpm, and a pH of 5.5.

reported that *R. rubra* needs thiamine for growth, and it was observed in this work that the yeast grew poorly with ammonium sulphate alone.

Wort, which contains amino acids and other soluble nitrogen compounds, is an adequate source of nitrogen (Prescott and Dunn, 1949). In this work, the highest dry biomass concentration (4.68 g/L) was obtained in molasses medium supplemented with 4 % (v/v) wort as the nitrogen source (Figure 3.1.1.3.). Growth of yeast on wort is more rapid than in a medium in which an ammonium salt is used as the nitrogen source, although according to Thorne (1949), ammonium salts are still better sources than any amino acid alone. Yeast can assimilate about 40 - 50 % of the total nitrogen in wort, which is mostly amino nitrogen (Suomalainen and Oura, 1971).

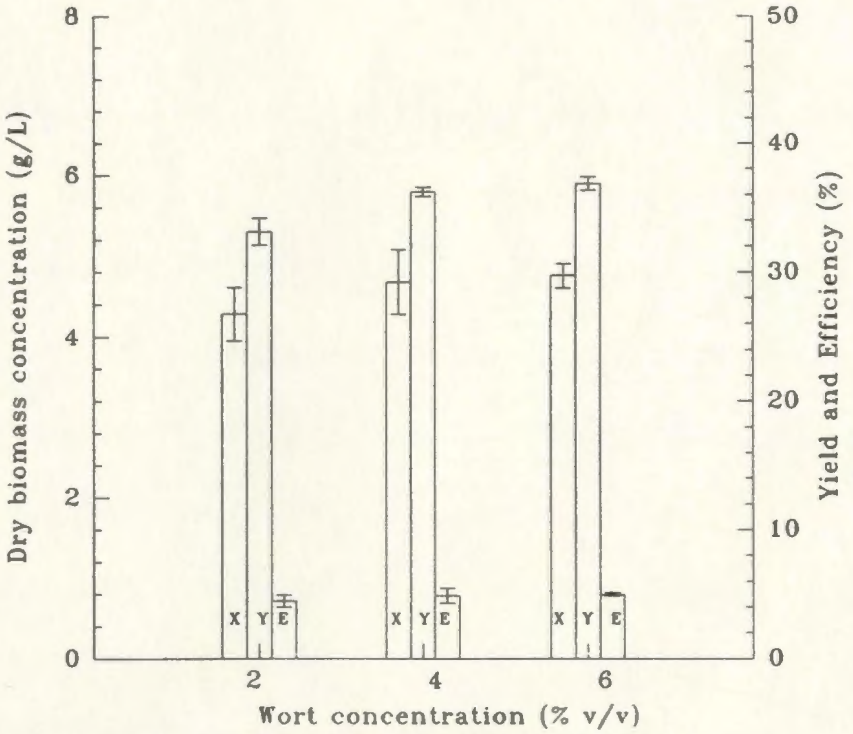
3.1.1.4 Effect of increasing concentrations of nitrogen on the yeast growth

It was observed that increased concentrations of nitrogen had a positive effect on the growth of *R. rubra*. There was a smooth increase in the growth when the concentrations of yeast extract and ammonium sulphate in the semi-synthetic medium were increased (Table 3.1.1.3a). Maximal growth values were obtained with 1.5 g/L yeast extract combined with 4 g/L ammonium sulphate in the semi-synthetic medium. There was no significant difference ($P < 0.05$) between the growth parameters at 3 and 4 g/L ammonium sulphate combined with 1.5 g/L of yeast extract. Higher concentrations of the yeast extract produced an increase in

Table 3.1.1.3b Effect of nitrogen source on the on the growth and β-carotene synthesis of *Rhodotorula rubra* [1]

$(NH_4)_2SO_4$ ($g \cdot L^{-1}$)	Yeast extract ($g \cdot L^{-1}$)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)	β-carotene production ($\mu g \cdot g^{-1}$)
0	5	4.35 ± 0.10^a	70.92 ± 0.69^a	29.02 ± 0.66^a	405.2 ± 79^a
4	1.5	2.65 ± 0.40^b	30.89 ± 0.90^b	17.65 ± 0.90^b	722.4 ± 50^b

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium with $15 g \cdot L^{-1}$ TCH, at a temperature of 22°C, a fermentation time of 4.0 d, an agitation speed of 200 rpm, and a pH of 5.5.



X– Dry biomass concentration (g/L)
Y– Yield (%)
E– Efficiency (%)

Fig. 3.1.1.3. Effect of wort supplementation on the growth of *R. rubra* in molasses medium in shake flask

the growth parameters; however the yeast had paler color, indicating that less carotenoids were produced under these conditions. The same observation was made for cells grown in molasses and wort medium: when the wort supplementation was increased to 40 % (v/v), no pink color was observed. This is in agreement with the findings of Goodwin (1980), that a medium with a low carbon to nitrogen (C:N) ratio will be much less effective for carotenoid synthesis by *R. rubra* than one with a high C:N ratio.

3.1.1.5 Addition of phosphorus to the peat extract medium for the growth of *R. rubra*.

The results obtained with K_2HPO_4, and KH_2PO_4, added as supplements to both the semi-synthetic medium (Table 3.1.1.5) and the peat extract medium, indicated that extra supplements of phosphorus in either chemical forms inhibited the growth of the yeast *R. rubra*. Supplementation of the semi-synthetic medium with either K_2HPO_4 or KH_2PO_4 resulted in dry biomass concentration, yield and efficiency values that were significantly lower ($P > 0.05$) than those obtained without supplementation. In peat extract medium, supplementation with either of these phosphorous sources resulted in the formation of much precipitate, with no growth observed. It was unexpected that potassium phosphate, added to either medium, would result in poor growth in *R. rubra*. Jacob (1991), using potassium phosphate as a supplement, obtained an increased dry biomass concentration of *R. gracilis*. Matelli *et al.* (1990) reported obtaining a dry biomass concentration of 15 g/L of *R. glutinis* when KH_2PO_4

was added at 5.5 g/L to sugar juice medium. Also, Quierzy *et al.* (1979), and McLoughlin and Küster (1972b), found that peat extract, when supplemented with phosphate, was a very good medium for microbial biomass production.

Yeast extract has a phosphorous content of 1.16 % (Anonymous, 1980*b*). When supplied to the peat extract medium and the semi-synthetic medium in this work, it gave a phosphorous concentration of 0.58 mM to both, corresponding to the condition described by Button *et al.* (1973), who reported that *R. rubra* is capable of extended growth at very low phosphate concentrations. A possible explanation of the response of *R. rubra* to phosphate supplementation, according to Button *et al.* (1973), is that the phosphate transport system in the cells is weakened or disabled. Therefore, it could be concluded that additional supplementation of phosphorous as either K_2HPO_4 or KH_2PO_4 inhibited growth of this species under these conditions. The carotene production in this experiment was not analyzed in either case due to insufficient amounts of biomass.

3.1.1.6 Effect of additional potassium on the growth and carotenogenesis of *R. rubra*

Due to the inhibitory effect of K_2HPO_4 and KH_2PO_4 on the growth of *R. rubra*, K_2SO_4 was tested as a potassium supplement to the peat extract medium. The results indicate that *R. rubra* has a specific requirement for potassium (Figure 3.1.1.6.). This is in agreement with the finding made by Atkin *et al.* (1949), that potassium is critical for the growth of yeast and for fermentation. Various

Table 3.1.1.5 Effect of phosphate on the growth of *Rhodotorula rubra* in synthetic medium [1]

Growth Parameter	Phosphate Source (3 mM P)	
	K_2HPO_4	KH_2PO_4
Dry biomass ($mg \cdot L^{-1}$)	2.002 ± 1.3	74.85 ± 16.4
Yield (%)	0.015 ± 0.09	0.57 ± 0.1
Efficiency (%)	0.013 ± 0.08	0.50 ± 0.1

[1] Values represent the mean of three determinations ± standard deviations.

concentrations of K_2SO_4 (2, 3, and 4 mM of $[K^+]$) were added to peat extract medium, along with the previously-determined optimal nitrogen supplementation, to optimize the growth and carotenogenesis of *R. rubra*. The results are presented in Figure 3.1.1.6. It was apparent that the addition of potassium sulphate to the peat extract medium enhanced the growth and carotene production.

The highest dry biomass concentration, 3.85 g/L, was obtained at 3 - 4 mM of potassium ion supplementation. This combination produced an increase in the biomass concentration of about 45 % over that produced without potassium supplementation. This is corresponds to the report of Jones and Greenfield (1984), that K^+ added to the medium at concentrations in the range of 2 - 4 mM stimulated the growth of yeast. However, there were no significant differences in growth or carotene synthesis with increasing supplementation of potassium ion concentration from 2 mM to 4 mM.

3.1.1.7. Addition of magnesium

It has been reported that magnesium is a necessary growth factor for yeasts (Morris, 1958). It is the most common enzyme activator, and is of particular importance in activating the large class of phosphate transferases, and a number of decarboxylases (Bowen, 1966). Therefore, the effect of magnesium on the growth and carotene synthesize of *R. rubra* was studied in this work. The results indicate that addition of magnesium sulphate to peat extract medium enhanced the biomass concentration, yield, efficiency, and carotene synthesis. Matelli *et al.*

(1990) added $MgSO_4 \cdot 7H_2O$ at 0.5 g/L as a nutrient supplement for the growth and β-carotene production of *R. glutinis*. Yoon and Rhee (1983) also added $MgSO_4 \cdot 7H_2O$, at 0.7 %, to synthetic medium for the growth of *R. glutinis*. In this work, various concentrations of $MgSO_4 \cdot 7H_2O$ (0.5, 1.0, 1.5 g/L) were added to peat extract containing the optimal supplementation levels of nitrogen (4 g/L $(NH_4)_2SO_4$ and 1.5 g/L yeast extract) and potassium (2 mM) as previously determined, and the results are summarized in Figure 3.1.1.7. It was apparent that the addition of magnesium sulphate to the peat extract enhanced the growth and β-carotene synthesis of the yeast.

The highest dry biomass concentration, 4.57 g/L, and the highest β-carotene production, 1112.6 μg/g, were obtained with the addition of 0.5 g/L of $MgSO_4 \cdot 7H_2O$. This supplementation produced an increase in the biomass concentration of about 21.8 % and an increase in the β-carotene production of about 36.5 %, over that produced without magnesium supplementation. No significant differences in yield and β-carotene production were observed for increasing the $MgSO_4 \cdot 7H_2O$ supplement concentration from 0.5 to 1.5 g/L.

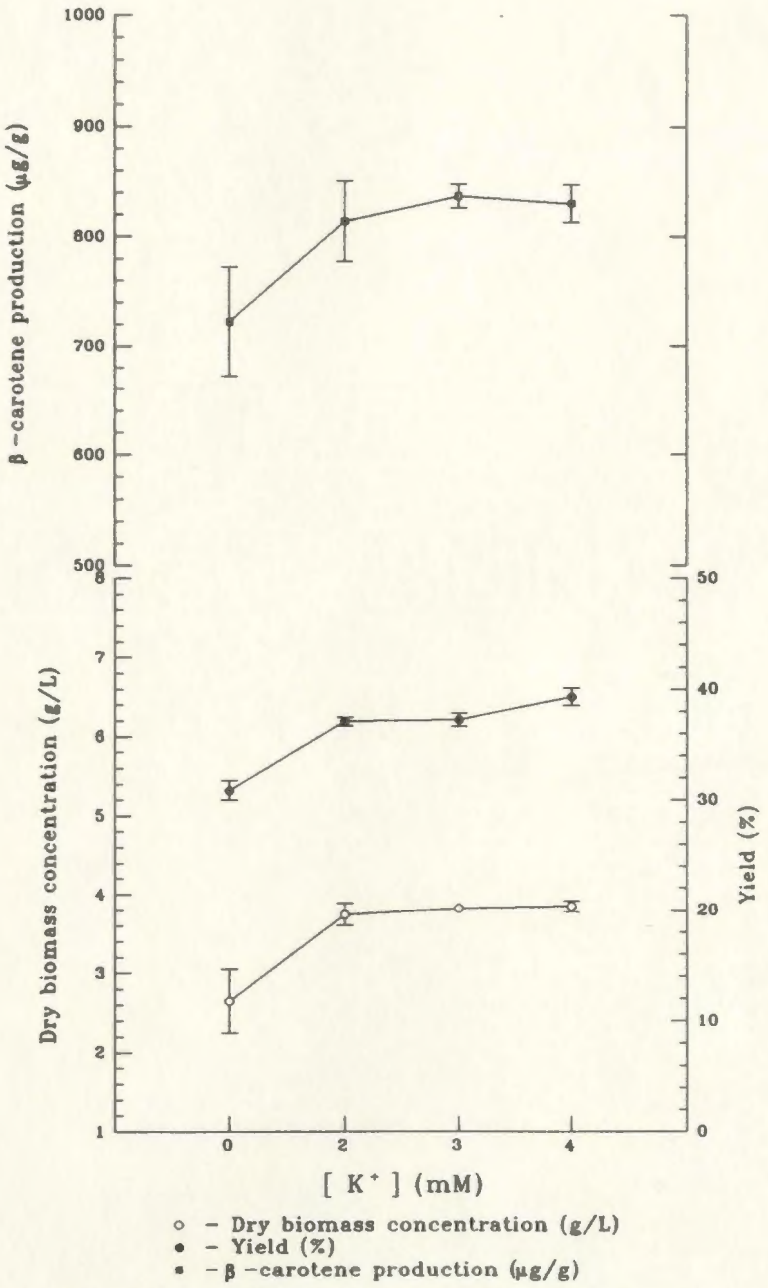


Fig. 3.1.1.6. Effect of potassium supplement on the growth and carotene synthesis of *R. rubra* in peat extract medium in shake flask.

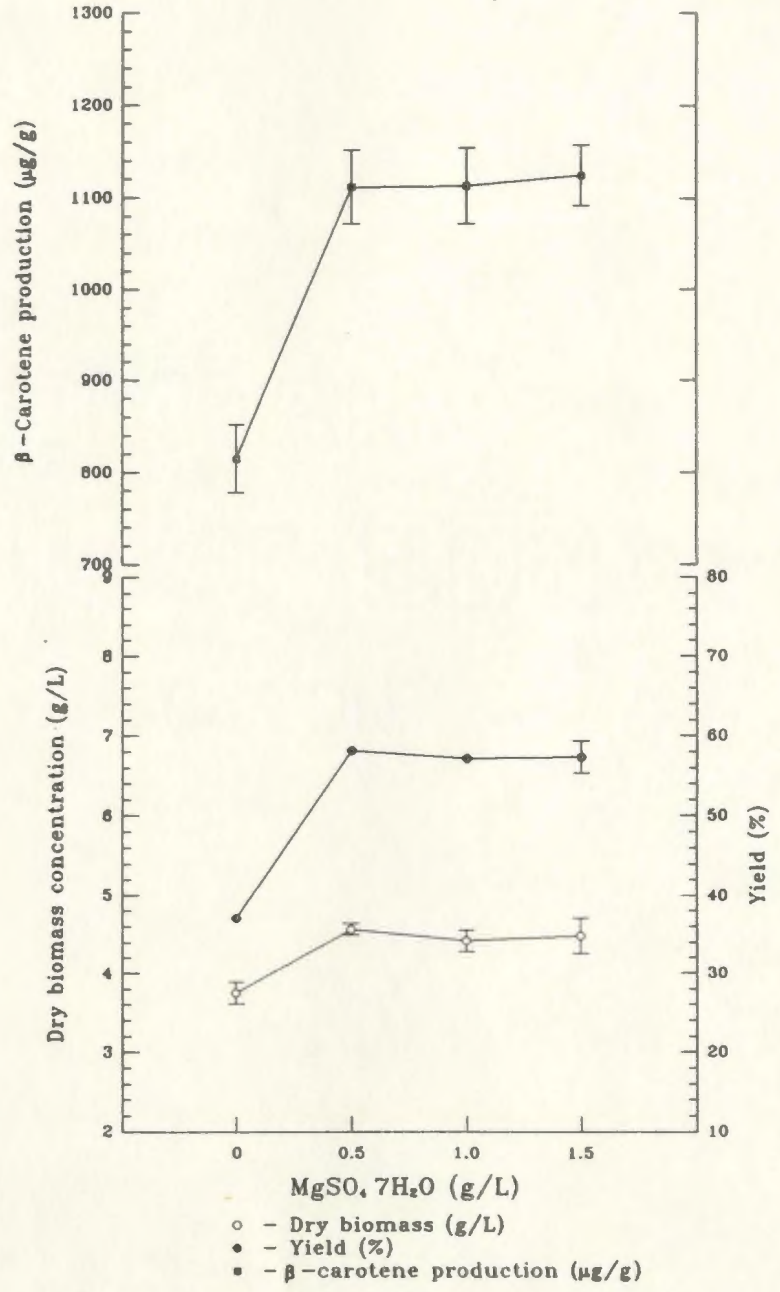


Fig. 3.1.1.7. Effect of magnesium sulphate (g/L) supplement on the growth and carotene synthesis of *R. rubra*.

3.1.2 Effect of operational conditions on the growth and carotenogenesis of *R. rubra* in shake flask experiments in peat extract medium.

3.1.2.1 Effect of initial pH value on the growth and carotenogenesis of *R. rubra* in shake flask experiments.

The optimal pH for carotenogenesis in *R. gracilis* is 5.0 - 5.6 (Goodwin, 1980). Costa *et al.* (1987), however, used a pH of 6.5 for the production of β-carotene in *R. glutinis*.

Initial pH values ranging from 3.0 to 6.0 were tested on the growth and carotenogenesis of *R. rubra*. It was observed that at the initial pH of 3.0, there was no growth, therefore the results obtained at this pH value are not presented. There was a great increase in the growth and carotene synthesis when the initial pH was increased to 4.5, and slight decreases in the growth and carotene production at initial pH values higher than 6.0.

Figure 3.1.2.1 shows clearly that the optimal initial pH range for the growth and β-carotene production of *R. rubra* was 4.5 - 5.5. There were no significant differences ($P < 0.05$) between the values of dry biomass concentration, efficiency, and β-carotene production obtained at pH 5.0 and those obtained at pH 5.5. Growth and β-carotene yield values decreased at pH 6.0.

The final pH value after fermentation was not notably different from the initial pH value when this was below 5.0 (Table 3.1.2.1). A significant rise in pH value was observed after fermentation when the initial pH value was higher than

Table 3.1.2.1 Initial and final pH values of peat extract fermentation medium

Initial pH value	Final pH value
4.0	4.0 ± 0.1
4.5	4.5 ± 0.0
5.0	5.2 ± 0.1
5.5	6.5 ± 0.0
6.0	7.3 ± 0.0

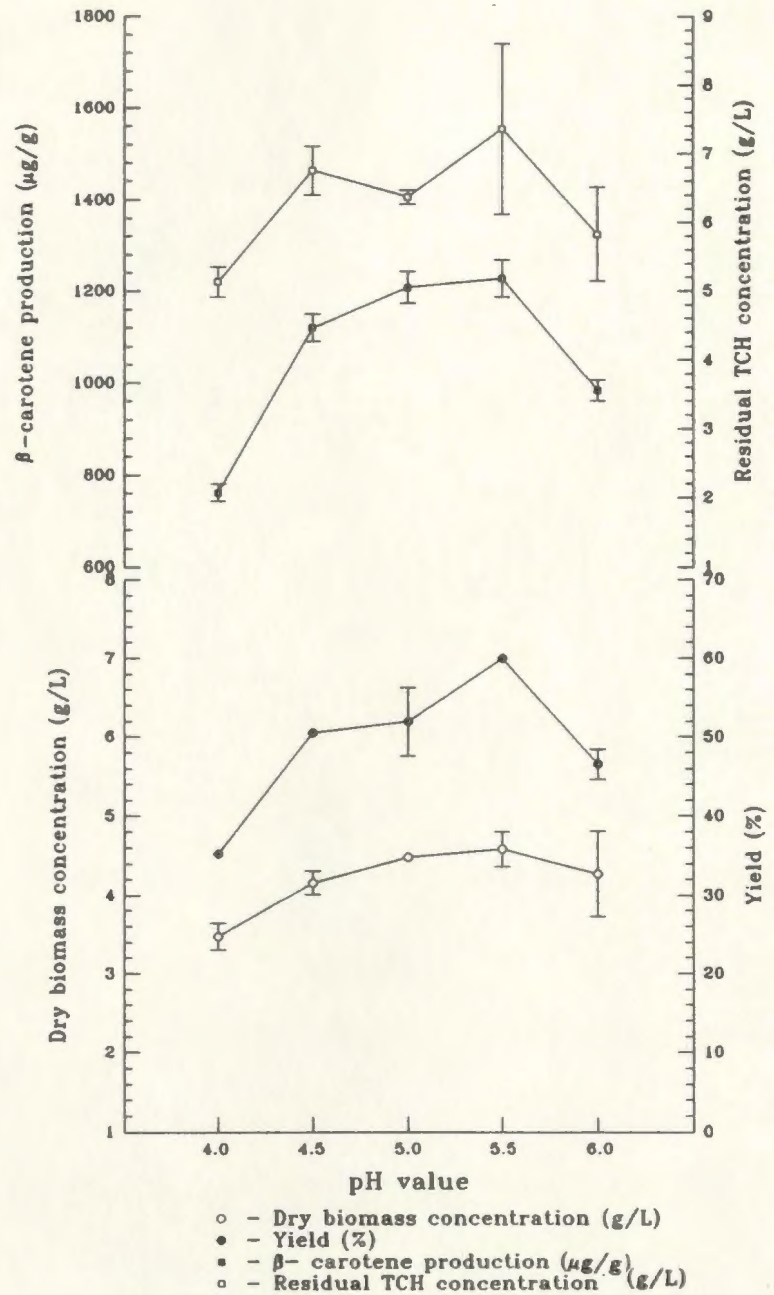


Fig. 3.1.2.1. Effect of initial pH of the medium on the growth and carotene synthesis of *R. rubra* in peat extract medium in shake flask.

5.0. This trend of pH value change corresponds to that reported by Bujak and Podgorska (1968). No significant residual TCH concentration changes ($P < 0.05$) were observed when the initial pH value was from 4.5 - 6.0 (Figure 3.1.2.1).

3.1.2.2 Effect of fermentation time on the growth and carotenogenesis of *R. rubra* in shake flask experiments.

The effect of fermentation time on the growth and carotenogenesis of *R. rubra* in the peat extract is shown in Figure 3.1.2.2. In general, there was a very short growth adaptation period, and afterwards, an accelerated growth phase that continued until 60 hrs after inoculation, followed by a decline in the growth rate. The highest dry biomass (4.84 g/L) and β-carotene (1255.56 μg/g) concentrations were obtained at 84 hrs of fermentation, with decreased dry biomass (4.6 g/L) and β-carotene (1227.36 μg/g) concentrations obtained at 96 hrs of fermentation. The two corresponding values, however, were not significantly different ($P < 0.05$). Yield values of growth and β-carotene synthesis decreased after 96 hrs of fermentation. The pattern of growth rate changes was comparable to that described by Goodwin (1980). The TCH concentration in the medium decreased sharply during the first 60 hrs of fermentation, and then the decrease became gradual until 96 hrs, when it became constant (Figure 3.1.2.2).

Yeast growth was observed within 60 hrs of fermentation. The turbidity of the peat extract medium increased during 84 hrs of growth. After 84 hrs, the pH rose from a little below 4.9 to about 5.5, after 120 hrs of fermentation (Table

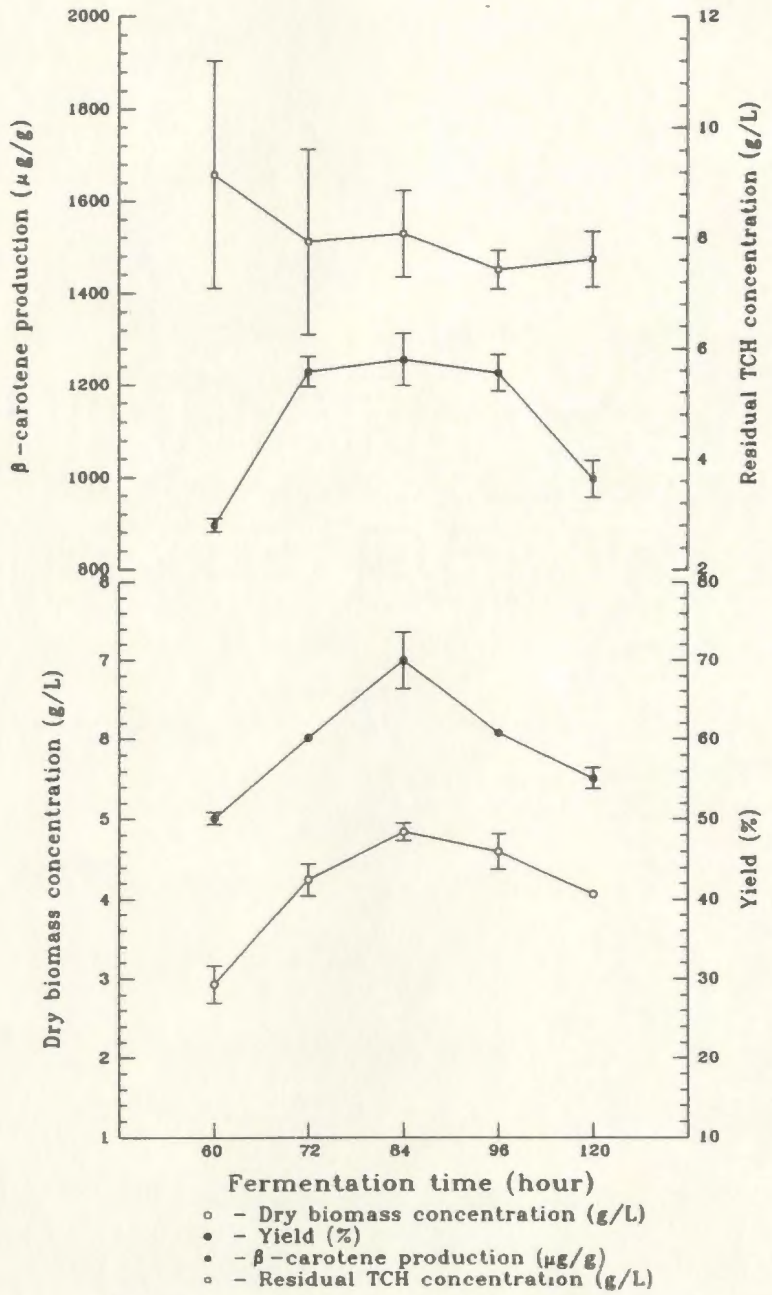


Fig. 3.1.2.2. Effect of fermentation time on the growth and carotene synthesis of *R. rubra* in peat extract medium in shake flask.

3.1.2.2.).

3.1.2.3 Effect of agitation speed on the growth and carotenogenesis of *R. rubra* in shake flask experiments.

It is apparent that the maximum growth and β-carotene production were observed at 200 rpm, with no significant difference ($P < 0.05$) between these values and those observed at 250 rpm, and with decreased growth beyond this range (Figure 3.1.2.3). The purpose of agitation in shake flask culture is to supply oxygen and nutrients to the growing cells (Calam, 1986). *R. rubra* is a strictly non-fermentative, or obligate, aerobe (Stewart and Russell, 1983). Therefore, it could be expected that growth would be higher at 200 rpm than at 150 rpm. However, decreased growth occurred when the agitation speed was higher than 250 rpm, the possible explanation is that stronger sheer force resulted from the higher agitation speed engendered stress that affected the microbial metabolism.

3.1.2.4 Effect of temperature on the growth and carotenogenesis of *R. rubra* in shake flask experiments.

The optimal growth temperature of the strain of *R. rubra* used in this work has been reported to be 20°C (Hari, 1992). Temperatures ranging from 18°C to 26°C were studied in this work for the growth and β-carotene production of *R. rubra*, and the results are presented in Figure 3.1.2.4. It may be observed that there was an increase in the growth parameters and β-carotene production with increasing temperatures from 18°C to 22°C. A further increase in temperature to

Table 3.1.2.2 The change of pH with fermentation time in peat extract medium

Fermentation time (hrs)	Final pH value
60	4.9 ± 0.0
72	4.9 ± 0.0
84	4.9 ± 0.0
96	5.2 ± 0.1
120	5.5 ± 0.1

26°C produced a significant reduction in the growth and β-carotene production. In contrast, Jacob (1991) used 28 ± 1°C for growing *R. gracilis* in solid-state fermentation, while Costa *et al.* (1987) used 27°C for β-carotene production of *R. glutinis* in a benchtop fermenter. In general, temperature changes between 5 - 25°C have no effect on the pigment production of *R. rubra* (Goodwin, 1980).

There was a significant increase in the carbohydrate utilization (the decrease in the residual TCH concentration) from 20 to 26°C. Maximum carbohydrate utilization was obtained at 26°C (Figure 3.1.2.4). The lowest carbon utilization (the highest residual TCH concentration) was obtained at 18°C, where the growth was also the lowest. This corresponds to that reported by Stokes (1971) that yeast growth is slow at low temperatures, and that most rapid growth occurs, usually, in the range of 20 - 30°C.

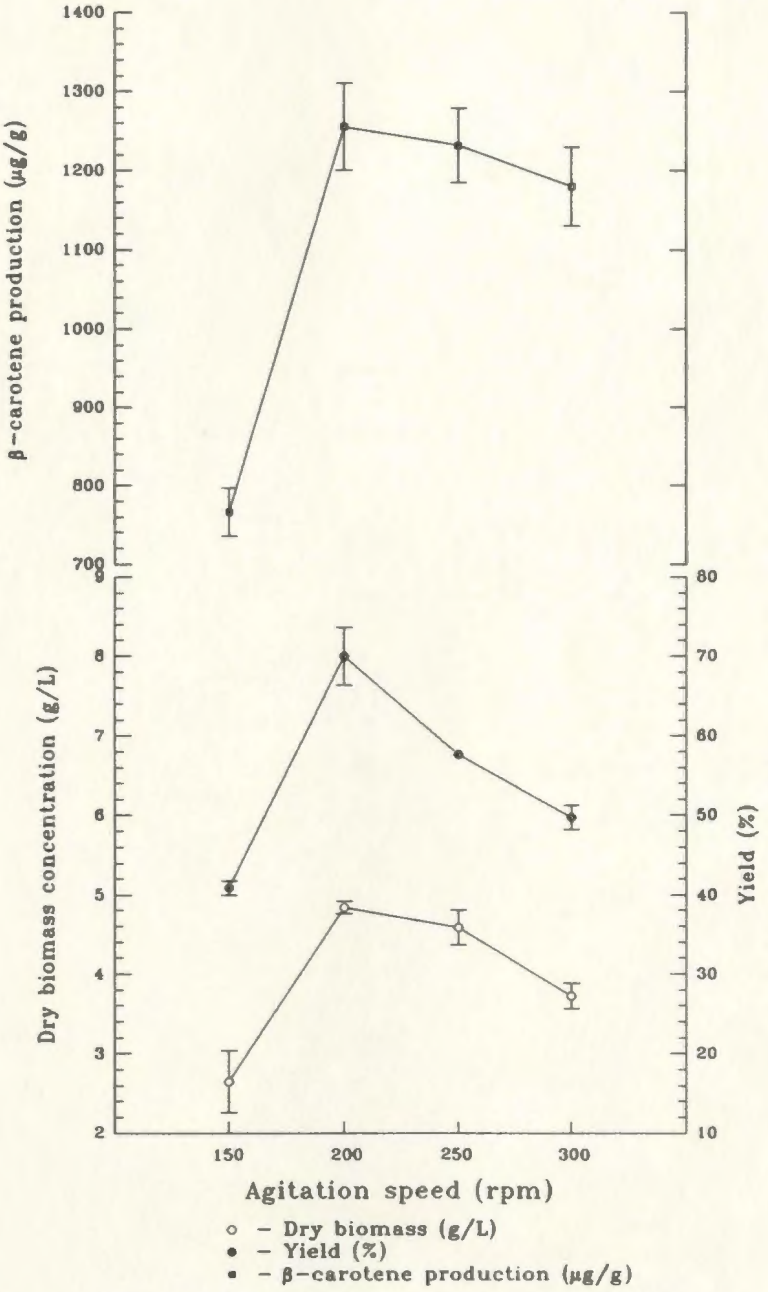


Fig. 3.1.2.3. Effect of agitation speed on the growth and carotene synthesis of *R. rubra* in shake flask.

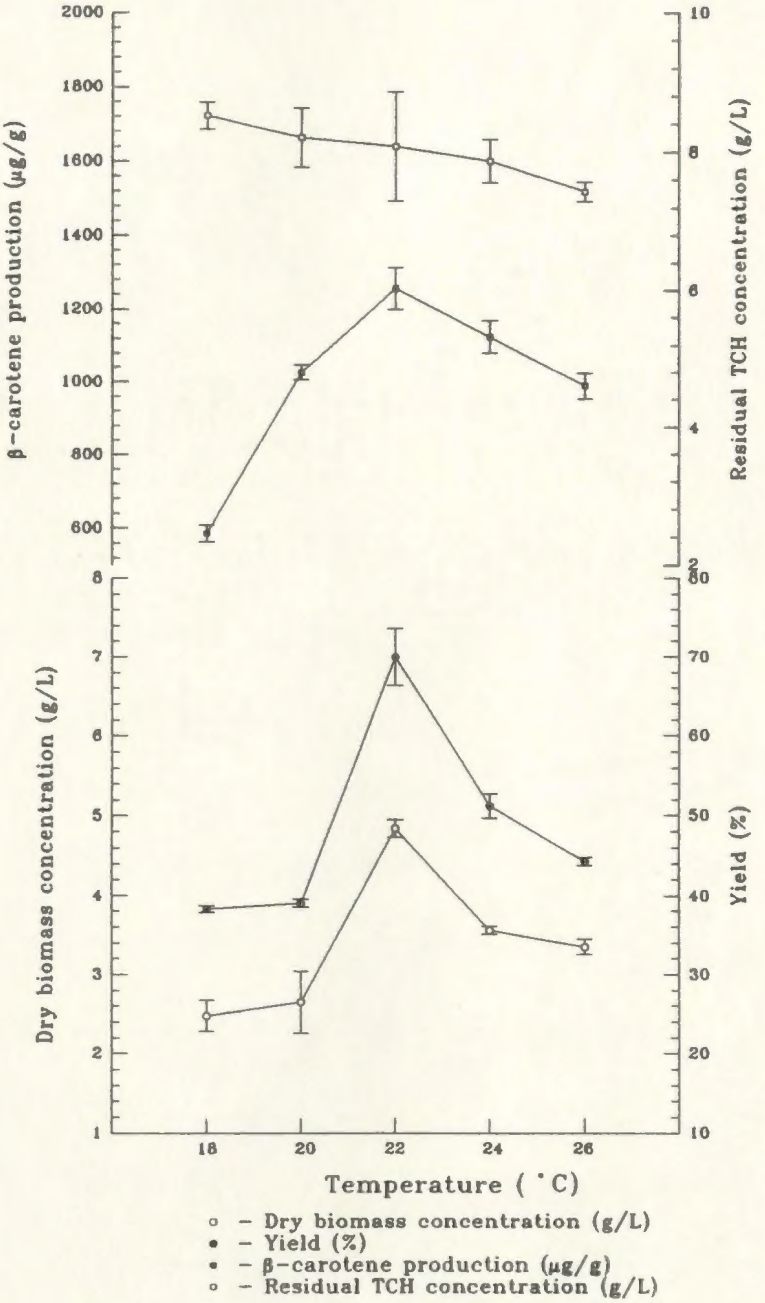


Fig. 3.1.2.4. Effect of temperature on the growth and carotene synthesis of *R. rubra* in peat extract medium in shake flask.

3.2 Batch fermenter experiments

Growth media A and B, described in Section 2.2.6.1, contained the best combinations of nutrient supplements as determined by the shake flask experiments. The amount of wort supplemented to the molasses for the optimal growth of *R. rubra* was used in the batch fermenter experiments.

3.2.1 Agitation and aeration

The effects of agitation speeds and aeration rates on the growth and carotenogenesis of *R. rubra*, in both peat extract medium and molasses and wort medium, were studied in batch fermenters. Maximum biomass yield and β-carotene production in both media were obtained at an agitation speed of 200 rpm with an aeration rate of 1.0 vvm.

The interaction of the effects of aeration rate and agitation speed on growth rate and carotene synthesis may also be observed. For example, when the aeration rate was 1.0 vvm, the dry biomass and β-carotene concentrations, obtained from both the peat extract and the molasses fermentations, were higher at 200 rpm than at 150 rpm (Figure 3.2.1a;b), while there was no significant difference ($P < 0.05$) in corresponding values obtained from the molasses fermentation at 200, 250 and 300 rpm, and no significant difference ($P < 0.05$) in the corresponding β-carotene concentration obtained from the peat extract fermentation at 200 and 250 rpm. However, highest yield values in both cases

were obtained at 200 rpm, indicating that the growth was, in general, better at 200 rpm.

The dry biomass concentration, yield, efficiency, and β-carotene production values obtained from both media were much higher at 1.0 vvm than those at 0.75 vvm when agitation speeds were 200 rpm. This conclusion is in agreement with reports by Bekers *et al.* (1969) and Zelie (1970), that strong aeration stimulates the growth and carotenogenesis of *R. gracilis*. Lower dry biomass concentration, yield, efficiency, and β-carotene production were obtained when the aeration rate was raised higher than 1.0 vvm at 200 rpm agitation speed in peat extract medium, in comparison to those grown at an aeration rate of 1.0 vvm and an agitation speed of 200 rpm, in the same medium (Figure 3.2.1c). However, the β-carotene production obtained with aeration rates equal to and above 1.0 vvm were not significantly different ($P < 0.05$) in molasses medium (Figure 3.2.1d). Higher dry biomass concentration, yield, efficiency, and β-carotene production values in both media were obtained at 1.0 vvm, indicating that 1.0 vvm was the optimal aeration rate for the growth and β-carotene synthesis of *R. rubra* in batch fermentation. This is in agreement with the conclusions of Bekers *et al.* (1969), and Zelie (1970), that a certain minimum dissolved oxygen concentration is required for the carotenogenesis of *Rhodotorula* spp.

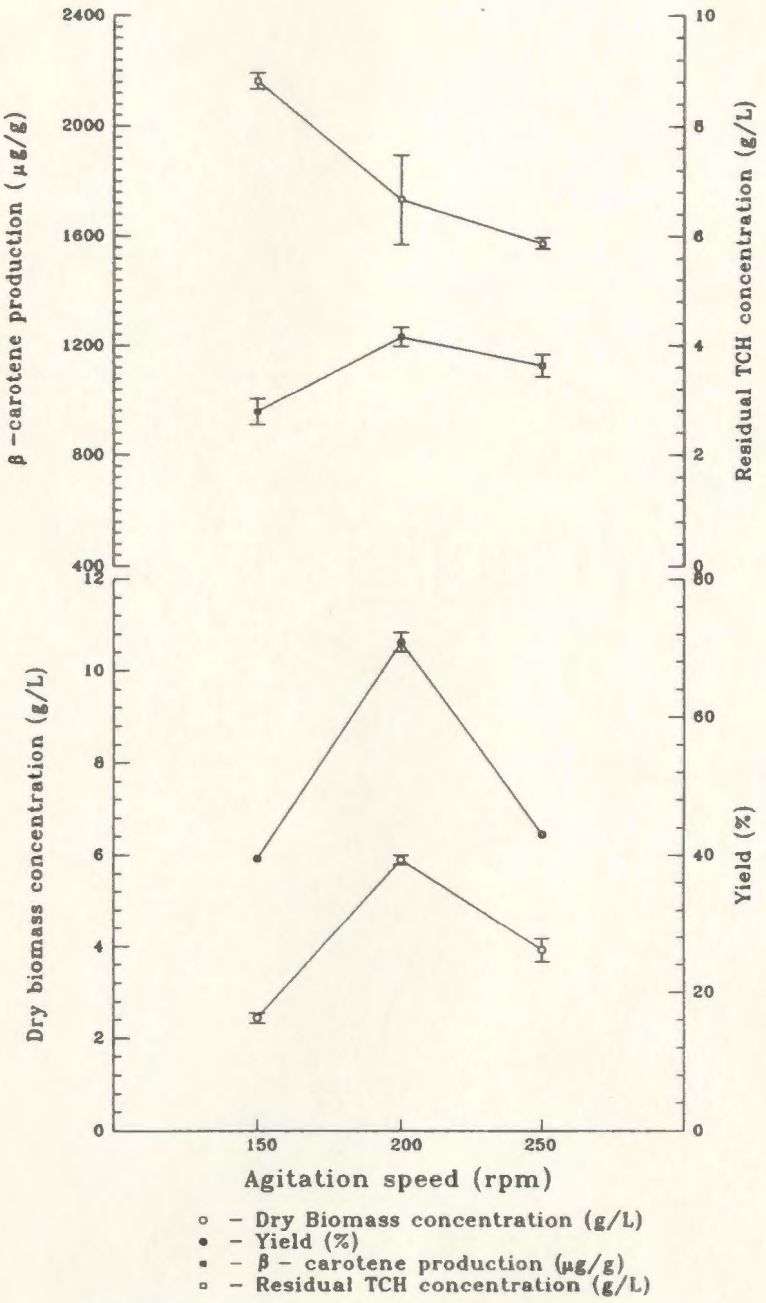


Fig. 3.2.1a. Effect of agitation speed on the growth and carotene synthesis of *R. rubra* in peat extract medium in batch fermenter at aeration rate of 1.0 vvm.

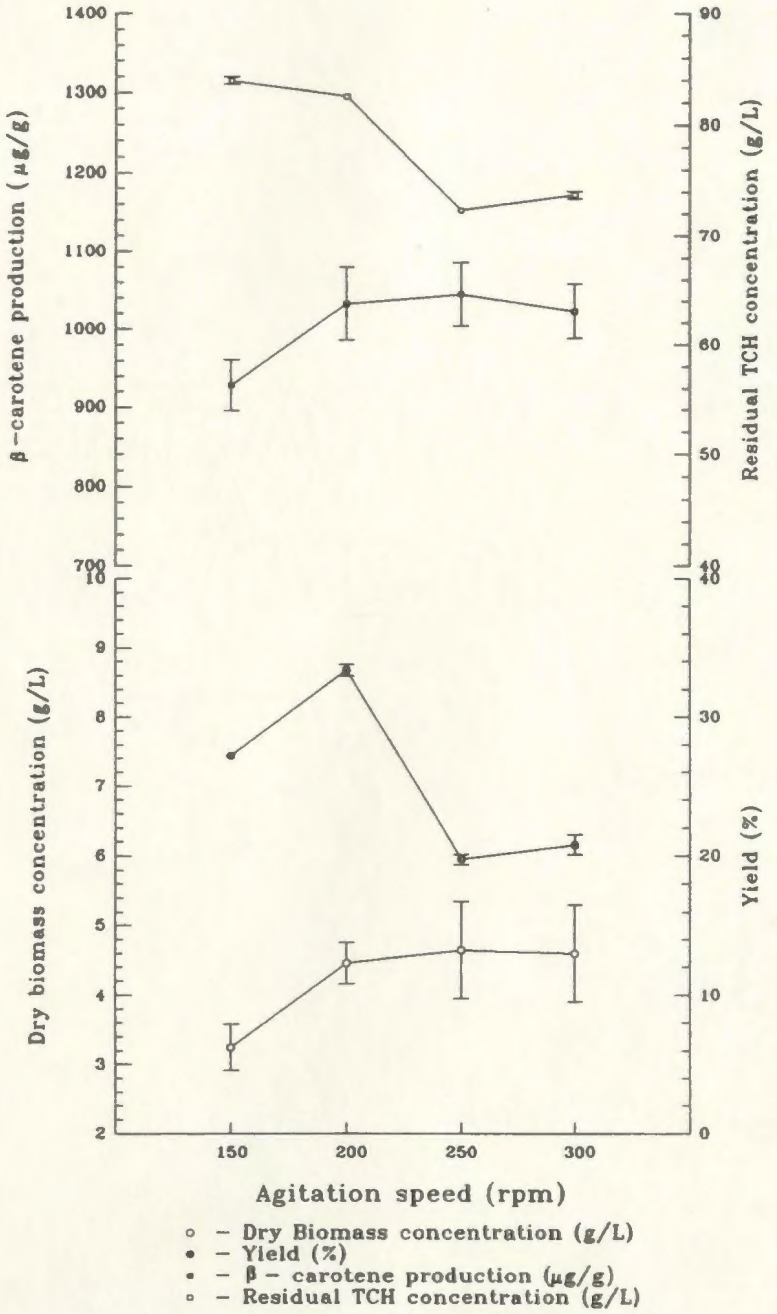


Fig. 3.2.1b. Effect of agitation speed on the growth and carotene synthesis of *R. rubra* in molasses medium in batch fermenter at aeration rate of 1.0 vvm.

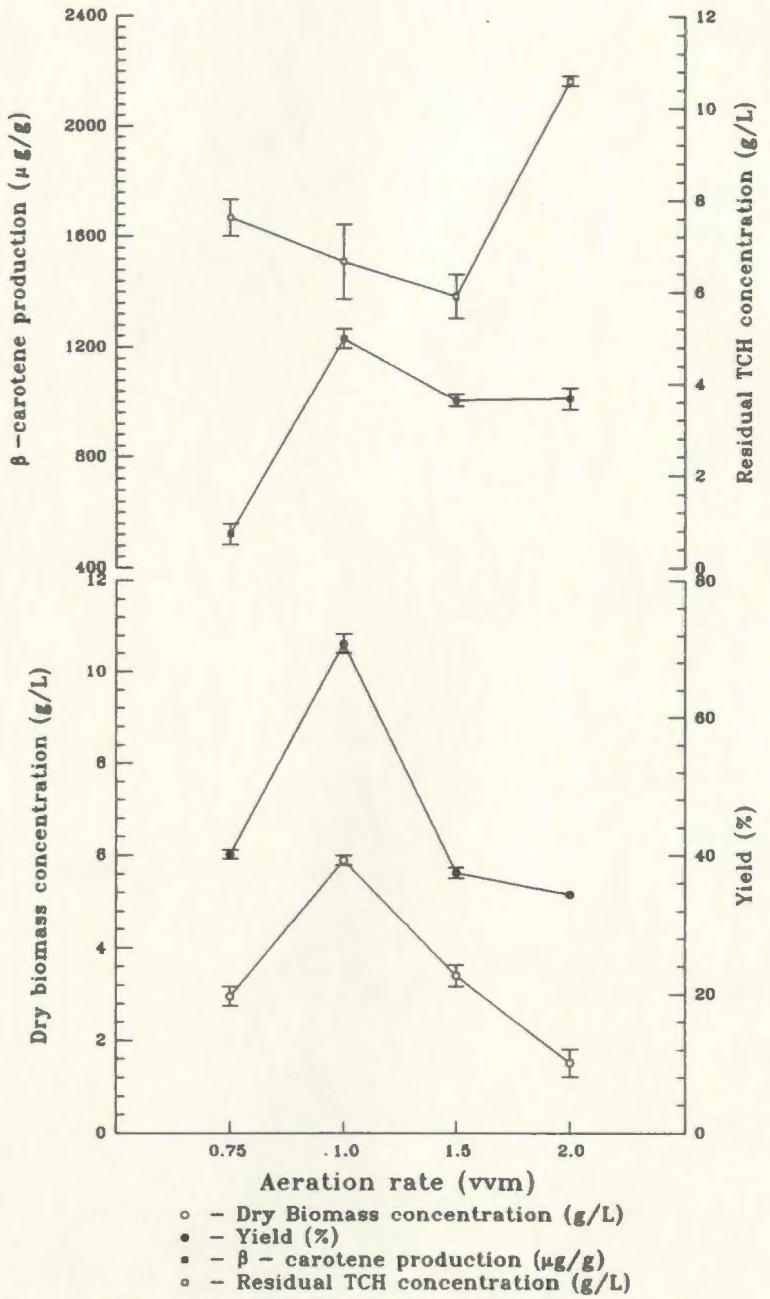


Fig. 3.2.1c. Effect of aeration rate on the growth and carotene synthesis of *R. rubra* in peat extract medium in batch fermenter at agitation speed of 200 rpm.

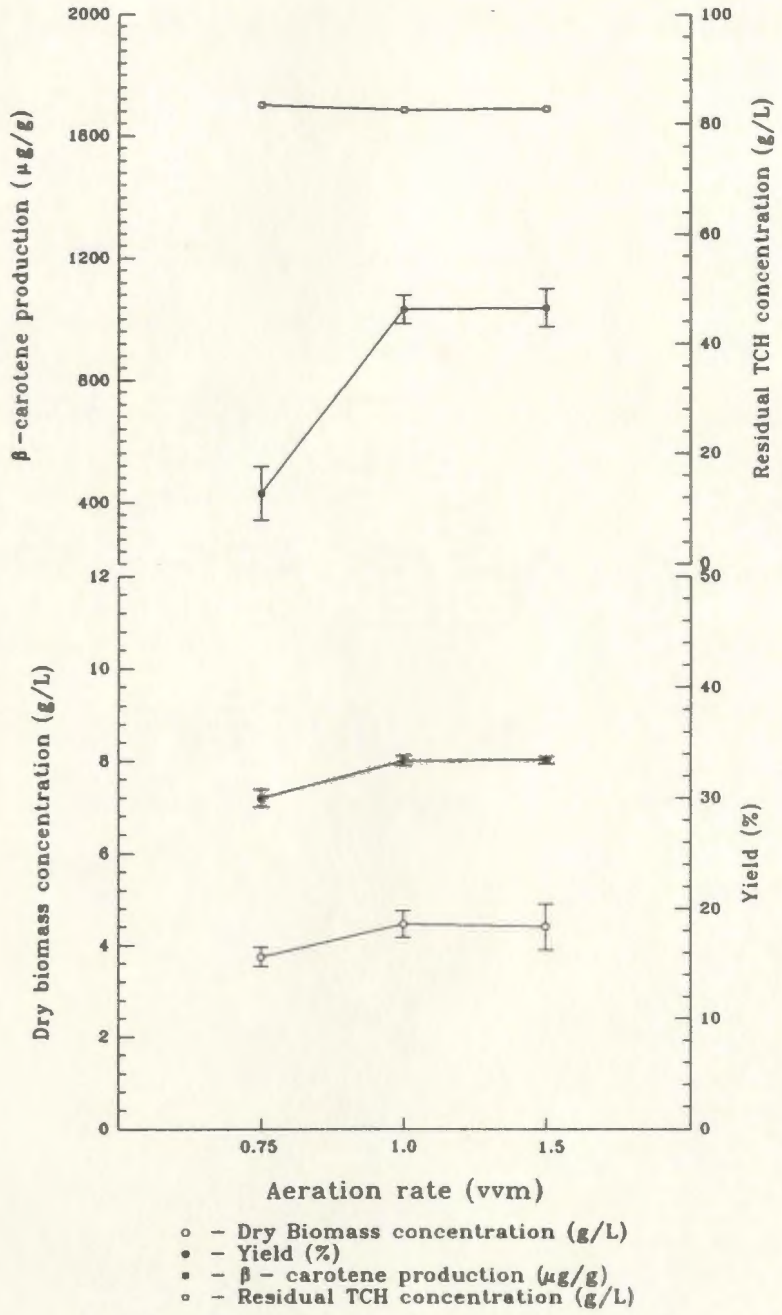


Fig. 3.2.1d. Effect of aeration rate on the growth and carotene synthesis of *R. rubra* in molasses medium in batch fermenter at agitation speed of 200 rpm.

3.2.2 Effect of agitation and aeration on substrate (TCH) consumption.

The relationship between the residual TCH concentration in the peat extract and molasses media and the aeration rates at various agitation speeds, and vice versa, are shown in Figures 3.2.1a,b and Figures 3.2.1c,d, respectively. It can be seen that the carbohydrates consumption rate follows a pattern similar to the growth parameters. That is, more carbohydrates were consumed at 200 rpm and 1.0 vvm, the optimal values for the growth of the yeast, than at other agitation speeds and aeration rates tested. This means that the process at 1.0 vvm was more productive, as it produced a higher yield.

3.2.3 General discussion: Effect of agitation and aeration on the growth of the yeast.

The agitation has four basic functions in a fermentation system: (1) It ensures homogeneity of the culture through macro-mixing. (2) It promotes interface mass transfer through micro-mixing. (3) It enhances heat transfer. (4) It provides gas-liquid interfacial area for gas-to-liquid and liquid-to-gas mass transfer. Aeration has basically two functions, to supply oxygen to the culture and to remove CO_2 and other volatile metabolites from the culture. With respect to the function of oxygen supplementation to the culture, the effect of airflow is rather complex and is closely interrelated with agitation. The interfacial surface area for mass transfer provided by bubble formation at the sparger in stirred tanks is usually too small to permit a sufficient oxygen transfer rate. Therefore, a fraction

of the mechanical energy imparted to the liquid by the impeller is used to break large bubbles into smaller ones, and thus increase the dissolved oxygen concentration by increasing the gas-to-liquid interfacial area. Aeration also decreases power consumption, mainly by decreasing the apparent density of the liquid in which the impeller rotates.

The dissolved oxygen concentration depends on the medium agitation speed and aeration rate, and higher levels of agitation or aeration will increase the oxygen transfer capability of the fermentation system. The higher the agitation speed and/or aeration rate, the higher the concentration of dissolved oxygen in the medium (Yoshida *et al.*, 1965; Martin and Bailey, 1985), which is essential for the production of biomass by aerobic microorganisms and for carotenogenesis by *Rhodotorula* spp. However, it was observed that the growth was reduced at 250 rpm and/or 1.5 vvm in peat extract medium, with no significant difference observed with the above growth conditions in molasses medium. In general, the carotenogenic yeasts are all obligate aerobes, and require a minimal amount of oxygen for growth (Simpson and Chichester, 1971). For example, Johnson and Lewis (1979) reported that a dissolved oxygen rate of 30 mmol O_2 L^{-1} h^{-1} is required for the growth and carotenogenesis of *P. rhodozyma*.

3.3 Kinetics of the growth of *R. rubra* in the peat extract medium

The optimal conditions of temperature, initial pH, inoculum ratio, substrate and nutrient concentrations, agitation speed, and aeration rate were employed to

study the growth characteristics of the *R. rubra* in a batch fermenter. The progress of batch cultivations over a fermentation time of 134 hours is shown in Figure 3.3. for the dry biomass concentration, residual TCH concentration, specific growth rate, and β-carotene production.

3.3.1 Lag phase

There was no appreciable growth within the first 5 hours of fermentation. This period could therefore be described as the lag phase. The lag phase arises from the need for cells in the inoculum to adapt to the prevailing conditions in the fermenter, although no built model incorporates any mechanism for this adaptation (Sinclair and Kristiansen, 1987). However, there was a rapid consumption of carbohydrates and a rapid utilization of the dissolved oxygen during this period. It has been observed that during the lag phase, the D.O. saturation (determined by a dissolved oxygen electrode, 900 series, New Brunswick Scientific Co., New Jersey, U.S.A.) was dropped from 100% of saturation to 80% within 24 hours. This could be due to the fact that enzyme activities such as oxygen consumption, carbon dioxide production and the evolution of heat were the same on a per unit of dry weight basis during the lag phase as during the exponential (logarithmic) phase (Luedeking, 1967). The adaption of the yeast to the batch fermentation conditions was quite good, resulting in a short lag phase. This could be due to the starter culture cells being in a period of exponential growth and being transferred into an medium with the same compositions as the starter culture medium.

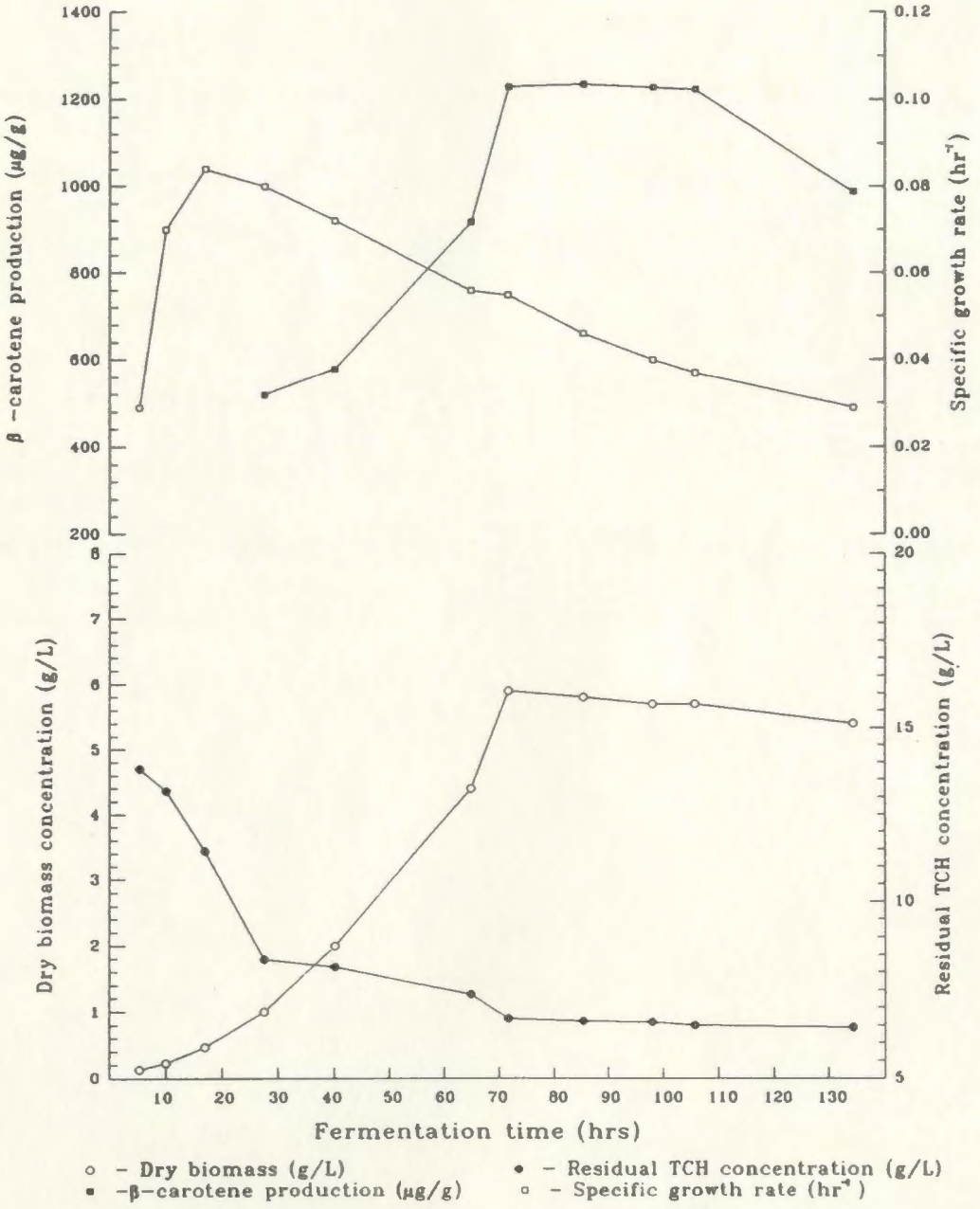


Fig. 3.3 Growth kinetics of *R. rubra* in fermenter in peat extract medium in batch fermenter.

3.3.2 Exponential phase

It was observed that there was a rapid increase in the cell mass after 5 hours of fermentation (Figure 3.3.). This period, after the lag phase, could be described as an exponential phase, in which all the numerous linked reactions comprising the metabolism of the cell had built up to a maximum rate (Luedeking, 1967). Therefore, with an excess of the substrate necessary for growth, there was a rapid increase in the specific growth rate. The maximum specific growth rate in the fermenter reached 0.084 hr^{-1} within 17 hrs. The maximum amount of cell mass produced by a unit amount of cells in a unit time (the maximum specific growth rate) was achieved during this period, which was also the period when β-carotene synthesis began. This is represented by the equations:

$$X = X_0 e^{\mu t}$$

or $$\mu = (\ln X - \ln X_0) / t$$

where: X is the concentration of organisms, usually expressed as dry weight per liter of culture, (g/L);

X_0 is the initial concentration of organisms at zero time;

μ is the specific growth rate during the logarithmic phase (hour $^{-1}$);

t is time in hours.

3.3.3 Stationary phase

After the exponential phase, a stationary phase occurred. The maximum dry biomass concentration and maximum β-carotene production in the fermenter reached 5.9 g/L and 1235.28 μg/g, respectively, during this phase (as compared to 4.85 g/L and 1225.56 μg/g, respectively, in the shake flask experiment), within 72 hrs of fermentation (as compared to 84 hrs in the shake flask experiment). This was followed, after 105.67 hrs of fermentation in peat extract medium, by a phase of constant dry biomass concentration (5.7 g/L).

The accelerated growth rate continued for only 40 hours after onset, and was followed by a decline in the growth rate associated with a constant residual TCH concentration value. The most probable cause of the declining growth rate would be a decrease of one of the required nutrients to a growth-limiting concentration (Righelato, 1975). Thus, a relationship between the cell concentration and the substrate concentration could be established during the stationary phase of growth. As can be seen from the following equation, the specific growth rate decreased as the limiting substrate concentration decreased.

The declining growth phase was associated with a constant value of the residual TCH concentration. Righelato (1975) has reported that oxygen (due to its low solubility in aqueous media) and carbon sources often become growth-limiting in fungal cultures. Therefore, the growth rate became a function of the substrate concentration (Monod, 1949), and is represented by the equation:

$$\mu = \frac{\mu_{max}\, S}{S + K_s}$$

where:

μ is the specific growth rate during the logarithmic phase (hour $^{-1)}$;

μ_{max} is the maximal specific growth rate (hour^{-1});

S is the limiting substrate concentration (grams / liter);

K_s is the substrate concentration (g/L) at which the maximum specific growth rate is reduced by one-half.

Brown (1988) concluded that the nature of the nutrient limitation will dictate the subsequent response of the cells to the nutrients remaining in the medium, thus, carbon limitation could depress the metabolism; oxygen limitation might cause autolysis, and; nitrogen limitation might result in the formation of carbohydrate storage compounds. Garraway and Evans (1984) concluded that nutrient limitation could result in unfavourable changes in such factors as oxygen tension or pH, and could result in the metabolites excreted by the organism becoming toxic. Eventually, growth of the population slows to the point at which there is no net increase in the number of cells; and a plateau or stationary phase is reached.

3.3.4 Decline phase

The biomass concentration declined after 105 hrs of fermentation. This could have had either of two causes; the endogenous metabolism of stored

carbohydrates within the cellular material, or the death of cells and the active decay of material due to autolysis (Brown, 1988). The death rate eventually increases and the population enters the phase of decline (Garraway and Evans, 1984).

3.3.5 Biomass Yield (Y)

As was defined elsewhere in this communication, the yield is given by the equation:

$$Y = (X - X_0) / (S_0 - S_f)$$

Where: S_0 is the initial substrate concentration (g/L);

S_f is the final substrate concentration (g/L);

X is the concentration of organisms, usually expressed as dry weight per liter of culture (g/L);

X_0 is the initial concentration of organisms.

The substrate was assumed to be the total carbohydrates (Solomons, 1975), because this was the carbon and energy source employed in this work. The yield value obtained, when expressed as a percentage (33.19 % in molasses and 70.85 % in peat extract) compares very well with the value (24.4 %) reported by Yoon and Rhee (1983) for *R. glutinis* grown in synthetic medium containing 1.5 % glucose.

The yield value obtained in this work is quite high. However, in a complex medium, there could be many other sources of carbon besides the carbohydrates present, such as amino acids, lipids, organic acids and higher-molecular-weight alcohols (Solomons, 1975). LeDuy (1981) has reported that about 80 % of the amino acids, 50 % of the non-volatile organic acids and 50 % of the hydroxymethylfurfurol in peat extract are utilized as carbon sources by microorganisms for the production of biomass. A yield value of 32.2 % was obtained by Raitsina and Evdokimova (1977), who cultivated *Rhodotorula* sp. on peat extract. High yield values have been reported by other investigators for some yeast cultivated in complex media. For example, a yield value of 54 % for *R. glutinis* grown in sugar cane juice was reported by Matelli *et al.* (1990).

The most accurate yield value can, therefore, be obtained if calculated as the amount of cell mass produced per gram of unit weight of carbon in the substrate consumed (Sinskey, 1978). Since the carbon source for cell biomass production is almost always the most expensive component supplied, Solomons (1975) has suggested that high yield factors could be of great importance in batch cultures.

3.3.6 General discussion: Growth kinetic parameters of the yeast produced by fermentation

Many papers on the growth of yeast refer to the biomass concentrations obtained after a certain number of hours of batch growth rather than to the growth

rate and the doubling time. In this work, in peat extract medium, the maximum yield of *R. rubra* in shake flask experiments (70 %) was obtained in 84 hrs, and was similar to that in batch fermenter experiments (71 %) in 71 hrs. In a molasses medium, the maximum yield (33.19 %) in batch fermenter was obtained in 72 hrs. Costa *et al.* (1987) using similar operational conditions (an incubation temperature of 27 °C, a pH range between 5.5 - 6.5, an agitation speed of 400 rpm, and an aeration rate of 1.0 vvm) obtained a maximum *R. glutinis* biomass yield of 49 % in 20 hours in a fermenter, and in 17 hours in shake flasks, in a sucrose semi-synthetic medium. Under the latter conditions, however, the cells were scarcely colored. The dry biomass concentration (5.9 g/L) obtained in 71 hrs in this work was not, however, superior to that of Costa *et al.* (1987), who obtained a dry biomass concentration of 11.1 g/L in the medium at an initial glucose concentration of 22 g/L. This could be due to the longer lag phase required for the yeast to adapt to a medium of complex composition, during which time the induction and / or depression of the enzymes to metabolize available nutrients takes place (Garraway and Evans, 1984). Also, inhibitory factors that exist in the peat extract (Martin and Bailey, 1985) may have hampered the yeast growth.

3.4 Composition of the biomass

3.4.1 Proximate composition

The concentrations of the main components of the yeast biomass are presented in Table 3.4.1, allowing comparison of the differences between biomass grown in the molasses and peat extract media. The protein content of *R. rubra* biomass obtained in this work compares favourably with those reported for this yeast by other investigators (Table 3.4.1.1). Total lipids contents were lower than the other investigators reported (Table 3.4.1.3).

3.4.1.1 Protein content

The crude protein content of the yeast produced in a molasses medium was 43.6 ± 0.7%, and that of yeast grown in the peat extract was not significantly different ($P < 0.05$) at 48.5 ± 0.5 %. These values compare favourably with those reported by other investigators. For example, Jacob (1991) reported that *R. gracilis* grown in wheat bran medium contained 21.6 % protein. Matelli *et al.* (1990) obtained a protein content of 38.2 % in *R. glutinis* grown by sugar cane juice fermentation. The protein contents from this work also compare favourably with that reported for *Candida* grown on peat extract (Fuchsman, 1980). Thus, both peat extract and molasses are favourable substrates for the protein synthesis of *R. rubra*.

The high protein content of *R. rubra* in this work is a significant characteristic. According to Reed (1982*a*), SCP refers to those cultured microbial

Table 3.4.1 Proximate composition of the *R. rubra* biomass (% of dry weight)

	Molasses	Peat extract
Moisture (%)	1.9 ± 0.2	1.7 ± 0.5
Crude protein (%)	43.6 ± 0.7	48.5 ± 0.5
Total lipid (%)	10.2 ± 1.2	5.7 ± 0.6
Total carbohydrate (%)	30.8 ± 3.1	27.7 ± 2.7
Ash (%)	3.8 ± 0.4	4.1 ± 0.1

Table 3.4.1.1 Protein production by *Rhodotorula* spp.

Species	Carbon source	Protein content (%)	References
R. rubra	molasses	43.6 ± 0.7	this work
	peat extract	48.5 ± 0.5	this work
R. glutinis	cane juice	38.2	Matelli *et al.* (1990)
R. gracilis	wheat bran	21.6	Jacob (1991)

cells that contain more than 40 % protein, and the proteins or protein concentrates that may be extracted from them. Thus, this new strain of *R. rubra* can be considered as a useful source of SCP for incorporation into feeds.

3.4.1.2 Amino acid composition

The amino acid profile of the yeast produced with a peat extract as the basic substrate revealed the presence of 10 amino acids including all the essential ones.

Table 3.4.1.2 compares the results of this work with those of other investigators, and with soya bean meal, fodder yeast, and an FAO protein standard. The content of most *R. rubra* amino acids compared well with those of the FAO standard, except for methionine, tryptophan and valine, which are much lower than the standard values. Nevertheless, the yeast may still be considered nutritionally valuable.

3.4.1.3 Lipids content

The total lipid content of *R. rubra* produced in molasses medium was significantly higher ($P > 0.05$) than that produced in peat extract medium. This could be because the molasses medium had a higher C:N ratio than the peat extract medium, and high C:N ratios favour lipid accumulation in yeast (Hammond *et al.*, 1981).

Table 3.4.1.2 Profile of essential amino acids ($g \cdot kg^{-1}$ protein) of *R. rubra* biomass grown in peat extract, and of other protein products

Amino acid	*R. rubra* grown in peat extract	Soya bean meal [1]	Fodder yeast[1]	FAO standard [2]
Arginine	38.5			
Histidine	16.9			
Isoleucine	25.9	42.0	53.0	42.0
Leucine	46.8	75.0	70.0	48.0
Lysine	39.1	64.0	67.0	42.0
Methionine	12.7	22.0	19.0	22.0
Phenylalanine	26.0	47.0	43.0	28.8
Threonine	32.3	36.0	55.0	28.0
Tryptophan	2.78	17.0	14.0	14.0
Valine	34.3	44.0	63.0	42.0

[1] Data taken from Moo-Young *et al.* (1979)

[2] Data taken from FAO/WHO (1973)

The total lipids content of *R. rubra* grown in either medium was lower than those reported by other authors for SCO (single cell oil) production by *Rhodotorula* spp. (Table 3.4.1.3)

3.4.1.4 Fatty Acids

The composition and content of the identified fatty acids in *R. rubra* are listed in Table 3.4.1.4. However, the structures of 51.5 % of the fatty acids produced by the new strain are uncertain. Estimated from the retard time, 21.24 % of the uncertain fatty acids have short carbon chains (with a carbon number less than 14). The retard times of a further 30.27 % of the uncertain fatty acids were between those of 17:0 and 18:0 fatty acids. Among the known fatty acids produced, 14:0 fatty acids are present in the highest amount. Ratledge (1981) reported that the major fatty acids produced by *Rhodotorula* species are 18:1, 16:0, 18:0, 18:2, 18:3, and 16:1 fatty acids, in that order, which are different from the major fatty acids produced in this work. Ratledge and Evans (1989) explained that the individual components that make up the total lipid content of the cell may vary in absolute as well as in relative abundance due to different growth conditions and to inadequate precautions to prevent the actions of lipases and phospholipases during extraction.

Table 3.4.1.3 Lipids produced by *Rhodotorula* spp.

Species	Lipid Content (% cell dry wt.)	References
R. glutinis	57	Yoon and Rhee (1983)
	58	Park (1974)
	66	Patent (1977)
	40	Allen *et al.* (1964)
	48	Steinberg and Ordal (1954)
	60	Enebo *et al.* (1946)
	72	Yoon and Rehm (1982)
R. graminis	41	Patent (1977)
R. mucilaginosa	28	Borisova and Atamanyuk (1980)
R. rubra	10.22	This work (molasses)
	5.7	This work (peat extract)

Table 3.4.1.4 Fatty acid contents of *Rhodotorula* spp.

Fatty acid	*R. rubra* (This work) (%)	*R. gracilis* (Ratledge, 1981) (%)
14:0	19.0	1
16:0	-	18
16:1	-	1
17:0	2.6	-
18:0	1.2	6
18:1	7.9	41
18:2	2.4	24
18:3n3	1.7	-
18:3	-	1
20:0	-	9
20:3	4.0	-
20:4	2.4	-
24:0	7.4	-

3.4.2 Carotenoid content

The β-carotene content of the yeast produced in the molasses and wort medium was 1034 μg/g of dry biomass, and in peat extract medium it was 1235 μg/g. The β-carotene contents of the yeast produced in both media are high, compared to the findings of other workers. For example, Simpson *et al.* (1964) reported that *R. glutinis* produced 257 μg/g of carotenoids in glucose medium, while Fuchsman (1980) reported that 153 μg/g of carotenoids were produced in peat extract by *R. glutinis*. However, Matelli *et al.* (1990) reported 1700 μg/g of β-carotene were produced by *R. glutinis* in sugar cane juice.

The high carotene content of *R. rubra* obtained in this work is a most significant characteristic. The values compare favourably with those reported for genus *Rhodotorula* grown in different media (Table 3.4.2.). Therefore, both media have beneficial effects on the carotenoids synthesis of *R. rubra*.

Table 3.4.2 Carotenoids produced by *Rhodotorula* spp.

Species	Carbon source	Carotenoid content (μg/g)	References
R. rubra	peat extract	1235 ± 57	this work
R. rubra	molasses	1034 ± 44	this work
R. glutinis	peat extract	153	Raitsina and Evdokimova (1977)
R. glutinis	glucose	257	Simpson *et al.* (1964)
R. glutinis	sugar cane juice	1700	Matelli *et al.* (1990)

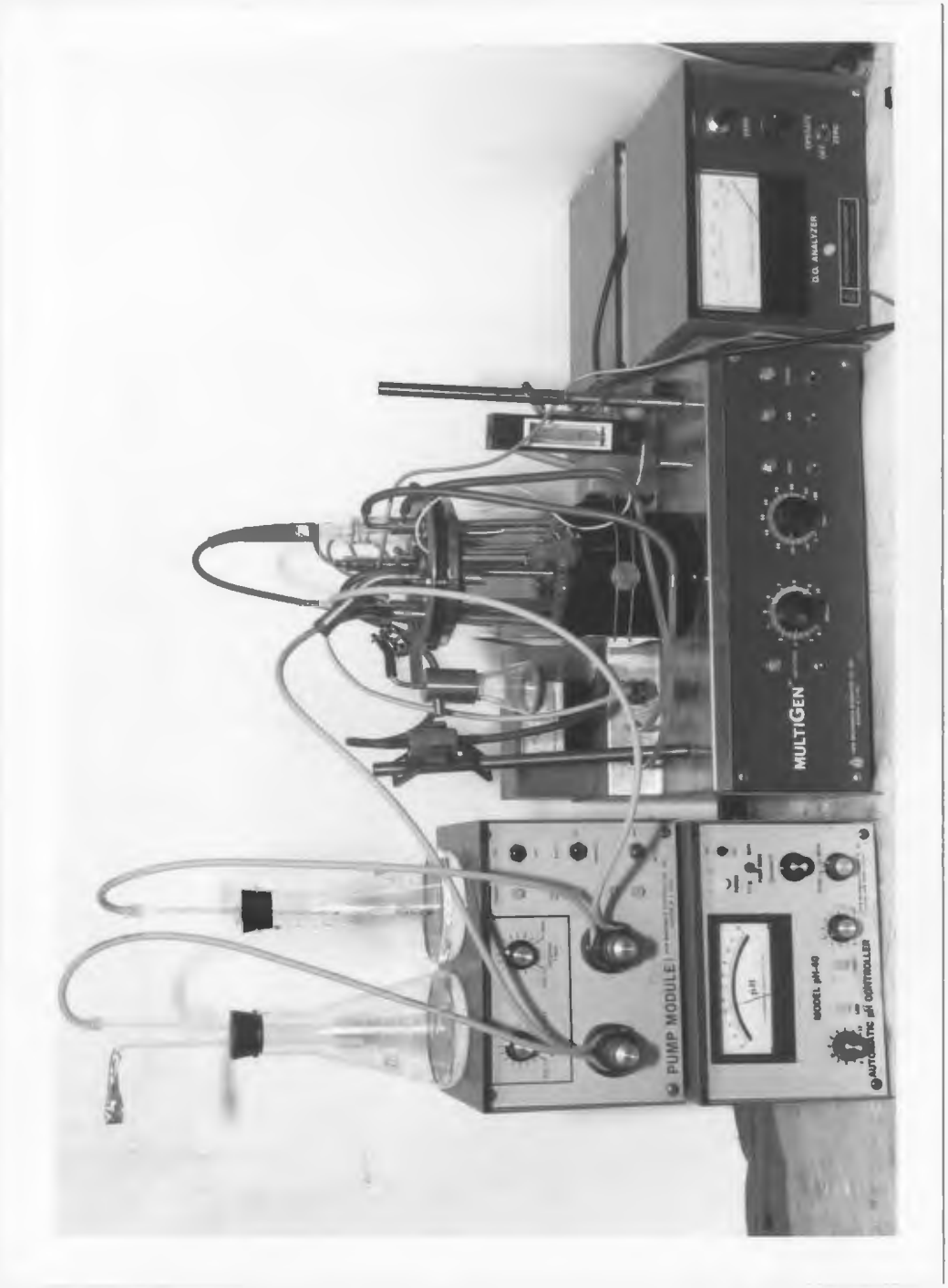
CONCLUSIONS

This work provides evidence that *R. rubra* can be cultured in acid peat extract medium supplemented with only nitrogen, potassium and magnesium, and that it can also be cultured in molasses media supplemented with only brewery wort. This work also shows that carotene yields can be obtained from this species in both media that are competitive with carotene yields obtained in other media. The use of peat extract as well as molasses as inexpensive sources of carbon and energy, and the simple nutritional requirements of *R. rubra*, make the use of peat-based and molasses-based media for the large scale production of the *R. rubra* biomass attractive possibilities.

This study has also established an optimal range of values for the most important operational variables involved in the batch fermentation of *R. rubra* in both peat-based and molasses-based culture media. The optimal operational conditions were the same in both media. These were as follows: an incubation temperature of 22 ± 1°C, a pH of 5.5 ± 0.1, an agitation speed of 200 rpm, an aeration rate of 1.0 vvm, an inoculum ratio of 2 % (v/v), and a fermentation time of 72 hours. The maximum specific growth rate of the yeast in fermenter by peat extract fermentation was 0.084 h^{-1}. The biosynthesis of carotenoids by *R. rubra* was found to occur after the cease of the yeast growth.

Supplementation of peat extract medium with ammonium sulphate, yeast extract, potassium sulphate and magnesium sulphate resulted in significant

increases in the biomass and β-carotene concentrations.



A.1. Arrangement of the pH controller, fermenter, and D.O. analyzer

Table B.1 Effect of total carbohydrate concentration on the growth of *Rhodotorula rubra* in peat extract medium [1]

Total carbohydrate concentration ($g \cdot L^{-1}$)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)
12.0	4.17 ± 0.30^{a}	62.18 ± 0.90^{a}	34.73 ± 0.50^{a}
15.0	4.35 ± 0.10^{a}	70.92 ± 0.70^{b}	29.02 ± 0.70^{b}
18.0	3.19 ± 0.21^{b}	48.28 ± 0.03^{c}	17.71 ± 0.20^{c}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The medium was supplemented with 5 g/L of yeast extract, and the experiments were conducted in shake flasks with a fermentation time of 96 h, an agitation speed of 200 rpm, and a pH of 5.5.

Table B.2 Effect of wort concentration on the growth of *Rhodotorula rubra* in molasses medium [1]

Wort concentration (% v/v)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)
2.0	4.29 ± 0.33^{a}	33.15 ± 1.05^{a}	4.47 ± 0.47^{a}
4.0	4.68 ± 0.40^{a}	36.24 ± 0.37^{ab}	4.88 ± 0.58^{a}
6.0	4.76 ± 0.15^{a}	36.87 ± 0.51^{b}	4.96 ± 0.11^{a}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in 10 % (v/v) molasses medium in shake flasks, with a fermentation time of 3.5 d, an agitation speed of 200 rpm, and a pH of 5.5.

Table B.3 Effect of K_2SO_4 supplementation on the growth of *Rhodotorula rubra* in peat extract medium [1]

mM K^+ added	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)	β-carotene production ($\mu g \cdot g^{-1}$)
0	2.65 ± 0.40^a	30.89 ± 0.90^a	17.65 ± 0.90^a	722.4 ± 50^a
2.0	3.75 ± 0.14^b	37.13 ± 0.40^b	25.01 ± 0.90^b	814.8 ± 37^a
3.0	3.82 ± 0.03^b	37.27 ± 0.60^b	25.47 ± 0.20^b	837.7 ± 11^a
4.0	3.85 ± 0.07^b	39.30 ± 0.80^b	25.65 ± 0.50^b	830.7 ± 17^a

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium, with 15g·L^{-1} TCH, 1.5 g·L^{-1} yeast extract, and 4.0 g·L^{-1} $(NH_4)_2SO_4$, with a fermentation time of 96 h, an agitation speed of 200 rpm, and a pH of 5.5.

Table B.4 Effect of $MgSO_4 \cdot 7H_2O$ supplementation on the growth of *Rhodotorula rubra* in peat extract medium [1]

$MgSO_4 \cdot 7H_2O$ added ($g \cdot L^{-1}$)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)	β-carotene ($\mu g \cdot g^{-1}$)
0.0	3.75 ± 0.14^{a}	37.13 ± 0.40^{a}	25.01 ± 0.90^{a}	814.8 ± 37^{a}
0.5	4.57 ± 0.07^{b}	58.13 ± 0.19^{b}	30.45 ± 0.52^{b}	1112.6 ± 40^{b}
1.0	4.42 ± 0.14^{c}	57.18 ± 0.08^{b}	29.43 ± 0.92^{c}	1113.7 ± 41^{b}
1.5	4.48 ± 0.23^{bc}	57.34 ± 2.01^{b}	29.88 ± 1.52^{b}	1125.1 ± 33^{b}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium, with $15 g \cdot L^{-1}$ TCH, $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, and $0.174 g \cdot L^{-1}$ K_2SO_4, and with a fermentation time of 96 h, an agitation speed of 200 rpm, and a pH of 5.5.

Table B.5 Effect of initial pH of peat extract medium on the growth and β-carotene synthesis of *Rhodotorula rubra* [1]

Initial pH of the medium	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
4.0	3.47 ± 0.17^{a}	35.18 ± 0.40^{a}	5.14 ± 0.22^{a}	761.65 ± 19^{a}
4.5	4.16 ± 0.15^{b}	50.50 ± 0.30^{bd}	6.76 ± 0.35^{b}	1120.65 ± 30^{b}
5.0	4.48 ± 0.04^{b}	51.95 ± 4.36^{abc}	6.38 ± 0.10^{b}	1208.2 ± 35^{bc}
5.5	4.58 ± 0.22^{b}	59.96 ± 0.10^{c}	7.36 ± 1.24^{ab}	1227.36 ± 41^{c}
6.0	4.27 ± 0.54^{ab}	46.56 ± 1.90^{cd}	5.829 ± 0.69^{ab}	983.1 ± 22^{d}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted with $15 g \cdot L^{-1}$ TCH, at an incubation time of 96 h, a temperature of 22°C, and an agitation rate of 200 rpm, and with nutrient supplementation of $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, 2.0 mM K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$.

Table B.6 Effect of fermentation time on the growth and β-carotene synthesis of *Rhodotorula rubra* in peat extract medium [1]

Fermentation time (hr)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
60	2.93 ± 0.23^{a}	50.10 ± 0.77^{a}	9.15 ± 2.06^{a}	896.5 ± 15^{a}
72	4.25 ± 0.20^{b}	60.17 ± 0.08^{bc}	7.94 ± 1.68^{a}	1230.2 ± 34^{b}
84	4.84 ± 0.11^{c}	70.02 ± 3.62^{b}	8.09 ± 0.78^{a}	1255.6 ± 57^{b}
96	4.60 ± 0.22^{d}	60.76 ± 0.27^{bc}	7.43 ± 0.35^{a}	1227.36 ± 41^{b}
120	4.07 ± 0.01^{abd}	55.16 ± 1.33^{c}	7.62 ± 0.51^{a}	997.5 ± 40^{a}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted with $15 g \cdot L^{-1}$ TCH, a temperature of 22°C, an agitation rate of 200 rpm, and a pH of 5.5, and with nutrient supplementation of $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, 2.0 mM K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$.

Table B.7 Effect of agitation speed on the growth and β-carotene synthesis of *Rhodotorula rubra* in peat extract medium [1]

Agitation speed (rpm)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Efficiency (%)	β-carotene ($\mu g \cdot g^{-1}$)
150	2.65 ± 0.39^{a}	40.89 ± 0.90^{a}	17.65 ± 0.60^{a}	765.9 ± 31^{a}
200	4.84 ± 0.11^{b}	70.02 ± 3.62^{b}	32.25 ± 0.70^{b}	1255.6 ± 57^{b}
250	4.58 ± 0.22^{c}	57.67 ± 0.45^{bc}	30.55 ± 1.45^{bc}	1232.4 ± 47^{b}
300	3.72 ± 0.16^{d}	49.71 ± 1.51^{d}	24.82 ± 1.05^{c}	1179.8 ± 50^{b}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted with $15 g \cdot L^{-1}$ TCH, $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, 2.0 mM K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and with a temperature of 22°C, a fermentation time of 84 h, and a pH of 5.5.

Table B.8 Effect of incubation temperature on the growth and β-carotene synthesis of *Rhodotorula rubra* in peat extract medium [1]

Incubation temperature (°C)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
18	2.51 ± 0.27^{a}	38.55 ± 0.4^{a}	8.49 ± 0.04^{a}	585.4 ± 22^{a}
20	2.65 ± 0.39^{a}	39.03 ± 0.5^{a}	8.21 ± 0.43^{ab}	1025.3 ± 21^{bd}
22	4.84 ± 0.11^{b}	70.02 ± 3.62^{b}	8.09 ± 0.78^{ab}	1255.6 ± 57^{c}
24	3.50 ± 0.18^{c}	48.88 ± 0.53^{c}	7.84 ± 0.61^{ab}	1123.4 ± 45^{bc}
26	3.35 ± 0.10^{d}	44.28 ± 0.51^{c}	7.43 ± 0.14^{b}	988.4 ± 31^{d}

[1] Values represent the mean of three determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium, with $15 g \cdot L^{-1}$ TCH, $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, $0.174 g \cdot L^{-1}$ K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and with a fermentation time of 84 h, an agitation speed of 200 rpm, and a pH of 5.5.

Table B.9 Effect of agitation speed on the growth and β-carotene synthesis of *Rhodotorula rubra* in batch fermentation in peat extract medium [1]

Agitation speed (rpm)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
150	2.44 ± 0.11^{a}	39.53 ± 0.33^{a}	8.82 ± 0.15^{a}	957.5 ± 47^{a}
200	5.90 ± 0.10^{b}	70.85 ± 1.40^{b}	6.67 ± 0.81^{ab}	1232.2 ± 35^{b}
250	3.92 ± 0.25^{c}	42.94 ± 0.31^{c}	5.88 ± 0.10^{b}	1127.3 ± 40^{b}

[1] Values represent the mean of two determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium in a batch fermenter, with $15 g \cdot L^{-1}$ TCH, $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, 2.0 mM K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and with an inoculum rate of 2 % (v/v), a fermentation time of 84 h, an aeration rate of 1.0 vvm, a temperature of 22°C, and a pH of 5.5.

Table B.10 Effect of agitation speed on the growth and β-carotene synthesis of *Rhodotorula rubra* in batch fermentation in molasses medium [1]

Agitation speed (rpm)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
150	3.25 ± 0.33^{a}	27.18 ± 0.18^{a}	83.95 ± 0.31^{a}	928.6 ± 33^{a}
200	4.46 ± 0.30^{b}	33.41 ± 0.43^{b}	82.56 ± 0.22^{b}	1033.5 ± 47^{ab}
250	4.65 ± 0.70^{b}	19.74 ± 0.35^{c}	72.35 ± 0.15^{c}	1045.5 ± 41^{b}
300	4.60 ± 0.70^{b}	20.77 ± 0.70^{c}	73.76 ± 0.33^{d}	1023.4 ± 35^{ab}

[1] Values represent the mean of two determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in molasses and wort medium (1.5 L), with 10 % (v/v) of molasses, 4 % (v/v) of wort, an inoculum rate of 2 % (v/v), a fermentation time of 84 h, and with an aeration rate of 1.0 vvm, a temperature of 22°C, and a pH of 5.5.

Table B.11 Effect of aeration rate on the growth and β-carotene synthesis of *Rhodotorula rubra* in fermenter in peat extract medium [1]

Aeration rate (vvm)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
0.75	2.96 ± 0.21^{a}	40.12 ± 0.62^{a}	7.62 ± 0.40^{a}	522.6 ± 37^{a}
1.0	5.90 ± 0.10^{b}	70.85 ± 1.40^{b}	6.67 ± 0.81^{abc}	1232.2 ± 35^{b}
1.5	3.40 ± 0.23^{c}	37.46 ± 0.81^{c}	5.92 ± 0.48^{b}	1007.3 ± 23^{c}
2.0	1.51 ± 0.30^{d}	34.38 ± 0.23^{c}	10.6 ± 0.11^{c}	1013.5 ± 40^{c}

[1] Values represent the mean of two determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in peat extract medium in a fermenter, with $15 g \cdot L^{-1}$ TCH, $1.5 g \cdot L^{-1}$ yeast extract, $4.0 g \cdot L^{-1}$ $(NH_4)_2SO_4$, 2.0 mM K_2SO_4 and $0.5 g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, and with an inoculum rate of 2 % (v/v), a fermentation time of 84 h, an agitation speed of 200 rpm, a temperature of 22°C, and a pH of 5.5.

Table B.12 Effect of aeration rate on the growth and β-carotene synthesis of *Rhodotorula rubra* in fermenter in molasses medium [1]

Aeration rate (vvm)	Dry biomass ($g \cdot L^{-1}$)	Yield (%)	Residual TCH conc. ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
0.75	3.75 ± 0.21^a	30.00 ± 0.81^a	83.40 ± 0.41^a	429.9 ± 87^a
1.0	4.46 ± 0.30^b	33.41 ± 0.43^b	82.56 ± 0.22^a	1033.5 ± 47^b
1.5	4.40 ± 0.51^b	33.43 ± 0.33^b	82.74 ± 0.17^a	1037.8 ± 61^b

[1] Values represent the mean of two determinations ± standard deviations. Values within the same column with the same superscript are not significantly different ($P < 0.05$). The experiment was conducted in molasses and wort medium (1.5 L), with 10 % (v/v) of molasses, 4 % (v/v) of wort, an inoculum rate of 2 % (v/v), and with a fermentation time of 84 h, an agitation speed of 200 rpm, a temperature of 22°C, and a pH of 5.5.

Table B.13 Growth kinetics of *R. rubra* in peat extract medium in batch fermenter.

Time (hr)	X ($g \cdot L^{-1}$)	μ (hr^{-1})	Y (%)	S ($g \cdot L^{-1}$)	β-carotene ($\mu g \cdot g^{-1}$)
5.3	0.133	0.029	11.18	13.81	-
10.0	0.23	0.070	12.64	13.18	-
17.0	0.47	0.084	13.20	11.44	-
27.5	1.0	0.080	15.07	8.366	520.33
40.08	2.0	0.072	29.21	8.154	579.25
64.83	4.4	0.056	57.65	7.368	918.24
71.66	5.9	0.055	70.85	6.673	1230.44
85.3	5.8	0.046	69.15	6.612	1235.28
98.0	5.7	0.040	67.74	6.585	1228.28
105.67	5.7	0.037	67.00	6.492	1224.22
134.42	5.4	0.029	63.08	6.439	987.4

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