STUDY OF THE GROWTH PARAMETERS OF THE YEAST *Phaffia rhodozyma* AND ITS PRODUCTION OF ASTAXANTHIN IN PEAT HYDROLYSATES

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by

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ABSTRACT

Salmonid aquaculture as a supplement to the world's protein resources increased substantially in the 1980s resulting an increase in the market for astaxanthin, the principal carotenoid of salmon. Consumer acceptance of farmed salmonids depends on the color of the flesh. Since fish are unable to synthesize these pigments, they must be supplied to the diet. Currently, synthetic astaxanthin and canthaxanthin are used as pigmenters but there is considerable interest in using biological sources of astaxanthin in the aquaculture industry.

The yeast *Phaffia rhodozyma* has the potential as an industrial pigment source but its use is limited by the low quantities of astaxanthin in the yeast and also the high cost of growth media. Peat is one of the most abundant and inexpensive resources of the world. The liquid extract from peat contains fermentable carbohydrates which have been used in the production of various microorganisms.

A comprehensive study on the efficiency and yield of the production of yeast biomass and astaxanthin in peat hydrolysate has been carried out by a biotechnological process using *Phaffia rhodozyma*. The peat hydrolysates used in the present study were prepared by acid and non-acid hydrolyses process at various temperatures ranging from

185 - 225°C. The sugars present in the peat hydrolysate were determined. The total reducing sugars constitute about 20.66% of the total carbohydrate concentration (TCH).

The substrate concentration that supported the best growth of *P. rhodozyma* was 30 $g \cdot L^{-1}$ for the non-acid hydrolysate and 15 $g \cdot L^{-1}$ for the acid hydrolysate. The non-acid hydrolysate produced a dry biomass concentration of 4.04 ± 0.11 $g \cdot L^{-1}$, a yield coefficient of 34.82 ± 0.98% and an efficiency of 13.48 ± 0.1% whilst the acid hydrolysate produced a biomass concentration of 4.30 ± 0.04 $g \cdot L^{-1}$, a yield coefficient of 36.74 ± 1.13% and an efficiency of 14.33 ± 0.1%. These results were obtained at a pH of 7.0, an incubation temperature of 18°C, and a fermentation time of 120 hours. The best inoculum ratio was 5% ($v \cdot v^{-1}$) and agitation speed was 200 r.p.m.

Of the six peat hydrolysates provided, the acid hydrolysate prepared at a temperature of 185°C (PH4-02-185) was found to support the best growth. The astaxanthin content of the yeast grown in the best acid and non-acid hydrolysates were determined. The acid hydrolysate produced an astaxanthin content of 1567 $\mu g \cdot g^{-1}$ yeast whereas the non-acid hydrolysate produced 1280 $\mu g \cdot g^{-1}$ yeast. These values compare favorably with those reported by other researchers.

The nutritional requirements of *P. rhodozyma* grown in the best peat hydrolysate were studied in an effort to enhance the growth and astaxanthin production by this yeast. Organic sources of nitrogen were found to promote the growth of and astaxanthin production by the yeast better than ammonium salts of inorganic acids. The addition of increasing concentrations of yeast extract resulted in increase in biomass concentration,

yield coefficient, efficiency and astaxanthin content. On the other hand, addition of potassium phosphate to the medium increased the dry biomass concentration but decreased the astaxanthin concentration of the yeast.

Experiments were conducted in 2 L fermenters to study the growth kinetics and astaxanthin production by the yeast in peat hydrolysate. The best growth produced a biomass concentration of 4.86 ± 0.12 $g \cdot L^{-1}$ and an astaxanthin content of 1079 ± 17 $\mu g \cdot g^{-1}$ yeast after 120 hours of fermentation. The optimum agitation speed was 250 r.p.m. The optimal aeration required for the production of astaxanthin was found to be 0.5 vvm. Higher agitation speeds reduced biomass production but had no effect on astaxanthin production. Similarly, aeration rates did not have any significant impact on astaxanthin production. The maximum specific growth rate, μ_{max} of the yeast grown in peat hydrolysate was found to be 0.038 h^{-1}.

The chemical composition of the *P. rhodozyma* cell biomass was analyzed. The crude protein content was found to be 47%, with 5% ash, 19% total lipids, and reasonable amounts of tryptophan, arginine, leucine phenylalanine, threonine, glycine and other amino acids. The yeast was found to contain high quantities of unsaturated fatty acids.

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Maame, may your soul rest in peace.

E. A. A. A.

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CHAPTER 1

INTRODUCTION

As a result of the dwindling of the fish resources in our oceans, the need for aquaculture as a supplement to the world's protein resources is well established. North America, Norway, Japan, and Chile have taken the lead in fish farming to cope with shortfalls in animal protein production. For aquaculture to be efficient, feed must be supplied to the fish. The feed must provide all the essential elements of the natural diet if the product is to compete with that harvested from the wild. Consumer acceptance of farmed salmonids is dependent upon the color of the fish (Meyers, 1977; Ostrander *et al.*, 1976). Since fish and other animals are thought incapable of a *de novo* synthesis of carotenoids, these compounds must be supplied in the diet whenever necessary.

The principal carotenoid found in wild salmonids is astaxanthin from ingested crustacea. Two main carotenoids, astaxanthin and canthaxanthin, are usually added to fish feed to obtain the desired coloration (Torrissen *et al.*, 1989). Synthetic astaxanthin and canthaxanthin are available but their inclusion is either not permitted or restricted by certain regulatory agencies in some countries, particularly the United States (Johnson *et al.*, 1980). Flesh of cultured salmonids can be pigmented by incorporating whole

crustacea or crustacean processing waste into their diets (Peterson *et al.*, 1966; Saito and Regier, 1971; Spinelli *et al.*, 1974). However, to achieve the required level of coloration, large amounts of these materials should be incorporated into the diets. This results in a moist diet which is nutritionally imbalanced, and feeding such diets to fish may induce mineral imbalances in them (Spinelli and Mahnken, 1978). Attempts to concentrate the crustacean-derived portion of the diet to reduce its bulk density results in the loss of significant amounts of carotenoids during drying (Spinelli *et al.*, 1974).

The economic importance of colored flesh in salmonids makes it imperative to find new sources of pigment suitable for incorporation into the feed of cultured salmonids (Torrissen *et al.*,1989). *Phaffia rhodozyma* is a Basidiomycetous yeast that is unusual in that it ferments carbohydrates and synthesizes the carotenoid, astaxanthin (Andrewes *et al.*, 1976). This yeast has been employed to impart red coloration to the flesh of salmonids, crustacea (Johnson *et al.*, 1977) and poultry (Johnson *et al.*, 1980). The production of *P. rhodozyma* on a large scale is therefore desirable, as it can provide natural astaxanthin and, as well, yeast can serve as a valuable protein source for the salmonid farming industry (Beck *et al.*, 1979). Also, the absorption and deposition of pigment in salmonid flesh seems to be more efficient with astaxanthin than with canthaxanthin (Torrissen *et al.*, 1986; Foss *et al.*, 1984; Tidemann *et al.*, 1984). Since *P. rhodozyma* was first identified, three basic issues have received attention in on-going research: the optimization of the astaxanthin yield; the search for more inexpensive growth media; and the extraction of the astaxanthin from the cells. Peat is one of the

most abundant and inexpensive resources of the world, and there is a growing interest in peat in Canada, where it is found throughout the wetlands. Hydrolysis of peat results in the extraction from the peat of sugars, which have been used as substrate for a variety of microorganisms and other fermentation processes (Martin, 1991; LeDuy, 1979, 1981a; Quierzy *et al.*, 1979; Boa and LeDuy, 1982; Martin and White, 1985, 1986) and the peat-bitumen fraction contains potential astaxanthin precursors such as carotenes. A novel, continuous thermomechanical method has been designed to fractionate peat, producing a hydrolysate rich in nutrients and growth stimulators (Overend and Chornet, 1987).

The objectives of the present study are:

1. To optimize the growth conditions of *P. rhodozyma* in peat hydrolysates prepared under different hydrolysis conditions.
2. To determine which of the various peat hydrolysates supports the best biomass production by *P. rhodozyma*.
3. To optimize the astaxanthin production by *P. rhodozyma* in the peat hydrolysate that supports the best growth.
4. To determine the nutritional requirements of *P. rhodozyma* in peat hydrolysate with the aim of increasing biomass concentration and astaxanthin content.
5. To study the growth kinetics of *P. rhodozyma* in peat hydrolysates.
6. To determine the proximate composition of the *P. rhodozyma* biomass produced in the peat hydrolysate.

CHAPTER 2

LITERATURE REVIEW

2.1 Carotenoids

Carotenoids are an important, chemically-related group of pigments that occur widely and abundantly in nature. They are primarily associated with cell membranes and organelles functioning in photosynthesis (accessory organs), photoprotection (free radical and singlet oxygen quenching), membrane stabilization, phototropism and phototaxis, vitamin A/retinoid metabolism, reproduction and photosynthetic electron transport (Gordon, 1972)

2.1.1 Distribution of carotenoids

Carotenoids are ubiquitous in nature. They are found in almost every plant and animal family, imparting the yellow, orange, and red colors to leaves, fruits, vegetables, flowers, dairy products, shrimp, lobster, and the plumage of exotic birds. It is estimated that 100 million tons of these pigments are produced annually in nature (Gordon, 1972). When carotenoids are complexed with proteins, blue and green colorations are achieved.

2.1.2 Chemical structure and classification of carotenoids

The structures of carotenoids are based on 5-carbon isoprene units ($CH_2=C(CH_3)CH=CH_2$). There are usually eight of such units which are linked so that the two methyl groups nearest the centre of the molecule are in positions 1:6 and all other lateral methyl groups are in positions 1:5. A series of conjugated C-C double bonds constitute the chromophoric system of carotenoids (Karrer and Jucker, 1950).

Classification of carotenoids can take several forms. Chemically, they may be divided into carotenes which are hydrocarbons, and xanthophylls (oxycarotenoids) which are oxygenated derivatives of carotenes. The oxygen contained in xanthophylls may occur as carboxyl, furanoxyl, hydroxyl, methoxyl, keto, or epoxy groups and esters. Carotenoids can also be subdivided under a different classification system into acyclic, monocyclic and bicyclic derivatives. They can further be divided according to their functions into provitamin A or vitamin A precursors, vitamin precursors which also perform a dual function as animal tissue pigmentors and compounds which do neither (Weedon, 1965; Zechmeister, 1962). As a classical example of the carotenoids, β-carotene is comprised of eight C_5-isoprene units to form a single C_{40} structure. C_{30} and C_{50} have also been elucidated. Those having less than 40 carbons are called apocarotenoids. An extensive literature exists on the chemistry, properties and the identification of carotenoids (Glover and Redfearn, 1954; Isler, 1971; Isler *et al*, 1965; Karrer and Jucker, 1950; Schwieter and Isler, 1967).

2.1.3 Commercial importance of carotenoids.

Carotenoids have a wide range of commercial applications. They are used as colorants for food and animal feeds. They impart distinctive orange-red coloration to the flesh of animals, thereby increasing their acceptance by the consumer (Torrissen *et al.*, 1989). Carotenoids have also been implicated in important metabolic functions in human beings and animals. They are said to play a role in enhancing the immune response, and in protection against such diseases as cancer by quenching oxygen radicals (Goodwin, 1986; Bendich and Olson, 1989). However, humans and animals are incapable of *de novo* synthesis of carotenoids, and therefore the pigments must be provided in the diet as a source of vitamin A.

2.1.4 Use of carotenoids for pigmentation of salmonid flesh

The flesh of anadromous salmonids (*Salmo* spp., *Oncorhynchus* spp.) is pink to red in color. This is a distinguishing feature that contributes to the elite image of these fishes. Because consumer acceptance of these fishes depend on this coloration, it is important that these animals, whether they originate in the wild or are farmed, are pigmented. These animals are incapable of synthesizing these pigments and therefore have to absorb them from their feed and deposit them in their flesh. Xanthophylls provide the necessary coloration.

Salmonid aquaculture is becoming increasingly important worldwide (Bjørndahl, 1990). Farm rearing of salmonids has grown dramatically, and in 1989 alone, over

200,000 tons of salmon were produced worldwide, with a large percentage coming from Norway (Bjørndahl, 1990). It is expected that farmed salmon will dominate the salmonid market by the year 2000, that the output of farmed salmon will by then exceed 460,000 tons yearly, and that, Norway, Canada, Chile and Japan emerge as the leading producers (Bjørndahl, 1990). In addition to this, several hundred thousand tons of trout are farmed worldwide each year (Rackham, 1988). These developments have resulted in a now dramatic increase in the amount of carotenoids used in the fish farming industry.

Over 6000 kg of carotenoids were used in the diets of farmed salmonids in 1986 alone, and every kg of synthesized carotenoid was worth over US $1000. This increased the cost of salmonid feed by approximately 10 to 15%. By 1990, it was expected that nearly 15,000 kg of carotenoids would be used in salmonid feeds to meet the predicted needs of the industry (Torrissen *et al.*, 1989).

2.2. Astaxanthin

Astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene, Fig. 2.1) is the principal carotenoid found in the flesh of salmon (Karrer and Jucker, 1950; Andrewes *et al.*, 1974; Kanemitsu and Aoe, 1958a, b; Simpson *et al.*, 1981). It is also the most costly component of salmonid feed (Torrissen *et al.*, 1989). The current market price for astaxanthin exceeds US $2000 per kg (marketed as "Carophyll Pink", Hoffmann-LaRoche, Inc., Basel, Switzerland) with a minimum astaxanthin content of 8% per kilogram (Johnson and An, 1991).

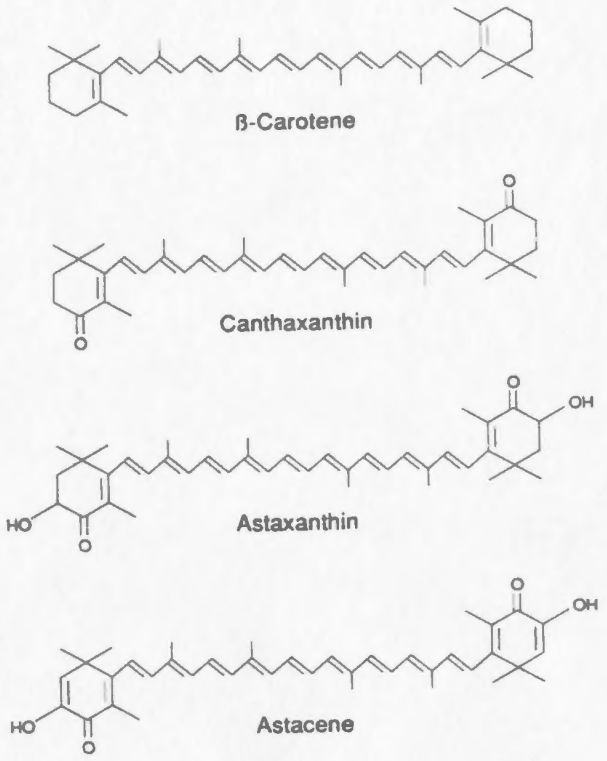

Fig. 2.1: Chemical structures of commercially important carotenoids (Taken from Johnson and An, 1991)

2.2.1 Chemical properties of astaxanthin

Astaxanthin is a xanthophyll (oxycarotenoid) with the molecular formula $C_{40}H_{52}O_4$ and a molecular weight of 596.86 (Foppen, 1971; Straub, 1976). Astaxanthin has two asymmetric carbon atoms at the 3 and 3′positions and can exist in four configurations including identical enantiomers (3R, 3′S, 3′R, and 3S, Fig. 2.2). Andrewes and Starr (1976) reported that astaxanthin isolated from the yeast *Phaffia rhodozyma* has the 3R,3′R -configuration; opposite to that of astaxanthin isolated from other sources previously investigated.

In *Haematococcus pluvialis* the 3S, 3′S configurational isomer is thought to be predominant (Andrewes *et al.*, 1974; Renstrom *et al.*, 1981). (3S, 3′S) Astaxanthin, (3, 3′R meso) and (3R, 3′R) astaxanthin have been isolated from wild salmon (Schiedt *et al.*, 1981; Matsuno *et al.*, 1984), lobster eggs (*Homarus gammarus*), shrimp (*Pandalus borealis*) and zooplankton (Renstrom *et al.*, 1980, 1981; Scheidt *et al.*, 1981; Foss *et al.*, 1987).

An extensive literature has been developed for the extraction, purification, identification and structural determination of carotenoids including astaxanthin (Isler, 1971; Davis, 1974; Davies, 1976; Liaaen-Jensen, 1978; Goodwin, 1984; Britton, 1985). Acetone is the most commonly-used solvent for the extraction of astaxanthin. Like any other carotenoid, astaxanthin is susceptible to light, acids, heat and oxygen. It is insoluble in aqueous solvents and in most polar organic solvents. However, it can readily dissolve in chloroform, dichloromethane, dimethylsulfoxide (DMSO), acetone and other non-polar

organic solvents at room temperature. It has a variable absorption spectrum, for example in chloroform it has a λ_{max} of 498 nm, and 480nm in acetone (Davies, 1976; Britton, 1967).

2.2.2 Analysis of astaxanthin

Generally, astaxanthin is extracted with water-miscible polar organic solvents. However, because of the possible presence of traces of HCl in chloroform/methanol, it is not recommended for this extraction (Johnson and An, 1991). For biological samples, mechanical disintegration is usually required to achieve quantitative extraction. Sedmak *et al.*, (1990) used hot DMSO to achieve quantitative extraction of astaxanthin from *P. rhodozyma* whereas Gentles and Haard (1989), Okagbue and Lewis (1985), and Johnson *et al.* (1980) have used enzymatic methods.

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) have been conveniently used for the separation of carotenoids after extraction (Britton, 1967). For quantitative analysis of astaxanthin, spectrophotometric methods have been used (An *et al.*, 1989; Johnson and Lewis, 1979). A wide range of extinction coefficients have been employed in quantifying astaxanthin in acetone:

$E_{1cm}^{1\%}$=1600 (Johnson *et al.*, 1977),

$E_{1cm}^{1\%}$ =2100 (Chen and Meyers, 1984),

$E_{1cm}^{1\%}$ =2200 (Saito, 1969).

Due to the lack of standards, these extinction coefficients are often calculated from other

more easily obtainable carotenoids (β-carotene) (Lamberstsen and Braekkan, 1971) and extrapolations are made to fit the solvents or expected ratios of stereoisomers (Torrissen *et al.*, 1989).

2.2.3 Pigmentation of salmonid flesh with astaxanthin

Salmonids are unable to oxygenate carotenoids, and instead deposit ingested carotenoids without modification (Steven, 1948; Hata and Hata, 1973). In the early 1900s, astacene, (3,3′-dihydroxy-2,3,2′,3′-tetradehydro-β,β-carotene-4,4′-dione) was thought to be the principal carotenoid in salmonids. However, this was later found to be a degradation product of naturally occurring astaxanthin. Pigmentation of farmed salmonids is influenced by several factors. These include nutrition, species of fish, sex, maturity, genetic variation and health of the fish (Torrissen *et al.*, 1989).

Environmental parameters can also affect pigmentation. For example, Vincent (1989), and No and Storebakken (1991) demonstrated that the total and specific carotenoids were affected by variations in water temperature. It has also been reported that the rate of carotenoid deposition in salmonids is affected by the growth rate and that there is a linear relationship between fish weight and flesh pigmentation (Spinelli and Mahnken, 1978; Torrissen, 1986).

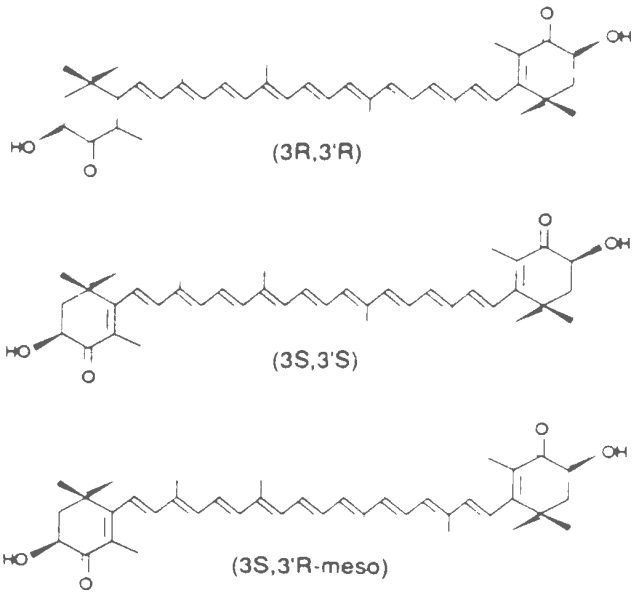

Fig. 2.2 Structures of configurational isomers of astaxanthin (Taken from Johnson and An, 1991)

2.2.4 Astaxanthin content of salmonid flesh

In adult Atlantic salmon (*Salmo salar*), the astaxanthin content is reported to be between 3 and 8 ppm, whilst the sockeye salmon may contain levels as high as 37 ppm. In the wild, the salmonids obtain astaxanthin and its esters from ingested zooplankton (Torrissen *et al.*, 1989). Ninety percent of the astaxanthin in salmonid flesh occurs in free (unesterified) form and esterified form in the skin and ovaries in the mature fish (Torrissen *et al.*, 1989).

Based on visual color impressions, astaxanthin levels of 4 mg per kg or above are reported to be the carotenoid concentration acceptable to the consumer. However, carotenoids may fade in salmonid flesh during handling and this must be compensated for by increasing levels a little above 4 mg per kg in the product to be marketed (Torrissen *et al.*, 1989).

2.2.5 Absorption of astaxanthin by salmon

Different types of animals seem to differ in their ability to absorb the various types of carotenoids. Fish and birds are known to preferentially absorb oxycarotenoids, whereas mammals absorb β-carotene better (Torrissen *et al.*, 1989). Astaxanthin and canthaxanthin are absorbed 10 to 20 times more efficiently by salmonids than are lutein and zeaxanthin, while chickens absorb zeaxanthin three times better than astaxanthin (Schiedt *et al.*, 1985). Goldfish and fancy red carp have also been reported to absorb carotenoids in a manner similar to that of chicken, astaxanthin being absorbed better than

either zeaxanthin or lutein (Hata and Hata, 1972, 1976).

2.2.6 Absorption of astaxanthin isomers

Since astaxanthin has two identical chiral centers, it exists in three configurational isomers in nature: (3S, 3′S), (3S, 3′R), and (3R, 3′R) (Fig. 2.2). All three of these optical isomers are found in marine fishes (Matsuno *et al.*, 1984) and all isomers are utilized to different extents by salmonids (Foss *et al.*, 1984). When fed a racemic mixture of astaxanthin, salmon and trout contained the same proportion of isomers as appeared in the feed (Torrissen *et al.*, 1989; Storebakken *et al.*, 1984; Foss *et al.*, 1984; Mori *et al.*, 1989). Experiments with rainbow trout and Atlantic salmon fed a mixture of all three optical isomers of astaxanthin dipalmitate have shown that the (3R,3′R) astaxanthin is deposited in the flesh to a greater extent than the (3S, 3′S) isomer. This is because the esterase responsible for the hydrolysis of astaxanthin dipalmitate has been reported to be more efficient in using the (3R, 3′R)-astaxanthin esters than the (3S, 3′S) esters as a substrate (Foss *et al.*, 1987). Similarly Katsuyama *et al.* (1987) reported that the (3R, 3′R) astaxanthin diester was deposited in the flesh of rainbow trout twice as efficiently as the (3R, 3′S) and four times more efficiently than the (3S, 3′S) diesters.

Free astaxanthin is also absorbed by salmonids to a greater extent than dietary astaxanthin esters (Torrissen *et al.*, 1989; Storebakken *et al.*, 1987). In a comparative study on rainbow trout, sea trout, and Atlantic salmon, it was reported that free astaxanthin was deposited to a much greater extent than astaxanthin palmitate (Foss *et al.*,

1987; Storebakken *et al.*, 1987). Furthermore, the flesh and plasma of salmonids have been found to contain only free astaxanthin. This is an indication that hydrolysis of esters of astaxanthin occur in the digestive tract, and that astaxanthin is absorbed in the free form (Steven, 1948; Khare *et al.*, 1973; Hata and Hata, 1975; Schiedt *et al.*, 1986)

2.3 Sources of astaxanthin for farmed salmon

Astaxanthin can be obtained from various sources among which are: chemical synthesis (which is also a source of canthaxanthin), crustacea and crustacean processing waste, plants and algae and such microorganisms as fungi and yeast.

2.3.1 Synthetic astaxanthin and canthaxanthin

In 1964, Hoffman-LaRoche (Basel, Switzerland) began the commercial production of synthetic canthaxanthin, marketed under the name "Roxanthin" or "Carophyll Red" for use as a pigmenter for foods and feeds (Isler, 1971).

Recently, free astaxanthin ("Carophyll Pink") has also been synthesized by Hoffman-LaRoche. This pigment is presently the principal source used in feeds, and it is apparently absorbed and deposited better than canthaxanthin by salmonids (Foss *et al.*, 1984, 1987; Storebakken *et al.*, 1987; Torrissen, 1986; Tidemann *et al.*, 1984). The level of astaxanthin in salmonids ranges from 3 to 37 mg per kg (Torrissen *et al.*, 1989). In some countries, synthetic astaxanthin and canthaxanthin are added to fish feed in levels ranging from 35 to 75 mg per kg dry feed (Torrissen *et al.*, 1989), but these compounds

are expensive and in the United States, they have not been approved by the Food and Drug Administration (FDA) for incorporation into salmonid feeds (Sinnot, 1988). Furthermore, chemical synthesis of astaxanthin is difficult owing to the four oxygen functions and the two chiral centres in the molecule (An *et al.*, 1991). Also, synthetic astaxanthin and canthaxanthin may contain unnatural configurational cis- and trans-isomers and carotenoid-like compounds (Storebakken *et al.*, 1984; Mayne and Parker, 1988). The cis-astaxanthin content should not exceed 2% (Johnson and An, 1991); however, Storebakken *et al.* (1984) reported about 15% cis-isomers in beadlets of synthetic astaxanthin. As well, Mayne and Parker (1988) reported an approximate all-trans to all-cis-canthaxanthin ratio of 3:1 in beadlets.

As a result of the increasing wariness by the farmer and consumer of the incorporation of synthetic chemicals in fish diets, there is a trend toward the use of natural sources of feed nutrients. All these factors have contributed to the search for natural sources of carotenoids.

2.3.2 Crustacean and crustacean processing wastes

The major carotenoid in many crustaceans is astaxanthin and several researchers have evaluated different crustacean products as pigment sources for farmed fish as reviewed by Torrissen *et al.* (1989). In Norway, shrimp (*Pandalus borealis*) wastes have been utilized as the traditional natural pigment source for farmed trout and salmon (Torrissen *et al.*, 1989). However, the level of carotenoid in most crustaceans is quite

low (Torrissen *et al.*, 1989; Lambersten and Braekkan, 1971). To achieve the desired level of coloration, about 10 to 25% by weight of the chitinous extract should be incorporated into the bulk diet. Crustacean wastes have low protein levels and high levels of ash, chitin, and moisture (Torrissen *et al.*, 1989). Therefore, incorporating large quantities of these materials into fish feeds would result in a moist diet that is nutritionally imbalanced, and may induce mineral imbalances in the fish (Spinelli and Mahnken, 1978). Attempts to concentrate the crustacean-derived portion of the diet to reduce its bulk density results in the loss of significant amounts of carotenoids during drying (Spenelli *et al.*, 1974). Torrissen *et al.* (1989) therefore concluded that crustacean waste has limited potential as an astaxanthin source for salmonids.

2.3.3 Plants and algae

The class Chlorophyceae includes some green algae that possess the ability to synthesize astaxanthin as their primary carotenoid (Nakayama, 1962; Wettern and Weber, 1979). *Chlamydomonas nivalis* has been named as the most likely and best-known astaxanthin-producing algae (Torrissen *et al.*, 1989). *Haematococcus pluvialis* is also known to produce high levels of astaxanthin depending on the culture conditions and method of cultivation (Droop, 1955; Goodwin and Jamikron, 1954). However, about 87% of its astaxanthin is esterified, which may affect its deposition and metabolism in some animals (Johnson and An, 1991). Kvalheim and Knutsen (1985) reported low deposition of astaxanthin in salmon fed an algae-based diet, giving the explanation that there was

low absorption of astaxanthin from the astaxanthin esters. Highly-pigmented algae occur in an encysted form surrounded by a thick cell wall, and this, according to Johnson and An (1991), could also impede the absorption of pigments. Chourbert (1979) reported no pigmentation in rainbow trout fed diets containing the filamentous blue-green algae, *Spirulina* spp. (division Cyanophyta), which is reported to contain high levels of carotenoids.

Several researchers have reported the use of various plants as possible pigment sources for fish and lobsters. For example, Isler (1971) and Peterson *et al.* (1966) reported the pigmentation of salmonid flesh with paprika which contains capsanthin as the major pigment (Torrissen *et al.*, 1989). D'Abrano *et al.* (1983) found paprika to be a possible source of pigment for lobster. Lee *et al.* (1978) observed increased amounts of canthaxanthin and lutein in the flesh of rainbow trout after they were fed diets containing extracts of marigold flowers (*Tagetes erecta*) and squash flowers (*Cucurbita maxima marcia*). Torrissen *et al.* (1989) concluded, however, that products from higher plants have little chance of being used as a source of pigment in practical diets.

2.3.4 The yeast *Phaffia rhodozyma*

The yeast, *Phaffia rhodozyma*, was isolated in the 1970s from exudates of deciduous trees in Japan, Alaska, and the former Soviet Union (Phaff *et al.*, 1972; Miller *et al.*, 1976). This yeast is strikingly different from other pigmented yeasts in that it produces the carotenoid astaxanthin (3′3′-dihydroxy-β,β-carotene-4,4′-dione) (Andrewes

et al., 1976a). Though astaxanthin is rarely found in fungi, it has occasionally been isolated from the Basidiomycetes *Peniophora* and *Perquercina* of the Aphyllophorales (Goodwin, 1972). *P. rhodozyma* produces the 3R-3R′ isomer of astaxanthin (Andrewes *et al*., 1976b) at a concentration of 1% or more of its dry weight (Haard, 1988; An *et al*., 1989).

When the mechanically-disrupted *P. rhodozyma* cells were added to the diets of pen-reared salmonids, astaxanthin was readily deposited in their flesh (Johnson *et al*., 1980). Currently, there is economic interest in *P. rhodozyma* as a biological source of astaxanthin because of its high astaxanthin content (approximately 800 - 900 µg per gram of yeast depending on the strain and culture conditions (Johnson and Lewis, 1979). When compared with *Haematococcus*, another possible candidate as a biological source of astaxanthin, *P. rhodozyma* has desirable qualities for use as an industrial pigment source including its heterotrophic metabolism, relatively rapid growth rate, ability to achieve high cell densities in industrial fermenters, nutritional quality and safety as food additive (An *et al*., 1991). Potential industrial use is, however, limited by the relatively low quantities of astaxanthin in certain strains of the yeast. The yeast also contains high levels of unsaturated fat, protein and vitamin that may contribute to the growth of the animal. These factors enhance the potential utility of the yeast as a source of astaxanthin in animal diets (Okagbue and Lewis, 1985).

2.3.4.1 Characteristics of *Phaffia rhodozyma*

P. rhodozyma was originally designated as *Rhodozyma montane* because all of the ten strains were isolated from broad-leaved trees in mountainous regions, either of Japan (nine strains) or of Alaska (one strain) (Phaff *et al.*, 1972). However, a Latin description was not given as required by the International Code of Botanical Nomenclature, hence the genus name was changed to *Phaffia* in honor of Hermann Jan Phaff for his contribution to yeast biology research (Miller *et al.*, 1976).

P. rhodozyma is unique among the pigmented yeasts because it synthesizes astaxanthin as its principal carotenoid (Andrewes *et al.*, 1976), and because, unlike other carotenoid producing yeasts which are strictly aerobic, all ten strains ferment glucose, maltose, sucrose and raffinose. Other sugars fermented by this yeast are D-L-lactate (latent), succinate, glycerol, α-methyl glucoside, D-mannitol (weak), 2-ketogluconate, ethanol (latent or negative), soluble starch (latent or negative), cellobiose and trehalose. It does not grow on lactose, galactose, glucosamine, D-ribose or D-arabinose and does not utilize nitrate, but does hydrolyse urea. It grows at temperatures from 0 to 27°C (Miller *et al.*, 1976).

2.3.4.2 Phylogeny of *Phaffia rhodozyma*

The genus *Phaffia* contains only one species (Miller *et al.*, 1976). The yeast is basidiomycetous in origin, but no sexual cycle has been determined. The most conclusive evidence of its basidiomycetous origin is its multi-layered cell wall, its enteroblastic

budding (Kreger-van Rij and Veenhuis, 1971), and the carbohydrate composition of its cell wall (Weijman *et al.*, 1988). The genus also produces cell surface-associated amyloid compounds, and a co-enzyme Q_{10} system.

Phaffia is phylogenetically related to other carotenoid-forming imperfect yeast including *Rhodotorula*, *Cryptococcus* and other hetero-basidiomycetous yeasts (Weijman *et al.*, 1988; Yamada and Kawasaki, 1989; Gueho *et al.*, 1989). The genus *Phaffia* is distinguished from the genus *Rhodotorula* by its ability to produce amyloid compounds and to ferment sugars, and from the genus *Cryptococcus* by its ability to ferment sugars. Recently, it has been found that both the genus *Phaffia* and the genus *Cryptococcus* contain xylose in their cells (Sugiyama *et al.*, 1985).

Recently, Weijman *et al.* (1988) studied the carbohydrate patterns of *Candida*, *Cryptococcus*, *Phaffia* and *Rhodotorula* species in detail. They distinguished *Phaffia* from *Cryptococcus* solely on the basis of the gaseous fermentation. It was observed (Van Dyken *et al.*, 1986) that non-fermentative species produce ethanol slowly with no visible gas production. In view of this, the genus *Phaffia* was regarded as a synonym of the genus *Cryptococcus* (Weijman *et al.*, 1988). However, Yamada and Kawasaki (1989) examined the partial sequence of the 18S rRNA of *Phaffia* and *Cryptococcus* with the aid of reverse transcriptase. By comparing the 18S rRNA sequences, they were able to clarify the phylogenetic relatedness of *Phaffia* and *Cryptococcus*. Having detected significant differences in the fingerprint region of the rRNA nucleotide sequences between *P. rhodozyma* and *C. laurentii* and *C. lusteolus*, Yamada and Kawasaki (1989)

concluded that the genus *Phaffia* is not closely related phylogenetically to the genus *Cryptococcus*. Hence the name *Phaffia* should be retained for the classification of these yeasts.

The composition of carotenoids is an important property that distinguishes *Phaffia* from other genera of related yeasts. Astaxanthin is the principal carotenoid in *P. rhodozyma* (Andrewes *et al.*, 1976a), and the predominant isomer has been determined to be 3R,3′R as opposed to 3S,3′S in lobster (Andrewes *et al.*, 1974). This makes the genus *Phaffia* unique. Also, a new type of carotenoid, 3-hydroxy-3,4′-didehydro-β-Ψ-caroten-4-one (HDCO), has been isolated from this yeast.

2.3.4.3 Culture conditions of *Phaffia rhodozyma.*

P. rhodozyma has been successfully cultivated in a wide range of carbohydrate sources (Miller *et al.*, 1976; Johnson and Lewis, 1979). Johnson and Lewis (1979) optimized such parameters as pH, aeration rate, nutrient requirements and the nature and concentration of the carbon source for biomass and astaxanthin production in this species. They reported that *P. rhodozyma* growth and pigment synthesis are both optimal at 20-22°C and pH 5.0, and with an oxygen concentration in excess of 50 mM per litre per hour. Astaxanthin concentration ranged from 379 $\mu g.g^{-1}$, when the yeast was grown in 1% L-arabinose, to 552 $\mu g.g^{-1}$ with D-cellobiose as the carbon source. Concentrations of glucose exceeding 1.5% decreased the astaxanthin content of the cells (Johnson and Lewis, 1979). A typical figure for biomass yield obtained with 6% glucose was 16 $g.L^{-1}$

(Johnson and Lewis, 1979). Haard (1988) studied the use of molasses as a carbon source for biomass and pigment production in *P. rhodozyma* and found that the replacement of glucose with 10% molasses resulted in a cell density of 14 $g.L^{-1}$, with a corresponding astaxanthin yield of over 1000 $\mu g.g^{-1}$ yeast. Johnson and Lewis (1979) observed that the addition of tomato waste, which supposedly supplies astaxanthin precursors, to the fermentation medium resulted in a substantial increase in pigment levels. Okagbue and Lewis (1984a) cultivated *P. rhodozyma* on alfalfa residual juice and reported that this medium effectively supported yeast growth but suppressed astaxanthin production. This inhibition effect was attributed to the presence of saponins in the juice (Okagbue and Lewis, 1984b).

Several factors hinder the commercialization of *P. rhodozyma*. Among these are the insufficient astaxanthin and biomass yields and the high cost of culture media (particularly yeast nitrogen base and sugars). Attempts to optimize the production of astaxanthin have so far proved unsatisfactory (An *et al.*, 1989) and attention is now focused on obtaining astaxanthin over-producing mutants (An *et al.*, 1989). Strains of yeast containing up to 2.5 mg of astaxanthin per gram of yeast were isolated when *P. rhodozyma* was grown in the presence of nitrosoguanidine and antimycin A, which are powerful inhibitors of the respiratory chain (An *et al.*, 1989). However, the mutants tended to grow more slowly and also to yield lower cell densities.

2.3.4.4 Pigment formation in *Phaffia rhodozyma*.

Unlike the yeast *Rhodotorula rubra*, in which carotenoid biosynthesis occurs mainly after growth has stopped (Goodwin, 1972; Goodwin, 1959), *P. rhodozyma* forms astaxanthin during its growth (Johnson and Lewis, 1979; An *et al.*, 1989) and continues to synthesize it after growth has stopped (Johnson and An, 1991). The availability of a carbon source during this non-growth phase is important for astaxanthin synthesis. Cells suspended in a medium or buffer without carbon do not increase in astaxanthin content, but do in media containing carbon or spent fermentation medium (Johnson and An, 1991). Light is important for the regulation of carotenoid biosynthesis in a wide variety of microorganisms. In *P. rhodozyma*, however, growth and pigment production was inhibited by high light intensities (Johnson and An, 1991).

2.3.4.5 Biosynthesis of astaxanthin in *Phaffia rhodozyma*.

Astaxanthin is produced from the mevalonate pathway (Fig. 2.3). Mevalonic acid (MVA) is the first important compound formed in this pathway from three molecules of acetyl-CoA. Two acetyl-CoA molecules condense to form acetoacetyl-CoA which in turn condenses with another molecule of acetyl-CoA to form β-hydroxy-β-methylglutayrl-CoA (HMG-CoA). HMG-CoA is then reduced by hydroxymethylglutaric acid (HMG)-CoA synthase and reductase (HMGS and HMGR) to form mevalonic acid (MVA). In the presence of adenosine triphosphate (ATP), MVA is converted to mevalonic pyrophosphate (MVAPP), which, also in the presence of ATP, is converted to a 5-carbon isoprene unit,

isopentyl pyrophosphate (IPP), through decarboxylation and dehydration reactions. The isopentyl pyrophosphate in turn undergoes a series of isomerisation and condensation reactions to give 5-carbon polymers. Products resulting from these reactions include polyrubbers (C_n), sterols (C_{30}), monoterpenes (C_{10}), sequiterpenes (C_{15}), gibberellins (C_{20}), and carotenoids (C_{40}). Compounds such as flavonoids, porphyrins, canabinoids, plastoquinones, ubiquinones and alkaloids are also produced from mixed biosynthesis (Goldstein and Brown, 1990).

Astaxanthin is derived from geranylgeranyl pyrophosphate, GGPP, (Fig. 2.3) which is in turn formed from the isomerisation of IPP to dimethylalyl pyrophosphate (DMP) that condenses with DMP to form geranyl pyrophosphate, a 10-carbon unit. By continued condensation with IPP, this 10-carbon unit yields farnesyl pyrophosphate and by further condensation forms geranylgeranyl pyrophosphate, a 20-carbon unit. By dimerisation, this 20-carbon unit forms phytoene, the basic 40-carbon acyclic carotenoid structure. The remainder of the pathway for astaxanthin production in *P. rhodozyma* has not been clearly elucidated. Chemical identification of various carotenoids in astaxanthin-producing microorganisms has been used to determine the biosynthetic pathway from phytoene to astaxanthin (Johnson and An, 1991). *P. rhodozyma* is said to synthesize astaxanthin from β-carotene using various intermediates (Andrewes *et al.*, 1976a). Recently, Johnson and An (1991) proposed two biosynthetic pathways for astaxanthin formation: one pathway, they claim, has a monocyclic precursor, and the other has a bicyclic precursor (Fig. 2.4). They reported that yeast cultures incubated with 2-methylimidazole (MI) or triethylamine

hydrochloride (TEA) accumulated the carotenoid 3-hydroxy-3,4′-didehydro-β-Ψ-caroten-4-one (HDCO). This, they concluded, indicates that astaxanthin may also be produced from HDCO (Fig. 2.4). Earlier, Andrewes *et al.* (1976a) had detected HDCO in *P. rhodozyma*, but could not explain its biosynthesis in their proposed pathway. Based on pigments isolated from *P. rhodozyma* they proposed a pathway with a bicyclic precursor. They postulated that neurosporene is converted to β-carotene, which in turn is converted directly to echinenone and this is then hydroxylated to 3-hydroxyechinenone (3-hydroxy-β,β-caroten-4-one). This then undergoes oxidation to form phoenicoxanthin (3-hydroxy-β,β-caroten-4,4′-dione). This compound is then converted to astaxanthin through hydroxylation at the C-3′. The presence of canthaxanthin and other possible intermediates such as 3,4′-dihydroxy-β,β-carotene-4-one in *P. rhodozyma* were not reported by these researchers. Johnson and Lewis (1979), on the other hand proposed a biosynthetic pathway for astaxanthin from neurosporene to β-carotene through γ-carotene.

Many environmental factors such as light, temperature, oxygen, carbon dioxide and nitrogen sources, and minerals have been reported to affect biosynthesis of carotenoids (Bramley, 1985). Furthermore, light has been reported to have a regulatory effect on carotenogenesis in fungi (Rau, 1983; Schrott, 1984). Other controlling factors that have been reported include regulation of oxidative metabolism, kinetic control of individual biosynthetic enzymes, compartmentation of enzymes and substrates, repression of regulatory genes by light and developmental processes (Tada, 1989; Tada *et al.*, 1990).

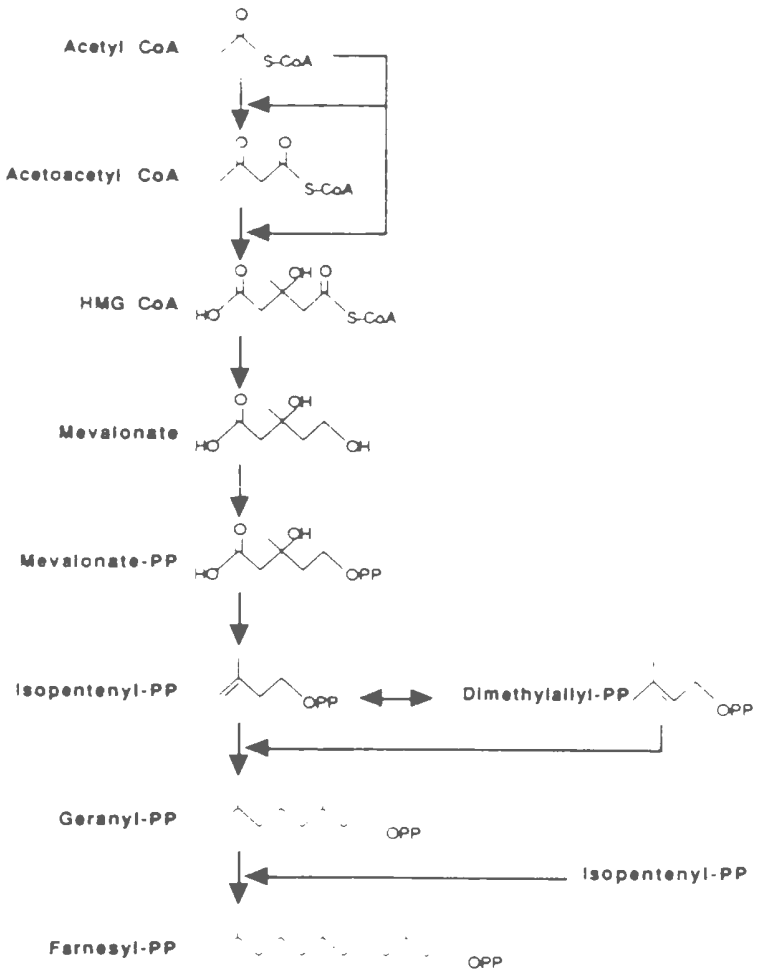

Fig. 2.3 The mevalonate pathway to farnesylpyrophosphate (Taken from Johnson and An, 1991)

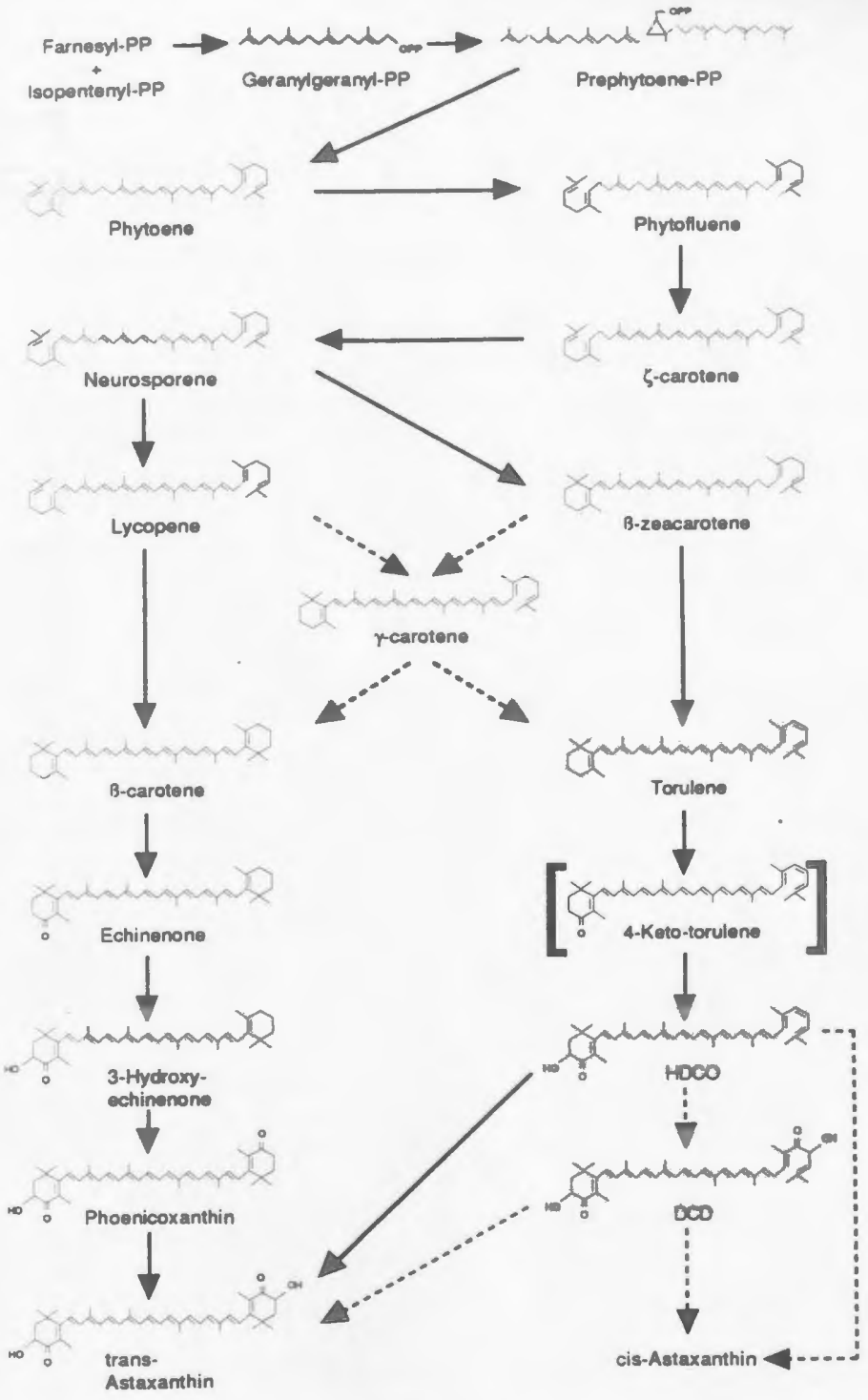

Fig. 2.4 The astaxanthin biosynthetic pathway proposed for *P. rhodozyma*. The left vertical pathway (phytoene → trans-astaxanthin) represents the pathway originally proposed by Andrewes *et al.* (1976). The right pathway (β-zeacarotene → torulene → HDCO → DCD → trans-astaxanthin) represents an alternative pathway based on the detection of the intermediate carotenoids (Taken from Johnson and An, 1991).

2.4 Peat

Peat is organic soil consisting of partially decomposed plant and vegetable material together with inorganic minerals. It accumulates as a result of partial decomposition of plant material by micro-organisms in water-logged environments where oxygen is limited or excluded (Chang, 1985). Peat contains a complex mixture of organic materials in which the more chemically stable residues of plant tissues predominate (Fuchsman, 1980). Peat can assume a wide range of colors. The highly decomposed peats are amorphous and black whilst the less decomposed are fibrous and brown. Peat has a high water-retention capacity, the most important feature of commercial *Sphagnum* peat moss ranging from 18 to 27 times the dry weight of the peat (Swinnerton, 1958).

2.4.1 Accumulation of peat

Climate has been cited as the most important single factor influencing the development of peats and peatlands. A positive water balance is required for the accumulation of peat. Plants growing under favorable conditions multiply rapidly and there is accumulation of large quantities of dead organic matter. This accumulated plant litter and the wetness limit air access to the underlying layers of decaying vegetation. Below the level of air penetration, only anaerobic decomposition occurs and the micro-organisms there depend on chemically stored oxygen in plant tissues. Peat develops as individual mires (also called bogs or swamps) in basins, hollows or valleys. In the absence of tree-cover and climatic inducement, peat may also form a thin blanket over

the land surface (Taylor and Smith, 1980).

It has been estimated that it takes 3000 to 4000 years to accumulate a meter of peat, and that peat continues to accumulate as long as bog plants continue to live and die at the surface of the deposit (Fuchsman, 1980)

2.4.2 Classification of peat

Peat can be classified according to its botanical, geological, and physiochemical characteristics. Botanical classification depends on the identification of plants that are predominant in the bog since they are most likely to be the same as the plants whose decomposed remnants make up the peat immediately underneath the surface (Fuchsman, 1980).

Geological classification is dependent on the relationship of the water in the peat deposit to the main groundwater system of the adjacent mineral soils, and can be divided into low-moor, transitional and high-moor peats. The bog is said to be low-moor or transitional if there is a continuation of the bog water system with the mineral and the groundwater system, with low-moor bogs being somewhat wetter and more frequently covered with water than the transitional-moors. In the case of high-moor bogs, their water system is significantly above the mineral soil groundwater system (Fuchsman, 1980). High-moor peat consists mainly of mosses since they are unable to thrive well in water that is poor in minerals. Low-moor peat consists mainly of woody plants that require nutrient-rich water.

Physiochemical classification is based on the degree of decomposition of the peat. Low-moor peat is about 25-45% decomposed and is usually more decomposed than high-moor peat, which has the structures of the original plant well preserved (Fuchsman, 1980). The degree to which the plants have undergone decomposition is expressed by the "humification" or H value. This value is expressed by a number on 1 to 10 scale. H-1 represents totally undecomposed plant material and H-10 represents completely decomposed peat (Tibetts, 1981).

2.4.3 Composition of peat

Chemically, peat is complex organic matter and its composition varies widely. It depends on the type of the constituent plants in the bog, and on the environmental conditions under which the peat was formed (LeDuy, 1979). Table 2.1 gives some of the chemical constituents of peat. The various types of compounds found in peat can be classified according to the ways they may be processed in a chemical plant (Fuchsman, 1980). "Bitumens" are substances that can be dissolved by suitable organic solvents; they include waxes and resinous materials. Those materials characterized by their solubility in aqueous alkaline media are named humic acids; their origin is not clear, but they have been reported to have a phenolic structure similar to lignin (Fuchsman, 1980). A third group includes carbohydrates related to cellulose and some protein-like substances; all of these can be dissolved in acid. Another group consists of the lignins; these serve as cement between cellulose fibres to give structural integrity to the leaves, stems and roots.

These substances are soluble in strong bases and are phenolic-like in their reactions. Other substances present are inorganic compounds (Table 2.2) and water. The inorganic fraction includes calcium, iron, magnesium, sulphur, as well as many micro-elements (Walsh and Barry, 1958).

Table 2.1: Chemical composition of high *Sphagnum* and low-moor sedge Peats (% dry weight of peat).

Components	*Sphagnum*	Sedge
Moisture	80.00 - 90.00	80.00 - 90.00
Bitumen	3.10 - 9.10	3.20 - 3.90
Hemicellulose	9.00 - 21.00	6.00 - 10.00
Cellulose	10.30 - 23.70	7.80 - 8.10
Lignin and humic substances	26.30 - 64.30	56.10 - 62.20
Protein (%N x 6.25)	5.60 - 6.90	10.00 - 13.80
Total reducing sugars	20.00 - 41.90	16.30 - 2.00
Total ash	1.50 - 3.00	7.70 - 14.50

From Fuchsman (1980).

Table 2.2: Elemental composition of peat (% dry organic material)

Organic Element	Slightly Decomposed *Sphagnum* peat	Highly Decomposed *Sphagnum* peat	Highly Decomposed Low-moor peat
Carbon	48.00 - 53.00	56.00 - 58.00	5.90 - 63.00
Hydrogen	5.00 - 6.10	5.50 - 6.10	5.10 - 6.10
Oxygen	40.00 - 46.60	34.00 - 39.00	31.00 - 34.00
Nitrogen	0.50 - 1.00	0.80 - 1.10	0.90 - 1.90
Sulfur	0.10 - 0.20	0.10 - 0.30	0.20 - 0.50

From: Fuchsman (1980).

2.4.4 World peat resources

It is estimated that peat covers about 1% to 2% of the earth's total surface (Tibbetts, 1981) or about 230 million hectares (LeDuy, 1979). About 95% of this occurs in Europe, Asia, and North America. The former Soviet Union has about 31% of the reserves whereas Canada has about 56% (Moore and Bellamy, 1974). Table 2.3 presents the overall picture of the peat resources of the world. The magnitude of the world's peat resources is known with very little accuracy since authors give different estimates (Fuchsman, 1980). Data published often describe either the reserves (exploitable quantity) or resources (total amount of peat present in an area of study). The methods used to classify organic soils as peat are also not well defined. Furthermore, many data on peat resources are published on the basis of undisclosed modes of computation (Fuchsman, 1980). For these and other reasons the estimates given in Table 2.3 may be misleading. For example, the Canadian figure is based on an assumption that peat is to be found throughout Canada's very extensive wetlands, but some studies indicate that Canadian peat reserves may be much less than had been previously suggested (Fuchsman, 1980). The figure for the former Soviet Union, however, is a relatively precise one based on a survey of exploitable deposits (Moore and Bellamy, 1974). The two figures are therefore not strictly comparable. However, many authors agree that the greatest areas of peatland in the world are found in Canada and the former Soviet Union, and these countries contain well over 80% of the world's peat resources (Moore and Bellamy, 1974; Fuchsman, 1980).

Table 2.3: World peat resources.

Country	Area Covered by Peat (Ha)
Canada	129 500 000
Former Soviet Union	71 500 000
Finland	10 000 000
U.S.A.	7 500 000
Norway	3 000 000
Germany	1 618 000
United Kingdom	1 582 000
Sweden	1 500 000
Poland	1 500 000
Iceland	1 000 000
Indonesia	700 000
Cuba	200 000
Japan	200 000
Ireland	172 000
Others	1 012 000

Modified from Moore and Bellamy (1974).

2.4.5 Uses of peat

Most of the peat produced in the world is used for fuel (LeDuy, 1979). The partially decomposed or humified type of peat is most suitable for this purpose because of its calorific value and its low ash content (LeDuy, 1979). In the former Soviet Union, about 70% of the peats harvested is used in the generation of electricity in 70 power plants, some of which have a capacity in excess of 300 megawatts. Other countries such as Ireland, Scotland, Finland and Germany also have generating stations that are powered by peat (LeDuy, 1979). The low ash content and sulfur levels, and the rapid burning characteristics of dehydrated peat, make it a potential substitute for oil in many utilities and industrial boilers (Rohrer, 1981). The slightly decomposed *Sphagnum* peat is generally used for horticultural purposes. It is used as soil conditioner in horticulture. The highly decomposed peat (sapric peat) is not suitable for use as fuel either due to its high ash content, but it has wide application in crop production (LeDuy, 1979). In Europe, some peatlands have been reclaimed for forestry, whereas others are used for cattle grazing and agricultural market gardening projects (Tibbetts, 1981). In parts of Canada, particularly Newfoundland, Quebec, Ontario and British Columbia, reclaimed peatlands are being used for the cultivation of blueberries and cranberries and for ornamental tree nurseries (Tibbetts, 1981).

Besides these uses, products of interest to the pharmaceutical and other biologically-oriented industries have been developed from peat. The principal medicinal product developed from peat is torfort, developed in the former Soviet Union. Torfort

is used for the treatment of ophthalmic diseases, myopia, myopic chorioretinis, opacification of the vitreous humor and early retinal degeneration. It has also been used for the treatment of anaemia, hepatitis, and various skin, gynaecological and neurological diseases (Fuchsman, 1980). Other products obtained are peat tars that have strong antiseptic and antibactericidal, germicidal and overall biocidal properties, and are used in wood preservation and agriculture (Fuchsman, 1980). Organic materials such as phenols, nitrogen-based and aromatic compounds useful for the production of plastics, and pharmaceutical products have also been obtained from peat. The liquid extract from peat, termed peat extract or peat hydrolysate, has been used for the submerged production of microbial cell biomass, also called single cell protein (SCP) (LeDuy, 1979).

2.4.6 Utilization of peat as a substrate for the production of single cell proteins

Poorly decomposed *Sphagnum* peat is rich in carbohydrates that are easily hydrolysed to yield monosaccharides accounting for 45 - 55% of the dry weight of the peats (Moore and Bellamy, 1974). The low molecular weight sugars produced provide a useful growth medium for various fermentation processes (LeDuy, 1979). Several workers have studied the utilization of the soluble and extractable components of peat as the main source of substrate for microbial biomass production (LeDuy, 1979; Martin, 1983; Martin and Bailey, 1983; Martin and White, 1985, 1986; Fuchsman, 1980; Quierzy *et al.*, 1979; Boa and LeDuy, 1983; Forsberg *et al.*, 1986). Table 2.4 gives a summary of the various fermentation processes that have utilized peat hydrolysate as a substrate.

The yeast, *Candida utilis*, is a well-known, high protein yeast that has received a great deal of attention. It is used for food and feed purposes and gives a high yield of biomass in fermentation processes (Quierzy *et al.*, 1979). This high yield is due to its ability to utilize both hexoses and pentoses, mostly glucose, xylose, arabinose and galactose, and various carbonaceous compounds that occur in peat hydrolysates (LeDuy, 1979). The yeasts *Sporobolomyces pararoseous T* and *Rhodotorula glutinis T-2* have been reported to produce high levels of carotenes when grown on peat hydrolysates (Raitsina and Evdokimova, 1977). *Candida humicola, Candida tropicalis* and *Lipomyces lipoferus* have also received attention as lipid yeasts for SCP because of their high concentrations of lipids, most of which are made up of unsaturated fatty acids (Raitsina and Evdokimova, 1977).

Martin (1986) reviewed the submerged production of mushroom mycelium in peat hydrolysate. A comprehensive review article on the utilization of peat hydrolysate for the production of single cell protein has been published by LeDuy (1979).

Table 2.4: Production of single-cell protein (SCP) from peat hydrolysates.

Microorganism	Production Scale	Reference
Bacillus polymyxa	5 L batch fermenter	Griffin and Forgarty, 1974.
Clostridium acetobutylicum	Shaker flask	Forgarty and Ward, 1970
Scytalidium acidophilum	Shaker flask	Martin *et al.*, 1980
Agaricus campestris	Shaker flask	Martin and Bailey, 1985
Pleurotus ostreatus	Shaker flask	Manu-Tawiah and Martin, 1988
Candida utilis	7 L batch fermenter	McLoughlin and Küster, 1972b
Candida tropicalis	Shaker flask	Zalashko *et al.*,1977

2.4.7 Methods for preparing peat hydrolysate.

Peat hydrolysates are produced by various means including soxhlet extraction with organic solvents, cold and hot water extraction and dilute acid and dilute alkaline hydrolysis (McLoughlin and Küster, 1972a; Fuchsman, 1980). Acid hydrolysis, which results in the production of simple carbohydrates, especially hexoses and pentoses from cellulose, is the most commonly-used method for the production of peat hydrolysates (Fuchsman, 1980). In addition, the hydrolysis technique results in the extraction of several organic substances some of which may be utilised as nutrients by microorganisms (Fuchsman, 1980). The rate of hydrolysis increases with temperature. There is thus an optimal reaction time for a given set of cellulose hydrolysis conditions. There is a decrease in yield due to excessive degradation of the glucose liberated by cellulose hydrolysis if the optimal reaction time is exceeded (Fuchsman, 1980). The amount of utilizable organic fraction obtained is also dependent on the hydrolysis process conditions which include temperature of hydrolysis, residence time, concentration and type of acid, peat:water ratio (LeDuy, 1981a, Quierzy *et al.*, 1979; Boa and LeDuy, 1982) and peat molecule size (Morita and Levesque, 1980).

Recently, steam explosion has been used to extract sugars from peat (Forsberg *et al.*, 1986). Cellulose can also be hydrolysed enzymatically using the cellulase produced by the fungus *Trichoderma viride*. However, there is no documentation of the application of the cellulase system to peat (Fuchsman, 1980). Quierzy *et al.* (1979) reported that water extracted during the drying of fuel-grade peat resembles peat hydrolysate and can

be used for culturing microorganisms.

2.4.8 Composition of peat hydrolysates.

Peat hydrolysates contain reducing substances, mostly glucose, up to 7.5% on a weight to volume basis (LeDuy, 1979). The carbohydrate portion of peat hydrolysate consists mainly of hydrolysed hemicelluloses, particularly glucose, xylose, galactose and arabinose (Küster *et al.*, 1968), as well as non-volatile organic acids (LeDuy, 1981a). The latter consists of uronic acids (glucuronic and galacturonic), hydrocarboxylic acids (lactic, malic, tartaric, citric, and glycolic) and C_2-C_5 dicarboxylic acids (LeDuy, 1981a).

The levels of nitrogen and phosphorus in peat hydrolysate are very low (Boa and LeDuy, 1982; LeDuy, 1979; Mulligan and Cooper, 1985). Nitrogen constitutes 1-3% of the dry weight of the peat but only a part of it, which is in the form of amino acids appears in the peat hydrolysate. Much of the nitrogen in peat is present in the form of polypeptides which are loosely bound to humic acids. The polypeptides, under appropriate hydrolysis conditions, yield amino acids. Other nitrogen-containing compounds, such as glucosamine, are also found in peat hydrolysate (Fuchsman, 1980). Peats are, however, rich in inorganic substances such as calcium, iron, magnesium and sodium which are present in higher quantities than cobalt, copper, potassium, manganese, nickel and zinc. Peat hydrolysates therefore, constitute a good fermentation medium in terms of oligo-elements (LeDuy, 1981a). They also contain other inorganic substances such as humic acids and bitumen (Chang, 1985; Fuchsman, 1983). Vitamin B_1 and B_2

are also present in small quantities in peat, but they are destroyed during hydrolysis (Fuchsman, 1980)

The concentrations of the various components of peat hydrolysates depend on the extraction procedure. Fuchsman (1980) stated that water and alcohol extracts of peat have low compositions of monosaccharide. The acid-catalyzed peat hydrolysates have higher total carbohydrate content (TCH) and concentrations of other organic nutrients than those obtained by other extraction methods (McLoughlin and Küster, 1972a; Quierzy *et al.*, 1979). Furthermore, it has been reported that acid hydrolysates produce more microbial biomass than non-acid hydrolysates (LeDuy, 1981a; McLoughlin and Kuster, 1972a). LeDuy (1981a) reported that hexose represents more than 50% of the total sugars that occur in peat hydrolysates. In sulfuric acid extracts, 43 $g.L^{-1}$ monosaccharides could be be obtained with glucose as the major sugar (about 33.50 - 39.80% of total monosaccharides) while in hydrochloric acid extracts, 13.20% of organic matter could be obtained as monosaccharides with xylose as the major sugar (LeDuy, 1981).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

The materials used in this work include peat hydrolysate prepared from peat moss, *Phaffia rhodozyma* culture, and different chemicals obtained from various sources. These materials and their sources have been itemized below.

3.1.2 Peat moss

The peat moss used in the preparation of the peat hydrolysate was obtained from Riviere-du-Loup, Quebec, and it was graded H_4 on the von Post scale (Tibbetts, 1981).

3.1.3 Organism used and its maintenance

Phaffia rhodozyma, American Type Culture Collection # 24202, was used for the fermentation studies. It was maintained on YM (Difco) agar plates at 4°C and transferred every month.

3.1.4 Chemicals

The chemicals used in this work and their sources were as follows:

Calf thymus DNA, RNA, sodium chloride, sodium hydroxide, hydroquinone, orcinol reagent, boric acid, and anthrone reagent were purchased from Sigma Chemical Company, St. Louis, MO. U.S.A.

Sulfuric acid, hydrochloric acid, ammonium sulfate, potassium nitrate, potassium hydroxide, petroleum ether, chloroform, ethanol, methanol, ammonium phosphate dibasic, potassium phosphate monobasic, urea, ammonium nitrate, acetone, hexane, manganese sulfate monohydrate, magnesium sulfate heptahydrate, acetic acid (glacial), D-glucose anhydrous, magnesium chloride, acetaldehyde, n-butanol, diphenylamine, potassium phosphate buffer, and hyperchloric acid were purchased from Fisher Scientific Company Ltd., Fairlawn, N. J. U.S.A.

The enzyme "Funcelase" was purchased from Yakult Honsha Company Ltd., Tokyo, Japan.

YM agar, YM broth, yeast extract, bactopeptone and yeast nitrogen base. were purchased from Difco Laboratories Ltd., Detroit, Michigan, U.S.A.

3.2 Methods

3.2.1 Preparation of peat hydrolysates

The peat hydrolysates used in this work were prepared at the University of Sherbrooke, Quebec, by the following procedure. About 6 kg of low-humified peat from Riviere-du-Loup, Quebec, graded H_4 on the von Post Scale, was mixed with 40 kg of water and macerated with a blade mixer. For producing acid hydrolysates, concentrated sulfuric acid was added until the mixture reached pH 2. The mixture was further macerated for 15 minutes to obtain a peat slurry that consisted of 7 to 8% solids. The slurry was then subjected to a pressure of 3000 psi and a temperature of either 185, 205, or 225°C for 2 minutes. The resulting solutions were centrifuged to remove suspended solids and the hydrolysate filtered through Whatman # 1 filter paper. The filtrates were then frozen and shipped to the Memorial University of Newfoundland for the fermentation studies.

Before being used in preparing the fermentation media, the peat hydrolysates were diluted with equal volumes of water, centrifuged at 10,000 × g for 30 minutes, and then filtered through a cellulose nitrate filter paper with a pore size of 0.45 μm (Sartorius GmBH, Germany) in a Megaflow Membrane Filtration Apparatus (Model TM-100, New Brunswick Scientific Co. Inc., Edison, New Jersey). The filtration apparatus was operated with a Watson-Marlow Peristaltic Pump (Model 502S, Watson-Marlow Ltd., England).

3.2.2 Inoculum preparation

A loopful of yeast was aseptically inoculated into 20 mL sterilized YM (Difco) broth and incubated in a Gyrotory water bath shaker (Model G76D, New Brunswick Scientific Co. Inc., Edison, New Jersey) at 22°C for 24 hours. About 1 mL of this culture was used to inoculate the experimental fermentation media.

3.2.3 Fermentation media preparation

Six different peat hydrolysates hydrolysed with or without added acid, and at various temperatures, were used. These hydrolysates are identified in this work as PH4-01-185, PH4-O1-205, PH4-01-225, PH4-02-185, PH4-02-205, and PH4-02-225. In this system of identification, 01 designates hydrolysates made without added acid (auto-hydrolysates) and 02 designates hydrolysates made with added acid. The last three digits in the codes, 185, 205, and 225, are the temperatures (°C) at which the hydrolysates were prepared.

For each experimental fermentation, 50 mL of peat hydrolysate, diluted to the required concentration and adjusted to pH 5.00 ± 0.10 with 10 M sodium hydroxide in a 125 mL Erlenmeyer flask, was supplemented with 0.68% ($w \cdot v^{-1}$) yeast nitrogen base (Difco) and 0.60% ($w \cdot v^{-1}$) bactopeptone (Difco). The medium was then sterilized at 121°C for 20 minutes and cooled. All incubations were done in a Gyrotory water bath shaker.

3.2.4 Optimization of growth conditions

Various growth conditions (substrate concentration, initial pH of growth medium, initial incubation temperature, fermentation time, inoculum ratio and agitation speed) were tested to determine the optimum parameters for the growth of the yeast.

3.2.4.1. Determination of the best substrate concentration for the growth of *Phaffia rhodozyma*

Preliminary studies were conducted to determine the best substrate concentration for the growth of the yeast. Two of the peat hydrolysates, PH4-01-185 and PH4-02-185 (non-acid and acid hydrolysates) were used for this purpose. In this work, the term "substrate concentration" refers to the total carbohydrate concentration (TCH) as determined by the Dreywood's Anthrone Method (Morris, 1948). The growth conditions employed were a pH of 5, a temperature of 22°C, an agitation speed of 300 r.p.m. and a fermentation time of 5 days (Johnson and Lewis, 1979). The initial substrate concentrations used were 15, 20, 25, and 30 $g \cdot L^{-1}$ for PH4-01-185 and 15, 30, and 45 $g \cdot L^{-1}$ for PH4-02-185.

3.2.4.2 Studies on growth conditions

Using peat hydrolysates PH4-01-185 and PH4-02-185 at substrate concentrations of 30 and 15 $g \cdot L^{-1}$, respectively (as previously optimized), the effects of various pH levels (4, 5, 6, 7 and 8), temperatures (16, 18, 20, 22, and 24°C), fermentation times (2,

3, 4, 5, 6 and 7 days), and agitation speeds (150, 200, 250, 300, and 350 r.p.m.) on the growth of *P. rhodozyma* were studied in shaker flask experiments.

3.2.4.3 Determination of the best peat hydrolysate for the growth of *Phaffia rhodozyma*

Having optimized the growth conditions for this yeast, it was grown separately in the six peat hydrolysates to determine which would support the growth best. An initial substrate concentration of 30 $g \cdot L^{-1}$, an initial pH of 7, an incubation temperature of 18°C, a fermentation time of 5 days and an agitation speed of 200 r.p.m. were the growth conditions employed.

3.2.5 Nutrient supplementation of peat hydrolysate

Peat hydrolysate PH4-02-185 obtained as supporting the best yeast growth was tested without addition of nutrients (non-supplemented), and with the addition of various sources of nitrogen, phosphorus, potassium, magnesium, and manganese alone and in combination. The growth conditions employed were a substrate concentration of 15 $g \cdot L^{-1}$, an initial pH of 7, an incubation temperature of 18°C, a fermentation time of 5 days, an inoculum ratio of 5% and an agitation speed of 200 r.p.m.

3.2.5.1 Supplementation of peat hydrolysate with various sources of nitrogen

A study was conducted to determine the effects of supplementing the peat hydrolysate with sources of nitrogen other than yeast nitrogen base and bactopeptone on the growth of and astaxanthin production by *P. rhodozyma*. The nitrogen sources tested were ammonium sulfate, ammonium nitrate, potassium nitrate and urea. The concentrations of these nitrogen sources were calculated so that they provided approximately 0.5 $g \cdot L^{-1}$ nitrogen to the peat hydrolysate.

In another study, the peat hydrolysate was supplemented with yeast extract to determine its effects on the growth of and pigment production by the yeast.

3.2.5.2 Supplementation of peat hydrolysate with potassium phosphate

Phosphorus and potassium have been reported to enhance yeast growth (Rose, 1987). Phosphorus and potassium were added in the form of potassium phosphate to study their effects on the growth of and pigment production by *P. rhodozyma*. Four concentrations (0.5, 1.0, 1.5, and 2.5 $g \cdot L^{-1}$) were tested.

3.2.5.3 Supplementation of peat with combinations of yeast extract and potassium phosphate

Yeast extract at various concentrations (0, 1.0, 2.0, and 3.0 g·L-1) was combined with potassium phosphate also at various concentrations (0, 0.5, 1.0, 1.5, 2.0 $g \cdot L^{-1}$) to supplement the peat hydrolysate. This was done to study the effects of these compounds in combination on the growth of and pigment production by the yeast (both of which separately enhanced the growth of the yeast).

3.2.5.4 Supplementation of the peat hydrolysate with magnesium and manganese

Magnesium sulfate and manganese sulfate were used to supplement the peat hydrolysate. The concentrations used for both compounds were 0, 1.0, 1.5, and 2.0 $g \cdot L^{-1}$.

3.2.6 Batch fermentation

Experiments were carried out in a 2 L fermenter to optimize agitation speed, aeration rate, and also to study the growth kinetics and astaxanthin production by *P. rhodozyma* in peat hydrolysate. Batch cultures were grown in a 2 L BioFlo fermenter (Model C30, New Brunswick Scientific Co., Edison, New Jersey) using 1 L and 1.5 L working volumes for the study of growth kinetics and astaxanthin production, respectively. Aeration rate, pH and agitation speeds were controlled. Agitation was provided by a turbine-type impeller (with four blades for mixing and mass transfer), and air was delivered to the culture by an external air source through a sterilized air filter and

a hollow agitator shaft. The pH was controlled by an automatic pH controller (Model pH-40, New Brunswick Scientific Co., Edison, New Jersey) fitted with a pump module (New Brunswick Scientific Co., Edison, New Jersey), which adjusted the pH by adding 10 M sodium hydroxide or 5 M sulfuric acid solution.

The growth conditions employed under sterile conditions were an initial pH of 7, an incubation temperature of 18°C, a fermentation time of five days and an inoculum ratio of 5% (unless otherwise stated).

3.2.6.1 Inoculum preparation

The inocula for the batch culture consisted of 24-hour culture grown in 250 mL Erlenmeyer flasks with 50 mL and 75 mL of growth medium for 1 and 1.5 L working volumes respectively. The growth medium consisted of peat hydrolysate with a TCH of 15 $g \cdot L^{-1}$, yeast extract (2 $g \cdot L^{-1}$), and potassium phosphate (1.5 $g \cdot L^{-1}$). The growth conditions were an initial pH of 7, an incubation temperature of 18°C, a fermentation time of 1 day and an agitation speed of 200 r.p.m. The culture flasks containing the microorganisms were incubated in a Gyratory water bath shaker.

3.2.6.2 Growth media

For the batch fermentations, the growth media consisted of peat hydrolysate with a TCH of 15 $g \cdot L^{-1}$, yeast extract (2 $g \cdot L^{-1}$), and potassium phosphate (1.5 $g \cdot L^{-1}$). This was the combination of nutrients that provided a balance between yeast dry biomass and

pigment production in the shaker flask experiments. The various components of the media were sterilized separately at 121 ± 1°C and 15 psi for 20 minutes and aseptically combined when needed.

3.2.6.3 Optimization of agitation speed and aeration rate

Three levels of agitation speed (150, 200, and 250 r.p.m.) and five levels of aeration rates (0, 0.5, 1.0, 2.0, and 3.0 vvm, *i.e.* volume of air per volume of medium per minute) were tested to determine their effects on the growth of and pigment production by *P. rhodozyma* in a fermenter. All the experiments were carried out separately and under the same growth conditions: an initial pH of 7, an incubation temperature of 18°C, a fermentation time of 5 days and an inoculum ratio of 5% ($v \cdot v^{-1}$).

3.2.6.4 Determination of growth kinetic parameters

The kinetics of growth, pigment production and TCH utilization by *P. rhodozyma* in peat hydrolysate were studied in 2 L fermenters with a 1.5 L working volume for 200 hours of fermentation. Dry biomass concentration, TCH concentration, and astaxanthin content were determined every 10 hours for each fermentation.

3.2.7 Analytical methods

Various analyses were conducted to determine the dry biomass concentration, the TCH of both the yeast cells and the peat hydrolysates, and the moisture contents of yeast and the peat, as well as the astaxanthin content, total lipid and fatty acid contents, nucleic acid content and amino acid profile of the yeast. Detailed descriptions of each analytical method are given below.

3.2.7.1 Determination of dry cell biomass

The biomass was harvested by centrifugation at 10,000 x g for 30 minutes, after which the cells were washed twice with a deionized water / 0.5 M sodium chloride solution and centrifuged again. The harvested cells were dried in pre-dried and weighed aluminum pans in an oven at 100°C for 18 hours. The dry biomass concentration, yield coefficient and efficiency were calculated as follows:

$$Dry\ Biomass\ Conc. = X - X_0$$

$$Yield\ Coefficient\ (\%) = 100\left(\frac{X-X_0}{S_0-S}\right)$$

$$Efficiency\ (\%) = 100\left(\frac{X-X_0}{S_0}\right)$$

where: X is final yeast biomass ($g \cdot L^{-1}$)

X_0 is initial dry biomass of inoculum ($g \cdot L^{-1}$)

S is final substrate concentration

S_0 is initial substrate concentration

3.2.7.2 Determination of the total carbohydrate concentration (TCH)

The total carbohydrate concentration of the peat hydrolysates and the yeast cell biomass was determined by two different methods as described below.

3.2.7.2.1 Determination of TCH of peat hydrolysate

The TCH of the growth medium before and after fermentation was determined by the Dreywood Anthrone Method (Morris, 1948). This method involved dissolving 2 g anthrone in 1 L 95% sulfuric acid. The reagent was stored at 4°C in a dark bottle. Five mL of the sample solution to be determined (diluted to appropriate range ca. 20-200 $mg \cdot L^{-1}$ carbohydrates) was measured into test tubes and 8 mL of the anthrone reagent was added with mixing. The absorbance at 540 nm was measured in a Novaspec II Spectrophotometer (LKB Biochrom Ltd., England) against a blank containing only water and the reagent. A series of glucose standards was used as the basis of comparison for determining the TCH. The results were expressed as equivalent glucose concentrations.

3.2.7.2.2 Determination of TCH of yeast cells

The TCH of the dry yeast cells was determined the method of Herbert *et al.* (1971). About 200 mg of anthrone was added to 5 mL absolute ethanol in a 100 mL volumetric flask and 75% sulfuric acid was added to give 100 mL of solution. About 0.5 - 1.0 mg quantities of dried yeast cells were mixed with 2.0 mL water in a thin-walled boiling tube. The tube was then cooled in a rack standing in a pan of ice-water in which the anthrone reagent was also cooled. After cooling, the tube was left standing in the ice-water and 5.0 mL of the anthrone reagent was added to the tube while the tube was swirled. The tube was then allowed to stand in the ice-water until its contents cooled to 0°C. The tube was then transferred to a vigorously boiling water-bath. After 10 minutes, the tube was returned to the ice-water and allowed to cool. The absorbance at 625 nm was measured in a spectrophotometer against a reagent blank and glucose standards.

3.2.7.3 Analysis of moisture content

The A.O.A.C. method (Anon. 1980) was used to determine the moisture content of the fresh yeast cells. For this, about 1 g of the sample was dried to a constant weight at 100°C under vacuum and the moisture content determined by weight difference.

3.2.7.4 Analysis of astaxanthin content

Freeze-dried cells were used for astaxanthin extraction, instead of oven-dried cells. A modified method of Gentles and Haard (1989) was used. It involved suspension of 300 mg of dried cells in 50 mL of water that had been previously adjusted to pH 5 with acetic acid and to which 3 µg of the enzyme "Funcelase" (Yakult Honsha Co., Ltd., 1-1-19 Higashi-Shinbashi, Minato-ku, Tokyo 105, Japan) had been added. The mixture was stirred at room temperature for 4 hours and then left sitting overnight. The digested cells were centrifuged at 10,000 × g for 30 minutes, and the resulting pelleted cells were washed with deionized water and then extracted twice with 50 mL quantities of acetone. The pooled acetone extracts, about 100 mL, were placed in a separatory funnel and 50 mL of petroleum ether added. The petroleum ether phase, containing the astaxanthin, was collected and saved. The acetone phase was further extracted with petroleum ether until colorless, and the collected ether phase was filtered through glass wool packed in a pasteur pipette to remove any suspended cell particles, and dried in sodium sulfate. The astaxanthin content in the petroleum ether was estimated by measuring the absorbance of samples at 474 nm in a Novaspec II Spectrophotometer. The total carotenoid content was calculated using the 1% extinction coefficient of 2100 and the formula developed by An *et al.* (1989). The formula is given as:

$$Total\ carotenoid\ cont. = \frac{V \times A_{474} \times 100}{21 \times W}$$

where: V is the volume (mL) of petroleum ether collected

A_{474} is the absorbance at 474 nm

21 is the 1% extinction coefficient

W is the dry weight of yeast used in grams.

3.2.7.5 Analysis of total nitrogen and crude protein contents of the yeast cells

The micro-Kjeldahl method (Anon., 1980) was used to determine the total nitrogen and the crude protein contents of the dried yeast cells. About 100-300 mg of sample was weighed on nitrogen-free paper. Each sample was transferred to a digestion tube and 2 Kjeltabs M pellets (mercuric oxide type) and 20 mL concentrated sulfuric acid were added to each tube. The mixture was then digested in a Büchi 426 Digestion Unit (Büchi Laboratoriums - Technik AG, Flawil, Switzerland) for a total period of 60 minutes. The digested solution was then allowed to cool and was then inserted in a Büchi 315 Distillation Unit (Büchi Laboratoriums - Technik AG, Flawil, Switzerland). Deionized water (100 mL) and 25% sodium hydroxide (150 mL) were then added to the digested solution and the mixture was subsequently distilled into 50 mL 4% boric acid until about 150 mL condensate was collected. The condensate was titrated with 0.1 M sulfuric acid using methyl red as an indicator, and the % N and crude protein content calculated as follows :

$$\% N = \frac{(V_1 - V_2) \times M \times 14.0067 \times 100}{W}$$

where: V_1 is the volume of titrant for the sample

water-soluble material from the lipid extract was completed by mixing the interface three times with 2 mL of a mixture of chloroform-methanol - 0.58% sodium chloride in water (3 : 48 : 47 by volume). A small amount of methanol was added to disperse the remaining rinse fluid into the lower phase. Chloroform-methanol (2 : 1 by volume) was added to the extract until the total volume was 30 mL and then it was allowed to separate in a separatory funnel. The chloroform layer was removed into a graduated cylinder and 10 mL aliquots of this were pipetted into pre-dried and weighed aluminum pans. These were evaporated to dryness in a vacuum at 50°C and the pans weighed to determine total lipids.

3.2.7.7 Analysis of fatty acids

The total lipids were extracted and purified by the modified method of Stewart (1975) as previously described. The extracts were stored at -60°C in chloroform to which 0.5% hydroquinone had been added. Aliquots of about 1 mL of the lipid extract were placed in 6 mL conical teflon-lined, screw-capped vials and evaporated to dryness under a stream of nitrogen. The residue was transmethylated by the addition of 2 mL of 6% sulfuric acid in 99.9 mol. % methanol to which 15 mg hydroquinone had been added as antioxidant. The mixture was incubated at 60°C overnight. Deionized water (1 mL) was then added to the mixture in each vial, thoroughly mixed, and then extracted three times with 1.5 mL of pesticide-grade hexane. A few more crystals of hydroquinone were added to each mixture at the first extraction with hexane. The hexane layers were removed,

combined in a clean tube and washed twice with 1.5 mL deionized water by vortexing. The hexane extracts were evaporated to dryness under nitrogen. The dried sample was dissolved in about 10-20 µL of ACS grade carbon disulfide and about 4 µL of the resulting solution was injected into a gas chromatography apparatus for analysis.

3.2.7.8 Analysis of nucleic acids

The nucleic acid content of the freeze-dried yeast cells was determined by the method of Herbert *et al.* (1971). The freshly harvested cell-suspension was cooled to 0°C, centrifuged, washed twice with 1 mM ice-cold magnesium chloride and finally resuspended in 1 mM magnesium chloride and freeze dried.

The freeze-dried cells were then suspended in 4 mL of hyperchloric acid, vortexed, allowed to stand in a water-bath at 70°C for 15 minutes with occasional shaking, and then centrifuged. The extraction was repeated twice with 3 mL of 0.5 M hyperchloric acid, each for 15 minutes and to the combined extracts was added 0.5 M hyperchloric acid to give a total volume of 10 mL. For RNA analysis, the sample was extracted with 0.5 M hyperchloric acid at 37°C for 90 minutes, centrifuged, and washed once with 0.5 M hyperchloric acid.

Determination of DNA was by the diphenylamine method of Burton (1968). This method involved measuring 1-2 mL of the extract and mixing with 2 mL of freshly prepared diphenylamine reagent containing acetaldehyde. Standards containing known amounts of DNA in 0.5 M hyperchloric acid, and a reagent blank containing 0.5 M

hyperchloric acid but no DNA, were also prepared. The tubes were incubated overnight at 30°C and the absorbance at 600 nm measured against the blank in a spectrophotometer.

The orcinol method was used for RNA analysis. About 1.0 mL extract was measured into graduated glass-stoppered test tubes. Freshly prepared orcinol reagent (3.0 mL) was added. A reagent blank and standard RNA solutions were prepared simultaneously. All the mixtures were heated in a boiling water-bath for 20 minutes, cooled in cold tap water, and made up to 15 mL with n-butanol. The absorbance at 672 nm was read in a spectrophotometer.

3.2.7.9 Determination of ash content

The A.O.A.C method (Anon., 1980) was used to determine the ash contents of the raw peat, peat hydrolysate, and the yeast cells. About 300-500 mg of the dried samples were weighed into previously ignited, cooled and weighed porcelain crucibles. The samples were ashed in a Lab-Heat Muffle Furnace (Blue M Electric Co., Blue Island, IL., USA) at 550°C for 16 hours (until only grey ash remained). The crucibles and their contents were allowed to cool in a desiccator. The ashes were moistened with deionized water to dissolve the soluble salts, dried slowly on a hot plate, and ignited again at 550°C to constant weight.

3.2.7.10 Amino acid analysis

The samples were hydrolysed with 6 M hydrochloric acid under vacuum for 24 hours at 110°C (Blackburn, 1978). They were then reconstituted with 0.6M lithium citrate buffer and analyzed with a Beckman 121 MB amino acid analyzer using a single column method (Mondino *et al.*, 1972; Ohara and Ariyoshi, 1979). Tryptophan was not determined because of its destruction by acid hydrolysis.

3.2.7.11 Statistical analysis

All data presented for shaker flask experiments, batch fermentations and proximate analyses of biomass represent mean values of at least three determinations ± standard deviations. A Statistical Analysis System (SAS Inc. 1990, North Carolina, USA) was used to perform Analysis of Variance and Tukey's Studentized range test (Snedecor and Cochran, 1980) to evaluate the significance of difference between means.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Shaker flask experiments

4.1.1 Preliminary studies

Preliminary studies were conducted to determine the best substrate concentration for the growth of *P. rhodozyma* in peat hydrolysates PH4-01-185 and PH4-02-185. Thereafter the effects of initial pH, initial incubation temperature, fermentation time, agitation speed and inoculum ratio on the growth of the yeast were studied using the optimal substrate concentrations determined.

4.1.1.1 Effects of substrate concentration on the growth of *Phaffia rhodozyma.*

Fig. 4.1a and 4.1b represent the results obtained when the yeast was grown in various concentrations of peat hydrolysates PH4-01-185 and PH4-02-185, respectively. The numerical values are also shown in Appendices A.1 and A.2, respectively. The results show that increasing the substrate concentration increased the biomass, but not proportionately. The highest biomass concentration was obtained at a substrate concentration of 30 $g \cdot L^{-1}$ for PH4-01-185 and 45 $g \cdot L^{-1}$ for PH4-02-185. However, in the case of PH4-02-185, the final yield per gram of substrate utilized at 45 $g \cdot L^{-1}$

concentration was lower than at the others. The best yield coefficient and efficiency were obtained at a substrate concentration of 30 $g \cdot L^{-1}$ for PH4-01-185 and 15 $g \cdot L^{-1}$ for PH4-02-185. The differences between the biomass concentrations, the yield coefficient and the efficiencies at each substrate concentration level were statistically significant ($P \leq 0.05$). The lower yield and efficiency values obtained at higher substrate concentrations in the case of PH4-02-185 could be due to inhibitory substances preventing the efficient utilization of substrate. It has been reported that in a complex industrial medium, nutrients are prevented from being freely available for microbial utilization by the chelating, sequestering and adsorbing action of various materials such as humic acids (Jones and Greenfield, 1984). Since peat hydrolysates are known to contain considerable amounts of humic acids (Fuchsman, 1980), it is possible that, at higher substrate concentrations, the microorganisms were prevented from utilizing the substrate efficiently as a result of the presence of higher concentrations of humic acids. Furthermore, it is possible that a non-carbohydrate nutrient such as nitrogen became growth-limiting at some point. Better growth and improved efficiency may be obtained with diluted peat hydrolysates because of reduced inhibition effects through dilution (Martin and Bailey, 1983).

Peat hydrolysates made from acid hydrolysis are better fermentation substrates and provide higher TCH levels than non-acid hydrolysates, according to McLoughlin and Küster (1972a) and LeDuy (1981a). In this work, the acid hydrolysate had higher TCH than the non-acid hydrolysates made at corresponding temperatures (*i.e.*, 84 $g \cdot L^{-1}$ as

compared to 120 $g \cdot L^{-1}$ for the non-acid and acid hydrolysates, respectively). It can therefore be expected that more fermentable carbohydrates will be present in an acid hydrolysate of a particular concentration than a non-acid hydrolysate of the same concentration. This is evident in the TCH of the residual substrate. The amount of TCH consumed by the yeast in the non-acid hydrolysate was about twice that consumed by the yeast in the acid hydrolysate. However, the biomasses produced in both cases were about equal. Hence, a higher TCH of a non-acid hydrolysate may be needed to give a biomass concentration comparable to that of an acid hydrolysate with a lower TCH. This may explain why the efficiency and yield were highest at higher TCH in the case of the non-acid hydrolysate.

The formation of less precipitates in PH4-02-185 at 15 $g \cdot L^{-1}$ TCH than at 30 and 45 $g \cdot L^{-1}$ TCH, and the comparative ease of removal of precipitates in the substrate at 15 $g \cdot L^{-1}$ were some of the practical considerations behind the decision to use 15 $g \cdot L^{-1}$ for subsequent studies.

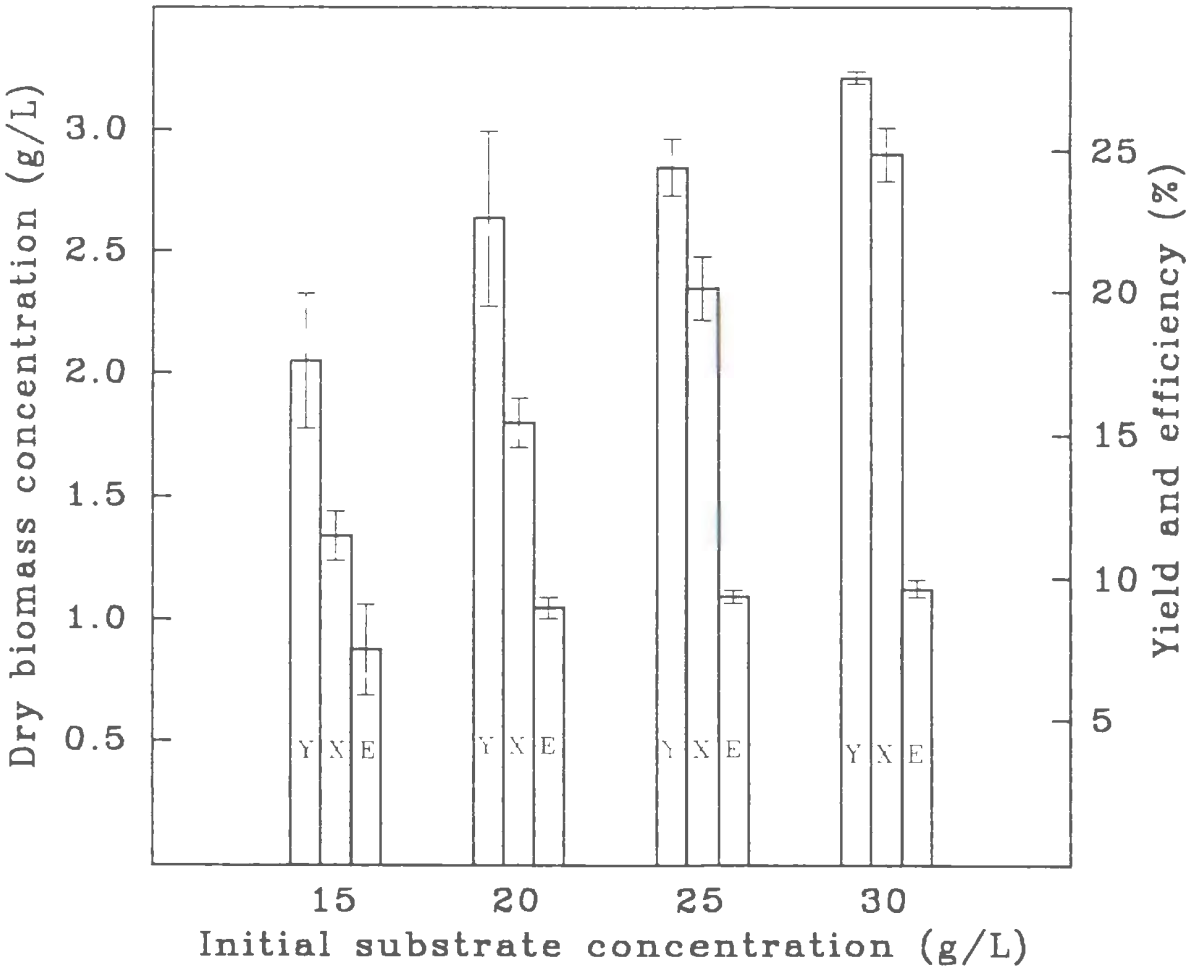

Fig. 4.1a. The effects of substrate concentration on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185. X = Dry biomass conc., Y = % Yield and E = % Efficiency.

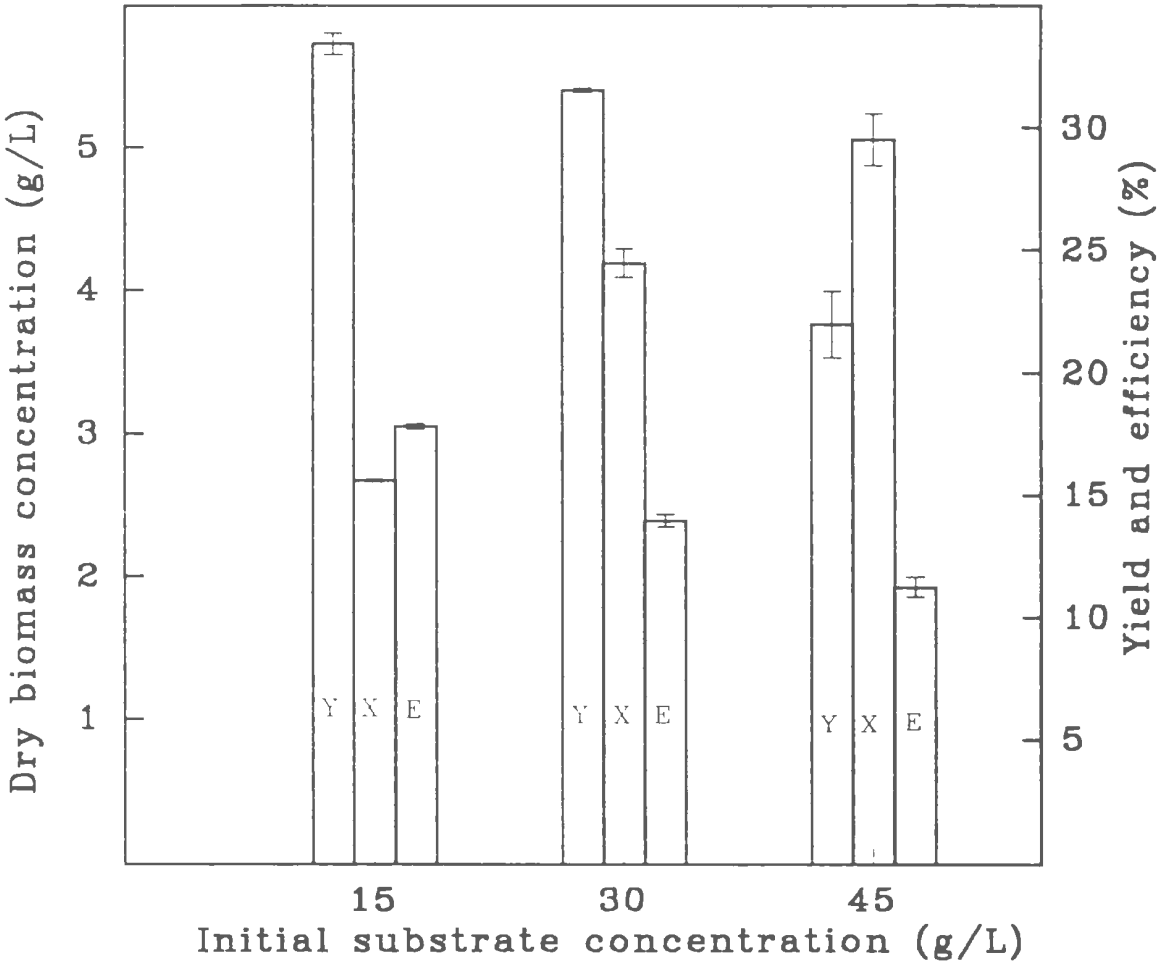

Fig. 4.1b. The effects of substrate concentration on the growth of *P. rhodozyma* peat hydrolysate PH4-02-185. X = Dry biomass conc., Y = % Yield and E = % Efficiency.

4.1.1.2 Effect of pH on the growth of *Phaffia rhodozyma*

Fig. 4.2a depicts the dry biomass concentration, yield coefficient and efficiency obtained when the yeast was grown in peat hydrolysate PH4-01-185 at various initial pH levels, and Fig.4.2b depicts the respective values for yeast growth in hydrolysate PH4-02-185. The numerical values for the dry biomass concentration, yield coefficient and efficiency for the yeast grown in PH4-01-185 and PH4-02-185 are shown in Appendices A.3 and A.4, respectively. The highest growth was obtained at a pH of 7 in both cases. However, there were no statistical differences in values obtained at pH 5, 6, and 7 ($P > 0.05$). Yeasts are known to grow best near pH 5, but will grow over a pH range of 2.5 to 8.5. The optimal pH reported for *P. rhodozyma* is around 5.0 (Johnson and Lewis, 1979). However, in a mixed culture of *P. rhodozyma* and *Bacillus circulans* WL-12, Okagbue and Lewis (1985) reported optimum growth at a pH around 6.5. Chang (1985) reported that the biomass yield of *Candida utilis* in high-pH peat hydrolysates was always higher than in low-pH peat hydrolysates, especially in sulfuric acid hydrolysates. This inhibitory effect at low pH was attributed to the presence of high concentrations of humic substances in low - pH peat hydrolysates. Numerous authors have shown that humic acids affect cellular metabolism in processes such as growth, respiration, photosynthesis and nitrogen fixation (Prakash and MacGregor, 1983; Flaig, 1968; Petrovic *et al.*, 1982; Khristeva, 1968; Nechutova and Tichy, 1970). However, there is much confusion surrounding the effects of humic substances on the growth and metabolism of microorganisms and plants. McLoughlin and Küster (1972c) reported no apparent effects

of humic acids on the metabolism of *Candida utilis*, whereas Dragunov and Popova (1968) reported that humic substances stimulate growth of sugar beet plants. Evdokimova *et al.* (1974) on the other hand, reported that humic substances have inhibitory effects on yeasts in peat hydrolysates, although they contended that when added to synthetic medium, humic substances have a stimulatory effect. Chintalapati (1987) also reported that humic acids concentrations of up to 0.20% were stimulatory to the growth of the fungus *Scytalidium acidophilum*, whereas concentrations above 0.20% were inhibitory. McLoughlin and Küster (1972c) reported that humic acids could have nutritive effects, and that the effects of the humic acids depended largely on the physiological state, nutritive requirements, and other parameters of growth relative to the study of the microorganism. Two effects, they suggested, may be working antagonistically to produce an appearance of no effects. They therefore attributed the contrary descriptions of different investigators of the effects of humic acids on plants and microbes to this.

Since humic acids are known to be soluble in alkalis and insoluble in acids (Fuchsman, 1983), it is possible that in this study, at high pH, the dissolution and degradation of humic acids might have reduced their concentration to levels that were stimulatory to the growth of the yeast. It was also observed in this study that there was precipitate formation at low pH. This might have been the precipitation of humic acids. McLoughlin and Küster (1972c) reported that very reactive oxidation-reduction agents affecting the cytochrome system result from precipitation of humic substances. It is possible therefore, that the precipitates might have interfered directly with the growth of

the yeast. Finally, the precipitates could have also removed valuable nutrients and ions from the growth media since humic acids have been reported to form chelating compounds with ions making them either available or non-available to the organism being cultivated, depending on the circumstances (McLoughlin and Küster, 1972c).

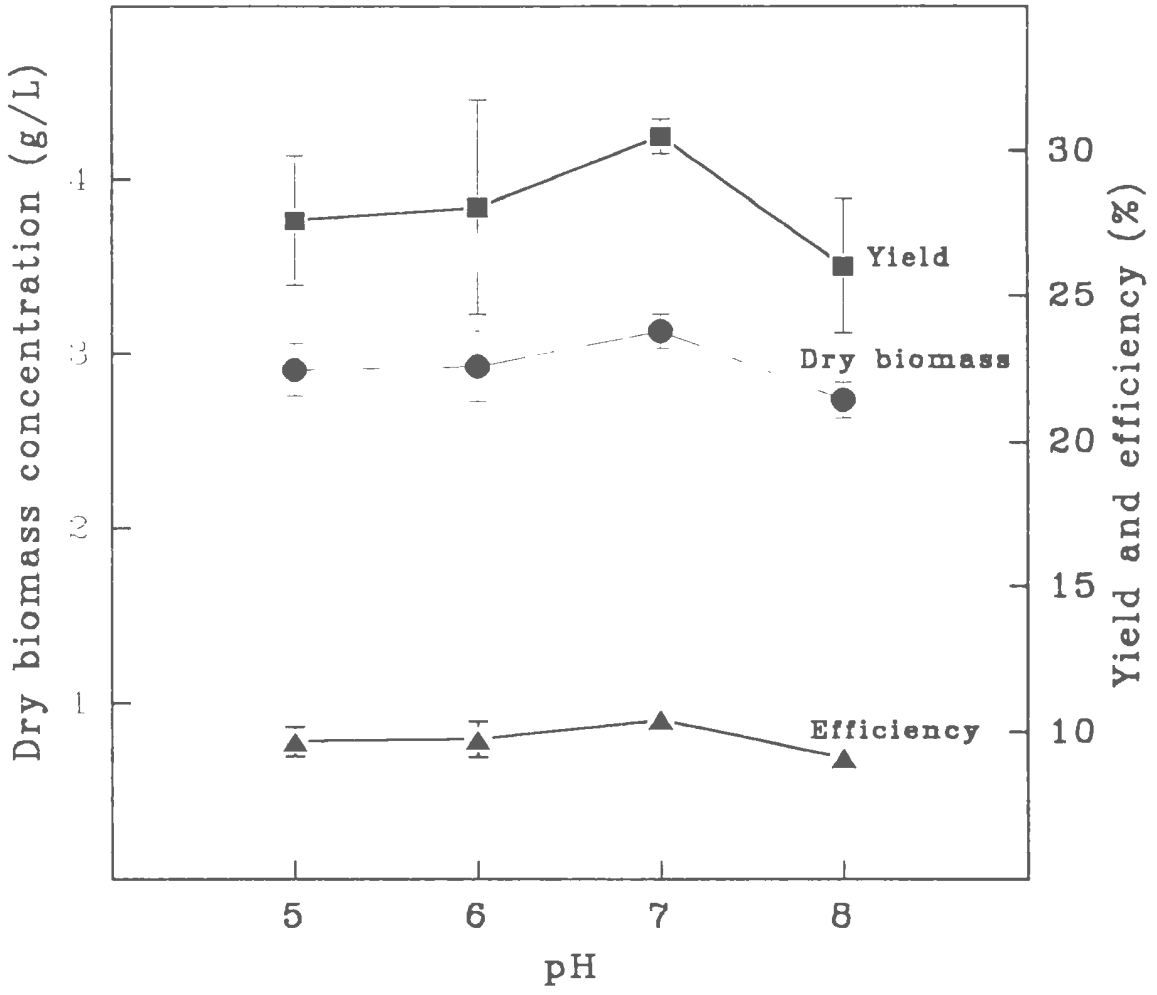

Fig. 4.2a. Effects of pH on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185.

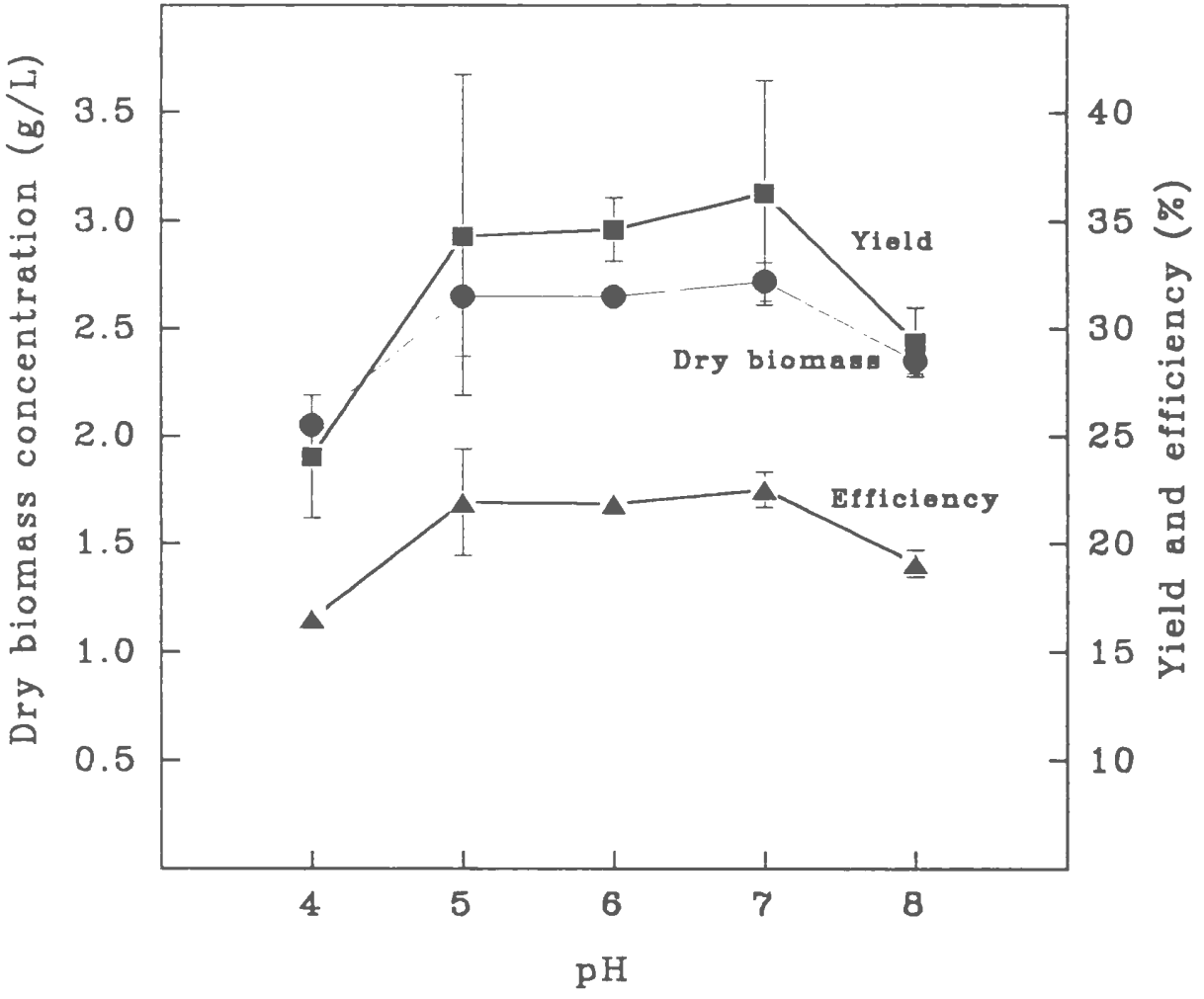

Fig. 4.2b. Effects of pH on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185.

4.1.1.3 The growth of *Phaffia rhodozyma* under various incubation temperatures

For *P. rhodozyma*, it has been reported that growth and pigment synthesis are both optimal at 20-22°C (Johnson and Lewis, 1979). Five temperatures (16, 18, 20, 22, and 24°C) were tested to determine their effects on the growth of *P. rhodozyma*, in peat hydrolysates PH4-01-185 and PH4-02-185 in separate experiments. Fig. 4.3a and 4.3b represent the results obtained for PH4-01-185 and PH4-02-185, respectively. The numerical values are also depicted in Appendices A.5 and A.6, respectively. The results demonstrate that growth in terms of dry biomass concentration, yield coefficient, and efficiency were highest at 18°C in both cases. However, there were no significant differences in biomass concentration obtained at temperatures 16-22°C, or in yield coefficient and efficiency at 16-20°C ($P > 0.05$). It was further observed that there was an increase in growth with increase in temperature from 16 to 20°C. A further increase in temperature to 22°C resulted in a decrease in growth, although this was not significant ($P > 0.05$). Johnson and Lewis (1979) observed that the final biomass yield of *P. rhodozyma* was relatively constant at temperatures of 22°C and below, but decreased significantly at temperatures above 22°C. They reported that the highest temperature at which growth could be observed was 27.5°C. At this temperature, the yeast increased its mass two fold after inoculation and then stopped growing (Johnson and Lewis, 1979). Okagbue and Lewis (1985) reported that optimum growth of *P. rhodozyma* in a mixed culture with *Bacillus circulans WL-4* occured around 20°C. They also reported that the

yeast grew well at a temperature of 15°C. The incubation temperature range 15 - 27.5°C has been used by many other researchers for culturing *P. rhodozyma* (Johnson *et al.*, 1980; Okagbue and Lewis, 1984a,b; Andrewes *et al.*, 1976; Miller *et al.*, 1976; An *et al.*, 1989; Johnson *et al.*, 1978; Haard, 1988) and the optimal in all cases has been reported to be in the range 20-22°C. Therefore the optimal temperature obtained in this study could be said to be in agreement with the values reported in the literature.

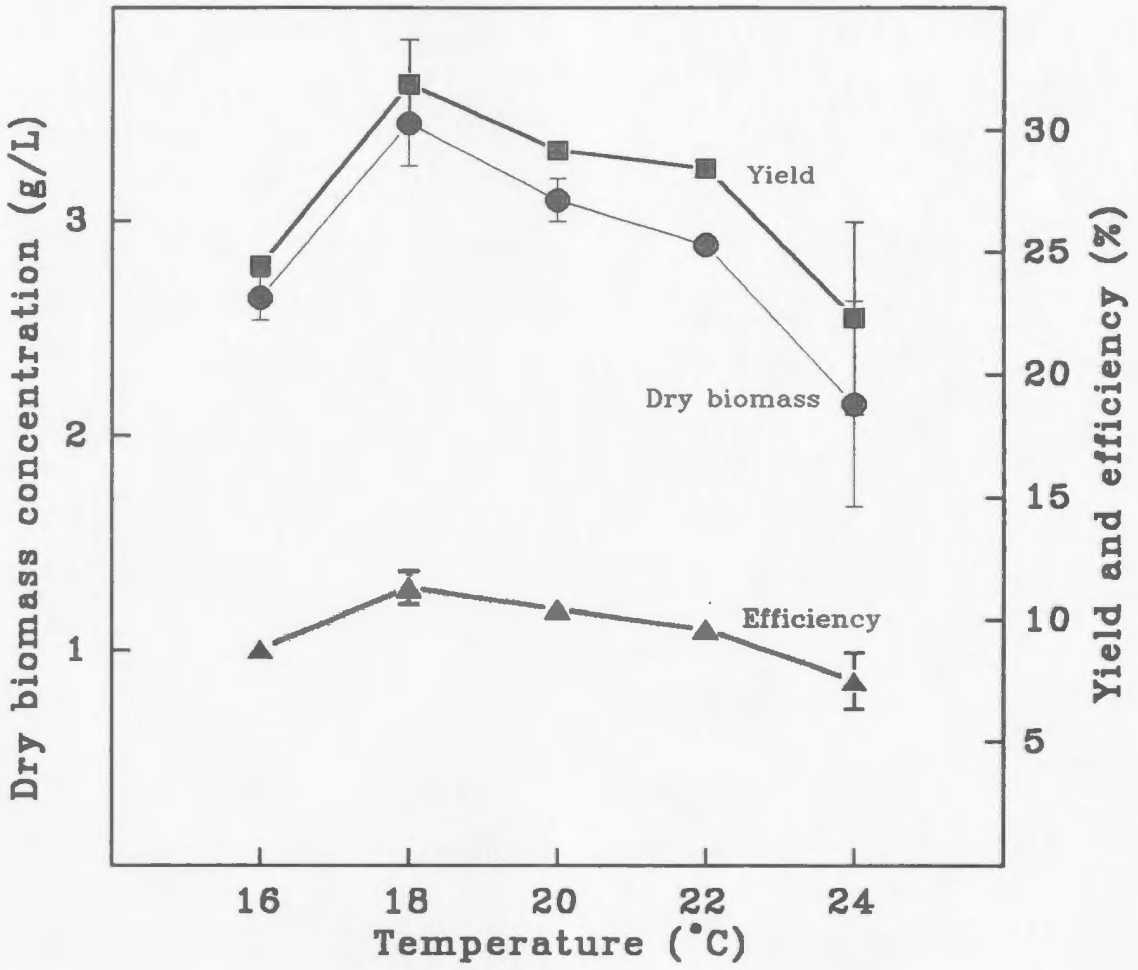

Fig. 4.3a. Effects of incubation temperature on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185.

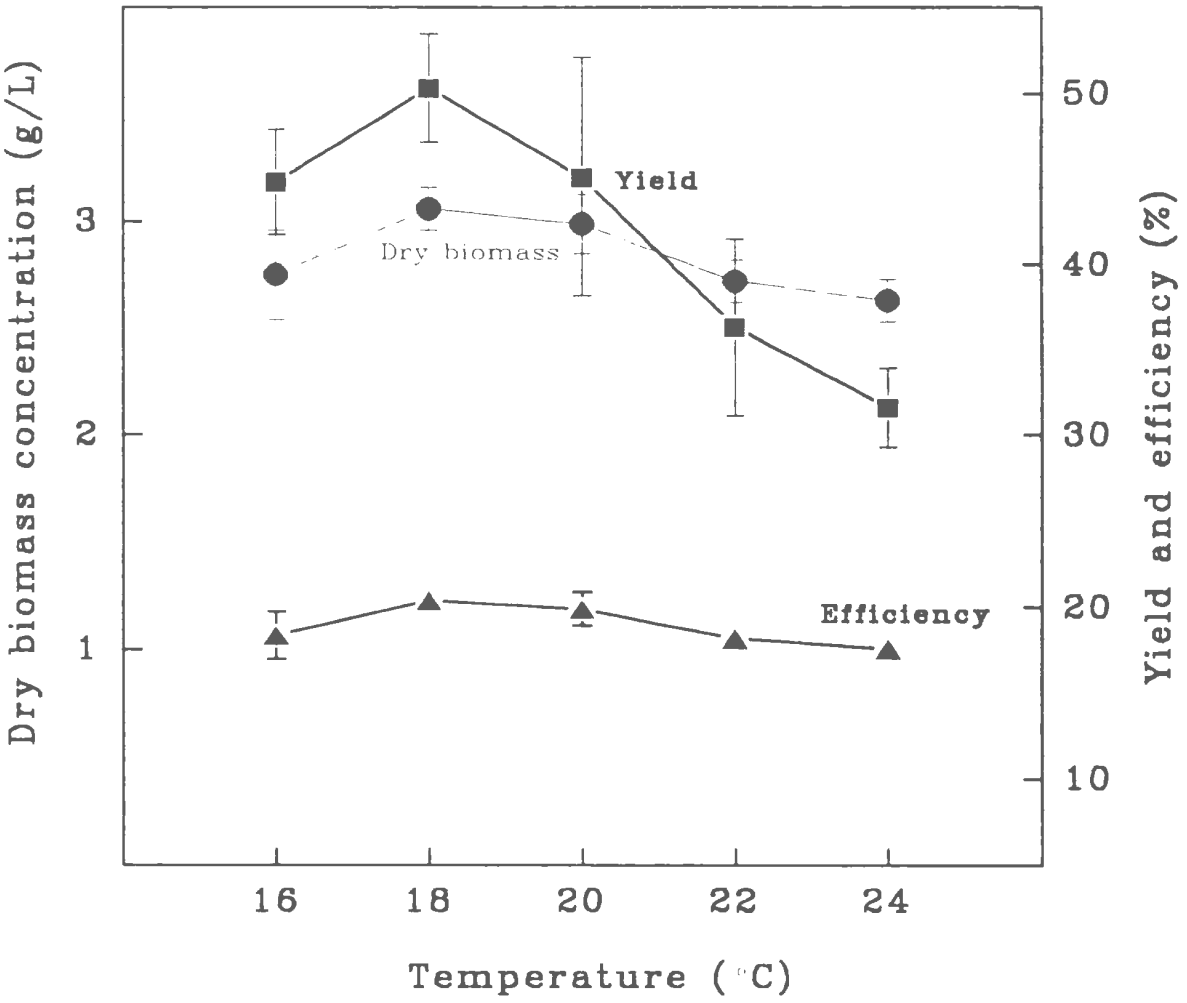

Fig. 4.3b. Effects of incubation temperature on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185.

4.1.1.4 Effect of fermentation time on the growth of *Phaffia rhodozyma*

The dry biomass concentration, yield coefficient, efficiency and residual substrate concentration obtained from a study of the effects of various fermentation times on the growth of *P. rhodozyma* in peat hydrolysates PH4-01-185 and PH4-02-185 are given in Fig. 4.4a and 4.4b, respectively. Appendices A.7 and A.8 represent the numerical values for the various growth parameters. There was no appreciable growth before 48 hours (and so results prior to this do not appear in the figures), but after this there was a gradual increase in biomass, which finally peaked at 120 hours. The yield coefficient and efficiency followed a similar pattern. Values reported at all levels of measurement differed significantly ($P \leq 0.05$). The accelerated growth phase occurred between 96 and 120 hours, after which there was a decrease in growth rate. The substrate was utilized rapidly in the first 72 hours, and then the rate of utilization decreased.

The monosaccharides detected in the peat hydrolysate and their relative abundance were arabinose 0.78%, xylose 4.47%, ribose 0.76%, rhamnose 2.84%, fucose 0.23%, mannose 1.88%, fructose 0.73%, and galactose 3.58%. The relative abundance of the various monosaccharides of the peat hydrolysate used in this study differs from the monosaccharide composition of the sulfuric acid extracts of various kinds of peat reported by Morita and Montgomery (1980) and Morita and Levesque (1980). These researchers reported glucose to be the predominant sugar followed by galactose, mannose, and xylose or arabinose. In this study glucose was found to be the predominant sugar but the others did not follow a pattern similar to that reported by the other authors. Only about 56%

of the TCH was utilized, indicating the possible presence of some carbohydrates that could not be utilized by the yeast. This assumption is in line with the views of Martin and Bailey(1983) and Quierzy *et al.* (1979), that not all of the carbohydrates present in peat can be assimilated. This percentage is low in terms of industrial fermentation where nearly complete utilisation is desirable. However, Forsberg *et al.* (1986) reported that because of the complex mixture of monosaccharides, disaccharides and longer oligosaccharides in peat hydrolysates several problems may be encountered by the microorganisms in the course of their growth. These include catabolite repression by glucose, of the utilization of such other sugars as arabinose and galactose. This may explain why only a fraction of the TCH was utilized by the yeast in this study. Also, the presence of a mixture of sugars, along with oligosaccharides, may have inhibited extensive hydrolysis of the oligosaccharides in the peat hydrolysate (Forsberg, 1986).

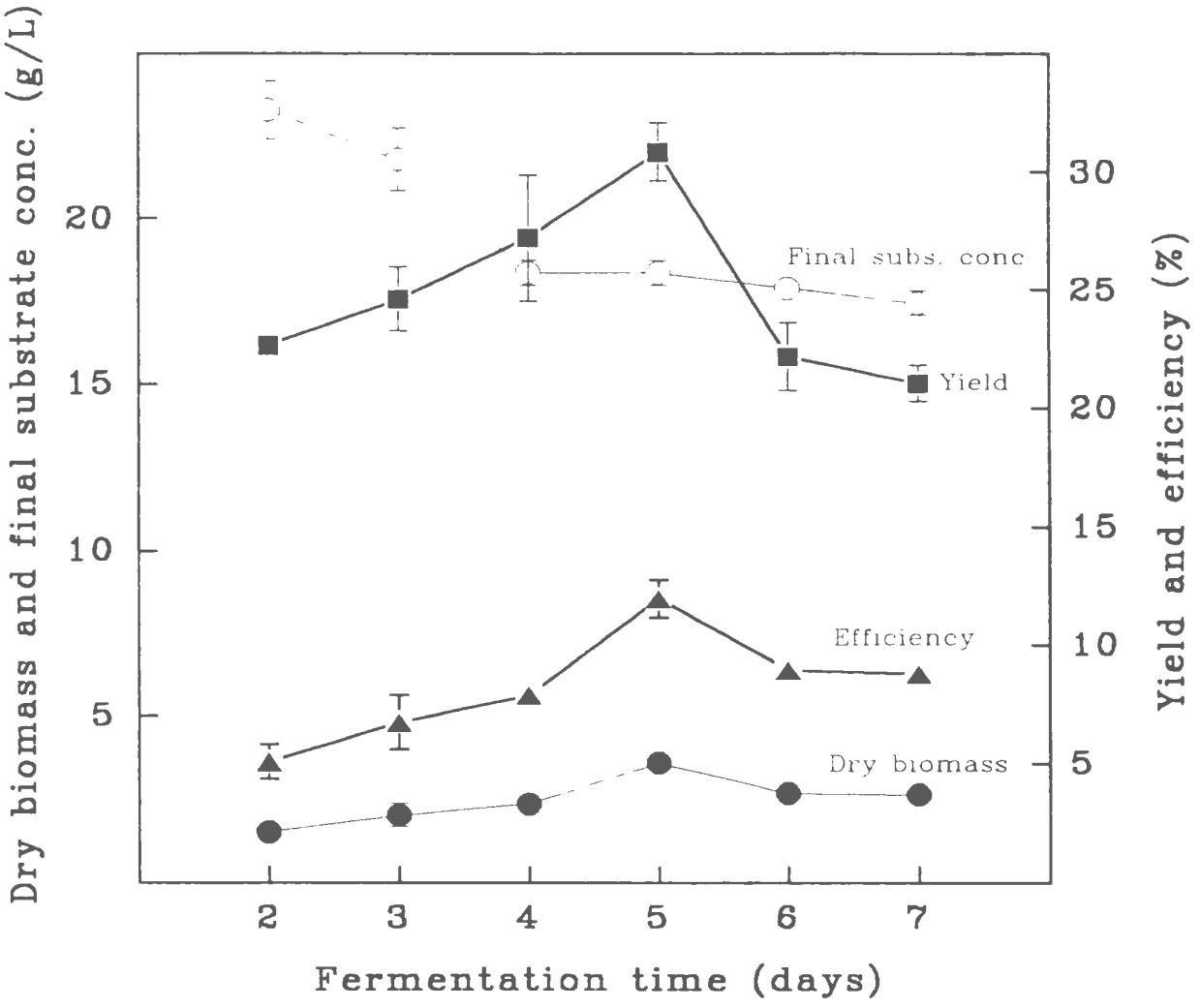

Fig. 4.4a. Effects of fermentation time on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185.

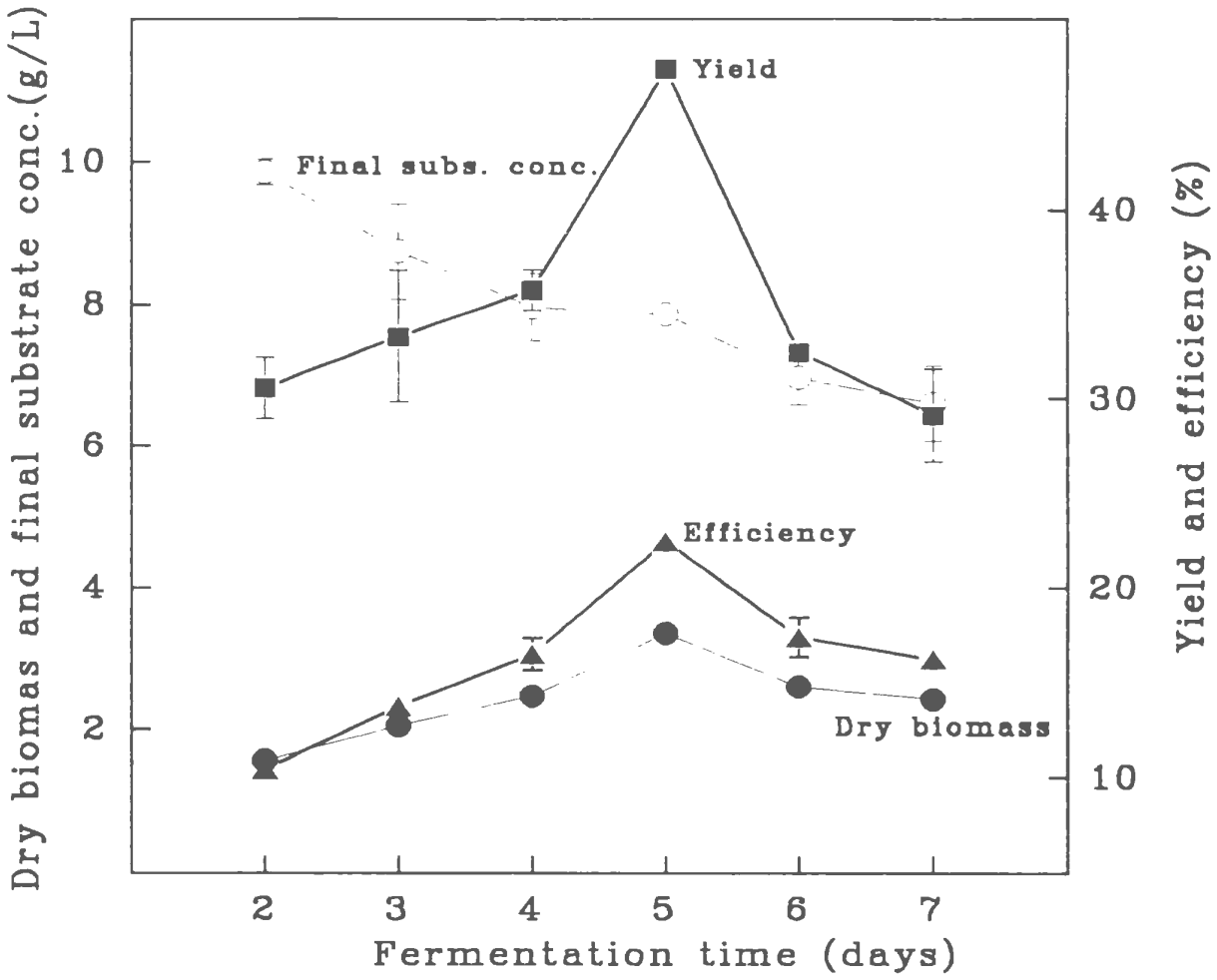

Fig. 4.4b. Effects of fermentation time on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185.

4.1.1.5 The effects of inoculum ratio on the growth of *Phaffia rhodozyma*

The results obtained from experiments on the effects of various inoculum ratios on the growth of *P. rhodozyma* are shown in Fig. 4.5a and 4.5b for peat hydrolysates PH4-01-185 and PH4-02-185, respectively. The numerical values for the dry biomass concentration, yield coefficient and efficiency for the yeast grown in PH4-01-185 and PH4-02-185 are shown in Appendices A.9 and A.10, respectively. It was observed that the quantity of the inoculum had a profound effect on the growth of the yeast. The highest dry biomass concentration, yield coefficient and efficiency were obtained at an inoculum ratio of 5% ($v \cdot v^{-1}$) for both peat hydrolysates. At higher and lower inoculum ratios, there were decreases in these growth parameters. However, values obtained for all measurements at 3%, 5%, and 7% were not statistically different from each other ($P > 0.05$). A similar observation was made by Kosaric and Miyata (1981) who found that an optimum biomass production was achieved with an inoculum ratio of 5-7.5% ($v \cdot v^{-1}$) when they cultivated morel mushroom in cheese whey. Similarly, Martin and White (1985) observed that 5% inoculum ratio produced better results than 10% ($v \cdot v^{-1}$) in a study with the acid-tolerant fungus *Scytalidium acidophilum*. Martin and White (1985) concluded that the adverse effects of the higher inoculum ratio were not due to the presence of inhibitory substances but rather due to production and accumulation of growth retarding metabolites that were transferred from the inoculum to the growth medium. It was observed in this study that not all the TCH were utilized by the yeast. This, coupled with the fact that there were higher TCH values at higher inoculum ratios, indicated that

the inhibition of microbial growth at higher inoculum ratios was not due to the exhaustion of nutrients. It is therefore reasonable to conclude that at higher inoculum ratios, higher concentrations of growth retarding metabolites were transferred to the culture medium from the inoculum. The lower growth at lower inoculum ratios, on the other hand, could be attributed to a greater lag in the microbial population. In *P. rhodozyma*, there has not been any previous systematic determination of the best inoculum ratio for the optimal growth of the yeast. However, most investigators have used an inoculum ratio of 2% (Johnson and Lewis, 1979; Haard, 1988).

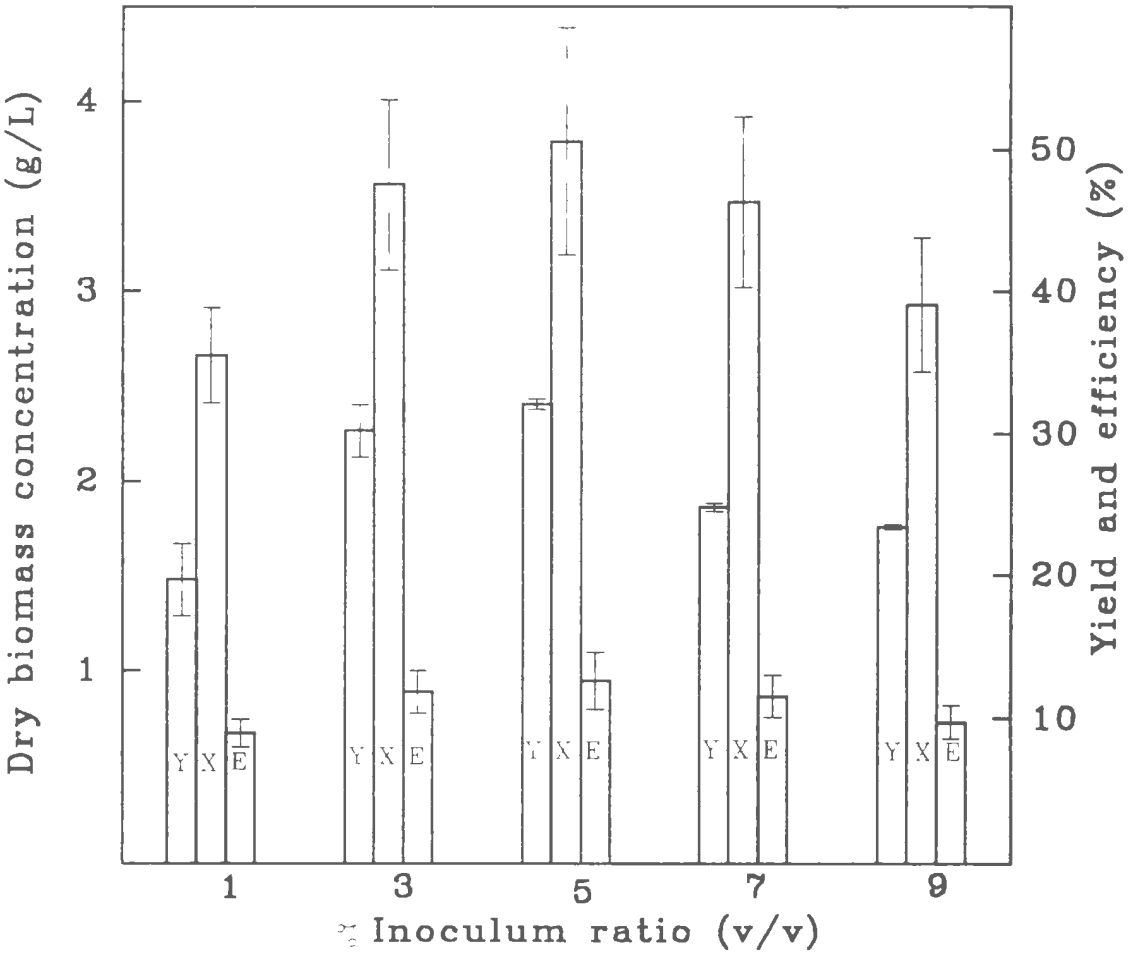

Fig. 4.5a. Effects of inoculum ratio on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-184-5. X = Dry biomass conc., Y = % Yield and E = % Efficiency.

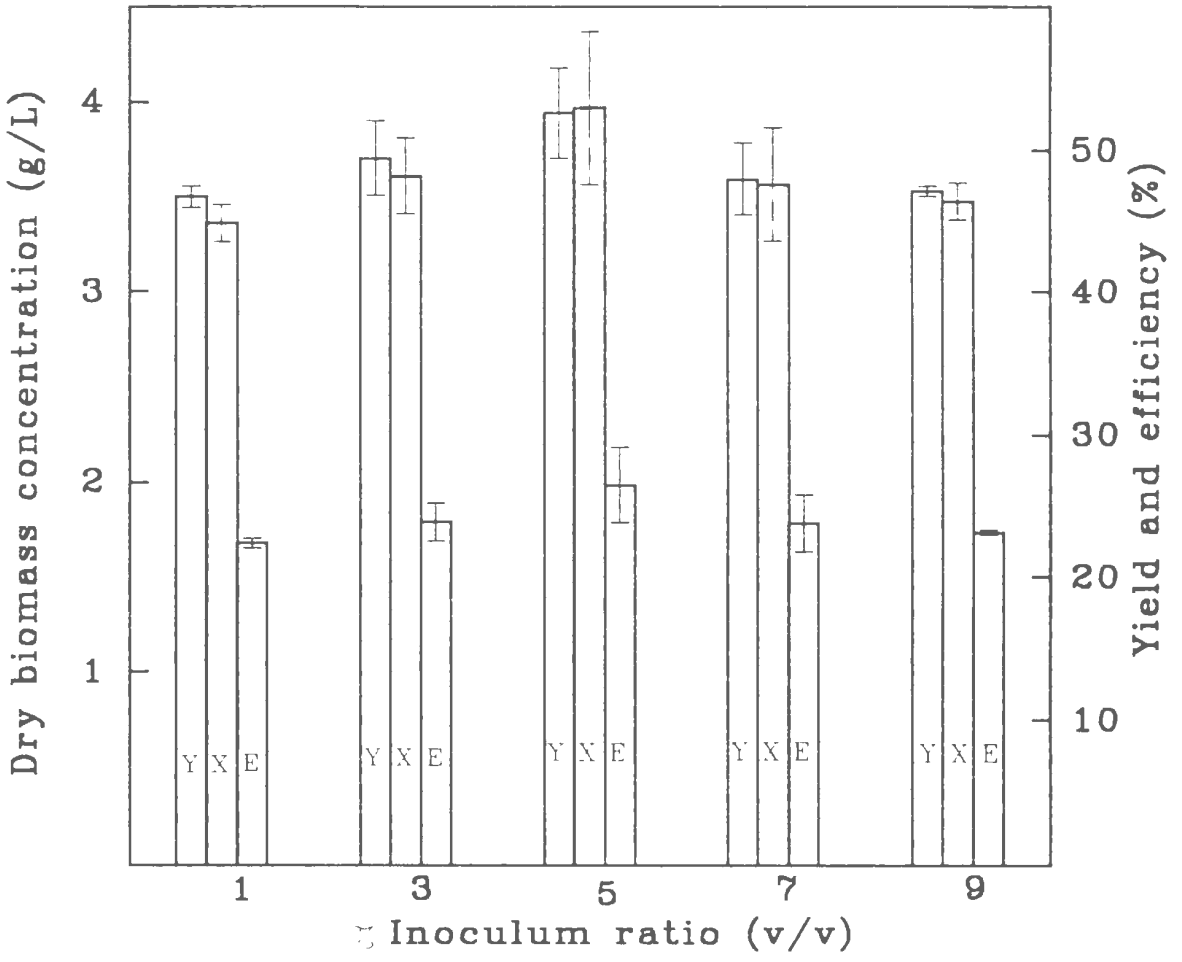

Fig. 4.5b. Effects of inoculum ratio on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185. X = Dry biomass conc., Y = % Yield, and E = % Efficiency.

4.1.1.6 Effects of agitation speed on the growth of *Phaffia rhodozyma*

Previous works with *P. rhodozyma* have involved agitation speeds ranging from 150 to 600 r.p.m. (Johnson *et al.*, 1978; Okagbue and Lewis, 1984a; Haard, 1988; An *et al.*, 1989; Okagbue *et al.*, 1985), but it has not been clearly stated which agitation speed is most suitable for the growth of the yeast. Five agitation speeds were tested to determine their effects on the growth of *P. rhodozyma* in peat hydrolysates PH4-01-185 and PH4-02-185. Fig. 4.6a and 4.6b, respectively, present the results obtained. The highest biomass, yield coefficient and efficiency were obtained at 200 r.p.m. The numerical values for these parameters are depicted in Appendices A.11 and A.12 for PH4-01-185 and PH4-02-185, respectively. There were no significant differences between these values and those obtained at 250 r.p.m., but the growth at these two agitation speeds differed significantly from the growth at the other speeds ($P \leq 0.05$). For fermentation broths in general, an increase in agitation speed will result in better mixing and better mass transfer. Therefore, one would expect higher values of biomass, yield coefficient and efficiency at higher agitation speeds. According to Boa and LeDuy (1986), this seems to be the case in fermentations utilizing simple substrates such as glucose and sucrose as the carbon source. However, in a complex medium such as peat hydrolysate, the opposite had been reported to happen. A possible explanation is that, in a complex peat hydrolysate medium, there are adverse effects from either increased shear or from increased levels of oxygen in the presence of certain chemical compounds, or both factors may have adverse effects (Boa and LeDuy, 1986).

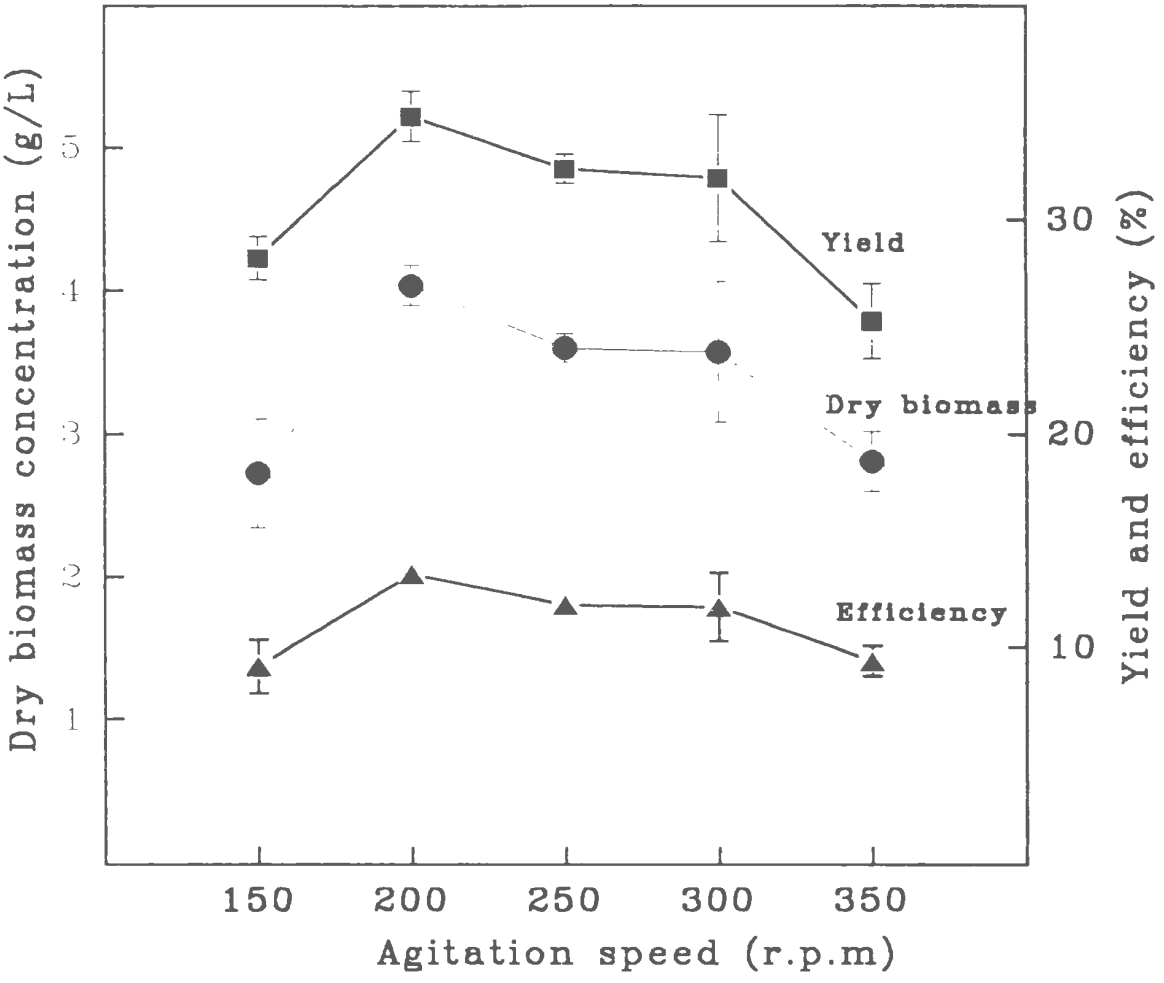

Fig. 4.6a. Effects of agitation speed on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185.

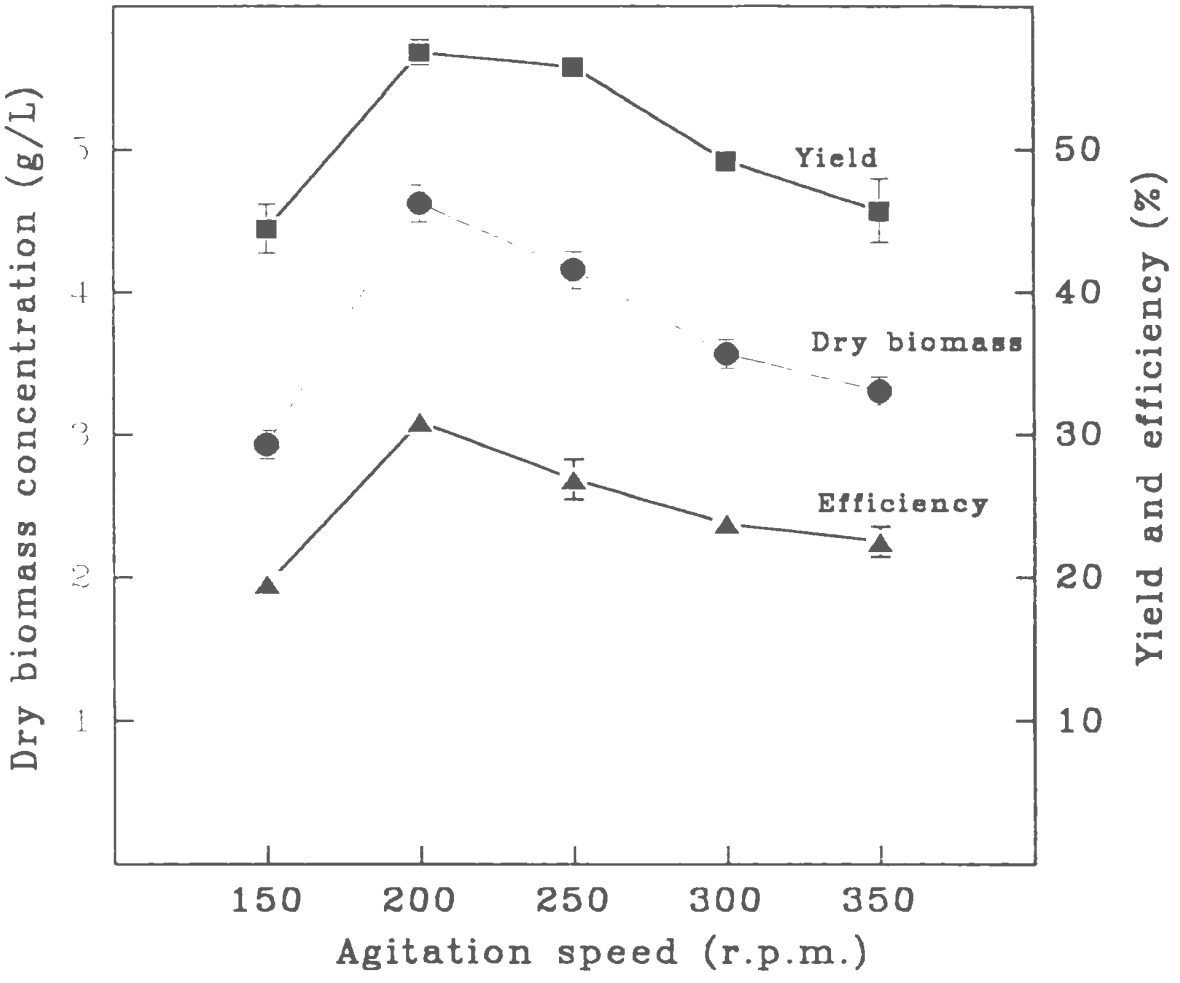

Fig. 4.6b. Effects of agitation speed on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185.

4.1.2 Peat hydrolysate evaluation for the growth of *Phaffia rhodozyma*

Having optimized the growth parameters in the non-acid and acid hydrolysates (PH4-01-185 and PH4-02-185, respectively), the yeast was grown in all the six peat hydrolysates to determine which one supported the growth best. Table 4.1 presents the results obtained. It is clear from the table that PH4-02-185 produced the best dry biomass concentration, yield coefficient and efficiency. The differences between these values and those for PH4-01-185 were not statistically significant. However, the results for PH4-01-185 and PH4-02-185 differed significantly from those obtained for the remaining four hydrolysates ($P \leq 0.05$).

Each acid hydrolysate produced more biomass than its non-acid counterpart made at the same temperature. This is in agreement with the findings of LeDuy (1981a) who stated that, in general, acid hydrolysates are better media than non-acid hydrolysates. McLoughlin and Küster (1972a) also reported that peat hydrolysates from acid hydrolysis gave a much higher *Candida utilis* biomass concentration than non-acid hydrolysates. This beneficial effect of acid hydrolysates on biomass production was attributed to the higher decomposition of peat hemicelluloses resulting in higher TCH concentration in the acid hydrolysates. Alkaline extracts, they concluded, were not suitable for biomass production, probably due to higher ionic content. However, PH4-01-185, a non-acid hydrolysate, produced better growth than the two other acid hydrolysates (PH4-02-205 and PH4-02-225) and, for both the acid and non-acid hydrolysates, as temperature of production increased, biomass production and yield decreased. This suggests that higher

temperatures of production could have destroyed some nutrients in the media vital for microbial growth, thereby decreasing their value as fermentation substrates. This is in agreement with Quierzy *et al.* (1979) who reported that temperature is very critical to the release of carbohydrates from peat. In peat hydrolysate preparation, for each selected value of holding time, there exists a critical temperature at which TCH yield is maximized. Below this critical temperature the TCH yield is low because of incomplete hydrolysis. Likewise, the TCH yield decreases as the temperature of production increases above the critical temperature, suggesting that the carbohydrates in the peat hydrolysate are destroyed at higher temperatures (Quierzy *et al.*, 1979). It was also observed in this study that increasing amounts of precipitates were formed in hydrolysates produced at higher temperatures when they were neutralized with sodium hydroxide. Chang (1983) reported that formation of colloid matter in peat hydrolysate during neutralization adversely affects the biomass and growth rate of *Candida utilis*. It is possible that precipitate formation removes nutrients from the medium. According to McLoughlin and Küster (1972b) and Fuchsman (1980) precipitates are themselves harmful to yeast growth, and their removal results in an increase in biomass. The precipitates formed in hydrolysates produced at higher temperatures could have resulted from a reaction between carbohydrate degradation products produced at higher temperatures and sodium hydroxide, and these might have had adverse effects on the growth of the yeast.

Table 4.1: Determination of the most suitable peat hydrolysate for the growth of *P rhodozyma* in shaker flask culture[1].

Peat Hydrolysate	Dry Biomass Conc. ($g.L^{-1}$)	% Yield	% Efficiency
PH4-01-185	4.04 ± 0.11^{a}	34.82 ± 0.98^{a}	13.48 ± 0.01^{a}
PH4-01-205	1.61 ± 0.06^{b}	$11.45 \pm 1.05^{b,c}$	5.36 ± 0.22^{b}
PH4-01-225	1.21 ± 0.02^{d}	8.97 ± 0.27^{d}	4.03 ± 0.05^{d}
PH4-02-185	4.30 ± 0.04^{a}	36.74 ± 1.13^{a}	14.33 ± 0.14^{a}
PH4-02-205	1.78 ± 0.09^{b}	12.28 ± 0.67^{b}	5.93 ± 0.31^{b}
PH4-02-225	1.28 ± 0.02^{c}	8.98 ± 0.27^{c}	4.29 ± 0.07^{c}

[1]Mean values of three determinations ± standard deviation. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

4.1.3 The concentration of astaxanthin in *Phaffia rhodozyma*

The dry biomass of the yeast grown in the most suitable acid and non-acid hydrolysates (PH4-02-185 and PH4-01-185) was analyzed for their astaxanthin content by an enzymatic method (Gentles and Haard, 1989). Table 4.2 compares data for the optimal production of astaxanthin in this work and those reported by other authors utilizing different substrates. The astaxanthin contents were 1567 ± 11 $\mu g \cdot g^{-1}$ yeast and 1280 ± 0.00 $\mu g \cdot g^{-1}$ yeast for *P. rhodozyma* produced in PH4-02-185 and PH4-01-185, respectively. These values compare favourably with those reported by other investigators (Table 4.2).

Cellobiose has been reported to produce more astaxanthin than other carbon sources (Johnson and Lewis, 1979). Forsberg *et al.* (1986) reported cellobiose in peat extract produced by steam explosion. Therefore the high content of astaxanthin obtained in this work might have been due to the combined effects of cellobiose and such astaxanthin precursors as carotenes that are known to be present in peat hydrolysates (Fuchsman, 1980). High levels of carotenoids have been obtained in the yeasts *Sporobolomyces pararoseous* T and *Rhodotorula glutinis* T-2 grown in peat hydrolysates (Raitsina and Evdokimova, 1977). The high yield of carotenoids with peat hydrolysate suggests that these carotenoid precursors may have entered the cell and enhanced carotenoid production (Johnson and Lewis, 1979).

Table 4.2 Comparison of astaxanthin contents of *P. rhodozyma* grown in various media.

Fermentation Media	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)	Reference
PH4-01-185	1280	This work
PH4-02-185	1567	This work
Cellobiose	1260	An *et al.* (1989)
Ethanol	540	An *et al.* (1989)
Glucose	421	Johnson and Lewis (1979)
Molasses	1086	Haard (1988)
Sucrose	508	Johnson and Lewis (1979)
YM Broth	2040	An *et al.* (1990)

4.1.4 Crude protein content of *Phaffia rhodozyma*

The crude protein contents (N × 6.25) of the yeast cultured in peat hydrolysates PH4-02-185 and PH4-01-185 are presented in Table 4.3 and compared with the protein contents of microbial biomass produced by other investigators. The crude protein contents were 47.10 ± 0.94% and 38.89 ± 2.58% for yeast grown in PH4-02-185 and PH4-01-185, respectively. Even though PH4-01-185 resulted in a significantly ($P \leq 0.05$) lower protein content than did PH4-02-185, both protein contents compare favourably with that produced by Fleischmann's commercial active dry yeast (LeDuy, 1981a) and the value Johnson *et al.* (1980) reported for *P. rhodozyma* (30.1%).

Table 4.3 Comparison of biomass protein contents from various microbial sources.

Biomass Source	% Protein (N × 6.25)	Reference
Phaffia rhodozyma from medium PH4-01-185	38.89 ± 2.58	This work
Phaffia rhodozyma from medium PH4-02-185	47.10 ± 0.94	This work
Phaffia rhodozyma	30.10	Johnson *et al.* (1980)
Fleischmann's commercial active dry yeast	41.40	LeDuy (1981a)

4.1.5 Nutritional requirements of *Phaffia rhodozyma*

4.1.5.1 Effects of different sources of nitrogen on the growth of and pigment production by *Phaffia rhodozyma* in peat hydrolysate.

When *P. rhodozyma* was grown in the unsupplemented peat hydrolysate, the dry biomass concentration and pigment production was low. The medium was therefore supplemented with yeast nitrogen base and bactopeptone to improve the biomass yield and pigment production. However, because of the relatively high cost of these ingredients, less-expensive chemicals were sought as replacements, and the effects of such substitution on the growth of and astaxanthin production by the yeast were determined.

Ammonium nitrate, ammonium phosphate, ammonium sulfate, potassium nitrate and urea were tested to determine their effects on the growth and pigment production by *P. rhodozyma*. Table 4.4 presents the results obtained. It is clear from the Table 4.4 that the growth and pigment production depended on the nitrogen source. The addition of ammonium sulfate, ammonium nitrate, potassium nitrate and urea had no significant effect on the final yeast biomass in comparison to the non-supplemented peat hydrolysate. Visual inspection of the cells revealed a very pale color, indicating a comparative lack of astaxanthin. Ammonium phosphate addition improved the biomass concentration from 1.81 ± 0.1 to 2.1 ± 0.72 $g \cdot L^{-1}$, but there was no statistical difference between these two biomass concentrations ($P > 0.05$). Similarly, the quantity of astaxanthin produced by the yeast grown in the ammonium phosphate supplemented peat hydrolysate, 579 ± 5 $\mu g \cdot g^{-1}$

yeast was not significantly different from that of the yeast grown in the non-supplemented peat hydrolysate, 575 ± 21 $\mu g \cdot g^{-1}$ yeast ($P > 0.05$).

Addition of increasing concentrations of yeast extract to the medium resulted in significant increases in the biomass concentration and astaxanthin content (Table 4.5). The biomass increased from 1.81 ± 0.1 to 4.21 ± 0.26 $g \cdot L^{-1}$ when the yeast extract concentration was increased from 0 to 3 $g \cdot L^{-1}$. This finding is in agreement with the observation of other investigators (Bukhalo and Solomoko, 1978; Litchfield, 1967) who stated that, generally, organic nitrogen sources are better than inorganic nitrogenous salts for microbial growth especially in an unbuffered medium. Johnson and Lewis (1979) reported that various concentrations of ammonium sulfate had little effect on the growth rate, final yeast biomass and carotenoid production by *P. rhodozyma* cultured in yeast nitrogen base medium supplemented with 1% D-glucose. These workers found further that neither ammonium phosphate nor peptone at various concentrations affected the dry biomass and pigment concentrations. In this work, it was observed that when ammonium salts were used as an inorganic source of nitrogen for the growth of the yeast, the pH of the medium dropped to a minimum of about 4.2 (Table 4.6). This might account for the poor growth of the yeast in this medium. Several researchers have reported a decrease in the pH of the medium when some fungal species are grown in a medium with an ammonium salt of an inorganic acid as the nitrogen source (Carels and Shepherd, 1977; Srivastava and Bano, 1970; Hashimato and Takahashi, 1974). The apparent decrease in the pH was attributed to the rapid utilization of the ammonium ions which rendered the

medium more acidic thus becoming poisonous to the microorganism. Nicholas (1965), on the other hand, argued that ammonium ions in the nutrient medium often reduces the activity of nitrate reductase, thus reducing the assimilation of nitrate. In addition, the activity of nitrate reductase and glutamase reductase, which are probably involved in the regulation of nitrogen uptake and metabolism, can also be inhibited by the lack of utilizable carbon in the medium (Hynes, 1973, 1974).

The enhanced growth obtained with the addition of ammonium phosphate to the medium could be due to the strong buffering capacity of the phosphate ions; which thereby maintain the pH at levels suitable for the growth of the yeast (Litchfield, 1967). Potassium nitrate was unable to support any good growth. Miller *et al.* (1976) reported that *P. rhodozyma* is unable to assimilate potassium nitrate and this may explain why a poor growth was observed in the medium supplemented with it. Yeast extract has been found to be a good source of nitrogen for the production of microbial cell biomass (Bukhalo and Solomoko, 1978; Sugimori *et al.*, 1971). Johnson and Lewis (1979) obtained enhanced biomass production in *P. rhodozyma* when yeast extract was used.

The concentration of pigments produced by *P. rhodozyma* was also affected in various ways by the addition of different nitrogen sources. Yeast extract at a concentration of 3 $g \cdot L^{-1}$ gave the highest astaxanthin concentration of 1572 ± 4 $\mu g \cdot g^{-1}$. The color and quantity of pigment produced by the yeast varied with the concentration of the yeast extract. In a similar nutritional study, Johnson and Lewis (1979) found that the addition of increasing concentrations of yeast extract to a vitamin free yeast nitrogen

base medium (YNB) resulted in an increase in the astaxanthin concentration from 156 to 524 $\mu g \cdot g^{-1}$ yeast. They also found that addition of ammonium sulfate, ammonium phosphate and bactopeptone had no effect on pigment production. Carels and Shepherd (1977) observed that addition of yeast extract to a medium increased pigment production and intensity of the red color of *Monascus* spp. in submerged shaken culture. It can therefore be concluded that yeast extract is the most suitable source of nitrogen for yeast growth.

Table 4.4 Influence of various sources nitrogen on the production of biomass astaxanthin by *Phaffia rhodozyma* in peat hydrolysate[1].

Nitrogen Source[2] (0.5 gL^{-1})	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	Astaxanthin Cont. ($\mu g.g^{-1}$ yeast)
None	1.81 ± 0.05^{a}	23.70 ± 0.70^{a}	575 ± 21^{a}
Ammonium Nitrate	1.45 ± 0.13^{a}	19.96 ± 3.33^{a}	nd
Ammonium Phosphate	2.10 ± 0.72^{a}	28.65 ± 11.05^{b}	579 ± 5^{a}
Ammonium Sulfate	1.66 ± 0.23^{a}	22.25 ± 3.65^{a}	nd
Potassium Nitrate	1.54 ± 0.22^{a}	20.63 ± 3.00^{a}	nd
Urea	1.48 ± 0.16^{a}	19.63 ± 1.99^{a}	nd

[1]Mean values of three determinations ± standard deviation. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

[2]The concentrations of these nitrogen sources were calculated so that they provided approximately 0.5 gL^{-1} nitrogen to the medium.

nd Not determined: Cells were not pigmented enough to warrant analysis for astaxanthin.

Table 4.5 Influence of different concentrations of yeast extract on biomass and astaxanthin production by *P. rhodozyma* in peat hydrolysate[1].

Yeast Extract Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
0	1.81 ± 0.05^{c}	23.70 ± 0.70^{d}	575 ± 21^{d}
1	3.20 ± 0.18^{b}	38.04 ± 1.88^{c}	761 ± 12^{c}
2	$3.63 \pm 0.59^{a,b}$	50.67 ± 3.78^{b}	1320 ± 11^{b}
3	4.21 ± 0.26^{a}	60.06 ± 1.63^{a}	1572 ± 4^{a}

[1]Mean values of three determinations ± standard deviations. Values in the same column with same superscripts are not statistically different ($P > 0.05$)

Table 4.6 Changes in the pH of *Phaffia rhodozyma* growth medium associated with various nitrogen sources[1].

Nitrogen Source[2] (0.5 gL^{-1})	Dry Biomass Conc. ($g \cdot L^{-1}$)	Final pH of Medium
None	1.81 ± 0.05^{a}	7.53 ± 0.31^{a}
Ammonium Nitrate	1.45 ± 0.13^{a}	4.21 ± 0.52^{c}
Ammonium Phosphate	2.10 ± 0.72^{a}	6.50 ± 0.61^{a}
Ammonium Sulfate	1.66 ± 0.23^{a}	4.40 ± 1.20^{b}
Potassium Nitrate	1.54 ± 0.22^{a}	6.93 ± 0.23^{a}
Urea	1.48 ± 0.16^{a}	7.13 ± 0.50^{a}

[1]Mean values of three determinations ± standard deviation. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

to have affected the extractability of the astaxanthin, since several acetone extractions could not remove all the pigments in the cells grown in medium supplemented with 0.5 $g \cdot L^{-1}$ potassium phosphate. In a study of *P. rhodozyma* culture conditions in alfalfa residual juice, Okagbue and Lewis (1984a) found that potassium phosphate alone or in combination with other nutrients enhanced cell biomass production, but the pellets were dark green in color, indicating that astaxanthin production was inhibited. These authors made a similar observation when they used phosphate buffer as diluent of the alfalfa residual juice.

Inorganic phosphate has long been reported to promote the growth of microorganisms but to inhibit the biosynthesis of secondary metabolites in these organisms (Demain, 1972; Weinberg, 1974, 1978). Dholakia and Modi (1984), on the other hand, found that, unlike the general phenomenon of phosphate suppression of secondary metabolite formation, β-carotene production in *Blakeslea trispora* was stimulated by higher phosphate concentrations in the growth medium. They conceded, however, that at concentrations higher than 1% ($w.w^{-1}$), phosphate inhibited carotenogenesis by inhibiting cellular metabolism. In *Ermothecium ashbyii*, a higher inorganic phosphate concentration was reported to enhance flavin mononucleotide formation, but decrease riboflavin synthesis (Mehta and Modi, 1982). In a bleached strain of *Euglena*, Blum and Begin-Heick (1967) found a three-fold stimulation of carotenogenesis by low concentrations of phosphate in the growth medium. In *Neurospora crassa*, it has been found that phosphate supplementation leads to the

accumulation of high concentrations of pyrophosphate (Piña *et al.*, 1972) which is a strong inhibitor *in vitro* of phytoene biosynthesis (Dogbo *et al.*, 1988). Inorganic pyrophosphate has also been found to be a strong inhibitor of terpenoids synthesis (Porter and Spurgeon, 1979; Ogura *et al.*, 1969). Thus, in view of the fact that one of the pathways proposed for synthesis of astaxanthin in *P. rhodozyma* is via phytoene (Andrewes *et al.*, 1976a), and also since it has been reported that some controls of carotenoid biosynthesis affect only the early steps in the pathway common to all isoprenoids, whereas others affect only later steps in the synthesis of specific carotenoids and related terpenoids (Johnson and An, 1991), there are reasons to believe that higher concentrations of inorganic phosphate might have increased the level of pyrophosphate in this study and as a result astaxanthin synthesis was adversely affected.

It is well known that phosphate readily forms precipitates with calcium, magnesium, iron, and other metals (Martin, 1977) that are known to occur in peat hydrolysates (Fuchsman, 1980). Therefore, the formation of the dark deposits on the surfaces of the yeast cells when the medium was supplemented with potassium phosphate could be due to the reaction between excess phosphate and other ions in the peat hydrolysate since no such observation was made when the cells were grown in a medium that was not supplemented with phosphate.

Table 4.7: Effects of different concentrations of potassium phosphate on the growth of *P. rhodozyma*[1].

KH_2PO_4 Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
0	1.81 ± 0.05^{b}	23.70 ± 0.70^{c}	575 ± 21^{a}
0.5	2.11 ± 0.10^{a}	25.00 ± 0.97^{c}	214 ± 27^{b}
1.0	2.26 ± 0.23^{a}	30.45 ± 1.69^{b}	nd
1.5	2.41 ± 0.22^{a}	32.56 ± 1.07^{b}	nd
2.0	2.52 ± 0.23^{a}	36.24 ± 1.69^{a}	nd

[1]Mean values of three determinations ± standard deviation. Values in the same column with the same superscripts are not statistically different ($P > 0.05$). nd Not determined: Cell pellets were not sufficiently pigmented to warrant analysis for astaxanthin.

4.1.5.3 Effects of the combination of different concentrations of yeast extract and potassium phosphate on *Phaffia rhodozyma* growth and pigment production

From the nutrient supplementation studies, it is clear that potassium phosphate and yeast extract can enhance biomass production but pigment production was inhibited potassium phosphate. It therefore appeared worthwhile to investigate the effects of combining different concentrations of yeast extract and potassium phosphate on the growth of *P. rhodozyma* in peat hydrolysate, and also to determine whether the yeast extract can counteract the inhibitory effects of phosphate on pigment production. Table 4.8 depicts the results obtained. It is clear from the table that a combination of different concentrations of the two nutrients increase the dry biomass concentration significantly ($P > 0.05$). However, the yeast extract could not counteract the inhibitory effects of the potassium phosphate on pigment production.

Table 4.8: Effects of the combination of different concentrations of yeast extract and potassium phosphate on the growth of *P. rhodozyma*[1]

Yeast Extract Conc. ($g \cdot L^{-1}$)	KH_2PO_4 Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
0	0	1.84 ± 0.10^{h}	575 ± 21^{h}
	0.5	$2.11 \pm 0.10^{g,h}$	214 ± 27^{i}
	1.0	$2.26 \pm 0.23^{g,h}$	nd
	1.5	$2.41 \pm 0.22^{f,g,h}$	nd
	2.0	$2.52 \pm 0.23^{e,f,g,h}$	nd
1.0	0	$3.20 \pm 0.18^{d,e,f,g,h}$	762 ± 12^{e}
	0.5	$3.35 \pm 0.13^{a,b,c,d,e}$	$717 \pm 9^{e,f}$
	1.0	$3.65 \pm 0.18^{b,c,d,e,f}$	$668 \pm 11^{f,g}$
	1.5	$3.90 \pm 0.56^{a,b,c,d}$	$608 \pm 9^{g,h}$
	2.0	$4.10 \pm 0.64^{a,b,c,d}$	578 ± 11^{h}
2.0	0	$3.63 \pm 0.59^{b,c,d,e,f}$	1322 ± 5^{b}
	0.5	$3.85 \pm 0.13^{a,b,c,d,e}$	1280 ± 28^{b}
	1.0	$4.20 \pm 0.51^{a,b,c,d}$	1150 ± 42^{c}
	1.5	$4.90 \pm 0.40^{a,b}$	$1101 \pm 7^{c,d}$
	2.0	$4.95 \pm 0.58^{a,b}$	$1083 \pm 5^{c,d}$
3.0	0	$4.21 \pm 0.26^{a,b,c,d}$	1572 ± 4^{a}
	0.5	$4.50 \pm 0.65^{a,b,c,d}$	1320 ± 16^{b}
	1.0	$4.71 \pm 0.84^{a,b,c}$	$1100 \pm 21^{c,d}$
	1.5	$4.97 \pm 0.60^{a,b}$	$1095 \pm 7^{c,d}$
	2.0	5.10 ± 0.54^{a}	1045 ± 11^{d}

[1]Mean values of three determinations, ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

nd Not determined: Cell pellets were not sufficiently pigmented to warrant analysis for astaxanthin

4.1.5.4 Effects of magnesium and manganese on the growth and pigment production by *Phaffia rhodozyma*

The effects of magnesium and manganese on yeast growth and metabolism have been reviewed (Jones and Greenfield, 1984). Magnesium is essential for the activation of glycolytic enzymes, the stimulation of essential fatty acid synthesis, the regulation of cellular ionic levels, the activation of membrane ATPase and together with K^+, is involved with phosphate uptake. It also has a structural role and is bound to ribosomes, cell membranes and nucleic acids (Jones and Greenfield, 1984). The addition of manganese to the medium on the other hand results in a higher cell nitrogen content, increased protein synthesis, and an increase in cell yield.

The effect of different concentrations of magnesium on the growth of and astaxanthin production by *P. rhodozyma* is shown in Table 4.9. It may be observed that the addition of magnesium sulfate concentrations of 0.8 - 1.4 $g \cdot L^{-1}$ to the medium was inhibitory to the yeast growth but that it has no apparent effects on pigment production. However, above 1.2 $g \cdot L^{-1}$ magnesium sulfate, the pigment production was reduced. There was no significant difference between the values for the biomass concentration obtained with the medium supplemented with the magnesium sulfate and the non-supplemented medium ($P>0.05$). The concentration of Mg^{2+} ions required for the optimal growth of yeast has been reported to be between 2 and 4 mM with a minimum requirement of 1.7 mM. Total inhibition of growth has been reported to occur at approximately 1 M (Jones and Greenfield, 1984). The 0.8 $g \cdot L^{-1}$ magnesium sulfate

provided approximately 3 mM Mg^{2+} to the medium. This concentration was well within the concentration range reported for optimal yeast growth. Peat hydrolysate has been found to contain moderate amounts of ions including Mg^{2+} (LeDuy, 1981). It is therefore possible that Mg^{2+} ion supplied to the medium might have increased the concentration of those already available in the medium to levels that were inhibitory to the growth of the yeast. Addition of manganese sulphate in the concentration range 0.05 to 0.2 $g.L^{-1}$ produced a non-significant difference in the dry biomass concentration and pigment synthesis ($P > 0.05$) (Table 4.10). Jones and Greenfield (1984) reported that between 2 to 4 μM of manganese is needed for the growth and metabolism of yeast, increased nitrogen content of the cells and increased protein synthesis. Growth was progressively inhibited at concentrations of 10 mM and above.

The enzymes catalyzing the synthesis of phytoene from isopentenyldiphosphate, the first C_{40} carotenoid formed during astaxanthin biosynthesis, have been found to have a bifunctional enzyme activity and to be strictly dependent on Mn^{2+} and no other divalent cation (Dogbo *et al.*, 1988). These investigators reported that in *Capsicum* chromoplasts, prephytoene and phytoene were formed by a manomeric protein (47,500 Da) which has a preference for Mn^{2+} over Mg^{2+} and that this preference may contribute to the preferential conversion of geranylgeranylpyrophosphate (GGPP) to carotenoids than other terpenoids. Thus Mn^{2+} ions are vital for biosynthesis of carotenoids and higher concentrations up to 4 mM are not inhibitory. However, the fact that in this study higher concentrations resulted in reduction of pigment synthesis implies that the optimal

concentration might have been exceeded.

Table 4.9: Effects of magnesium sulfate on the growth of and astaxanthin production by *P. rhodozyma*[1]

$MgSO_4$ Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
None	4.90 ± 0.40^a	71.32 ± 3.54^a	1101 ± 7^a
0.8	3.97 ± 0.42^a	61.92 ± 0.42^b	1098 ± 19^a
1.0	3.85 ± 0.25^a	59.97 ± 1.26^b	1100 ± 11^a
1.2	3.68 ± 0.29^a	54.40 ± 1.11^c	1103 ± 8^a
1.4	3.65 ± 0.55^a	53.08 ± 1.90^c	998 ± 26^b

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

Table 4.10: Effects of manganese sulfate on the growth and astaxanthin production by *P. rhodozyma* in peat hydrolysate.

$MnSO_4$ Conc. ($g.L^{-1}$)	Dry Biomass ($g.L^{-1}$)	% Yield	Astaxanthin Content ($\mu g.g^{-1}$ yeast)
None	4.90 ± 0.40^a	71.32 ± 3.54^a	1101 ± 7^a
0.05	4.96 ± 0.41^a	72.55 ± 3.58^a	1110 ± 12^a
0.10	5.05 ± 0.33^a	74.25 ± 0.74^a	1111 ± 10^a
0.15	4.85 ± 0.52^a	64.62 ± 0.64^b	1011 ± 15^b
0.20	4.70 ± 0.52^a	62.62 ± 2.67^b	1001 ± 17^b

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

4.2 Batch fermentation

4.2.1 Optimization of agitation speed and aeration rates in batch fermenters

To study the effects of agitation speed on the growth and pigment synthesis in batch fermenters, three agitation speeds - 200, 250, and 300 r.p.m. - were tested while the supply of oxygen to the medium was kept constant at 1 vvm (volume of air per volume of medium per minute). The results obtained are presented in Fig. 4.7 and the numerical values are shown in Appendix B.1. Of the three agitation speeds tested, 250 r.p.m. was found to be the best for the growth of the yeast. This finding contrasted with the earlier finding in the shaker flask where the optimum growth was obtained at 200 r.p.m. The difference between the two could be due to the volume of medium used in each case since vigorous agitation may be required to effect the necessary mixing and mass transfer in larger volumes than smaller ones. The yeast biomass concentration obtained at the optimal agitation speed, 250 r.p.m. and 200 r.p.m in the batch fermenter were 4.85 ± 0.44 and 4.65 ± 0.56 $g \cdot L^{-1}$, respectively. However, there was no significant differences between these values ($P > 0.05$). The astaxanthin content obtained at 200 and 250 r.p.m. were 1072 ± 26 and 1079 ± 26 $\mu g \cdot g^{-1}$, respectively. Likewise there was no statistical difference between these two values.

The aeration rate was also varied between 0 and 2.5 vvm whilst the agitation speed was kept constant at 250 r.p.m. This was done to produce a range of oxygen dissolution rates and also to determine the effects of aeration on the growth and

pigmentation of *P. rhodozyma*. The results obtained are depicted in Fig. 4.8 and Appendix B.2. At zero aeration rate (0 vvm), the biomass concentration was only 0.87 ± 0.20 $g \cdot L^{-1}$ and the yeast cells were grey with no indication of the presence of astaxanthin. However, when the aeration rate was increased to 0.5 vvm, the dry biomass concentration increased to 4.86 ± 0.49 $g \cdot L^{-1}$ and the astaxanthin content was 1078 ± 14 $\mu g \cdot g^{-1}$ yeast. Above 0.5 vvm, the biomass concentration and astaxanthin content were independent of aeration rate. Johnson and Lewis (1979) made a similar observation when they cultured *P. rhodozyma* in glucose medium. They reported that cell mass and astaxanthin concentration were independent of oxygen dissolution except at the lowest oxygen dissolution rates *i.e.* less than 30 mM oxygen L^{-1} h^{-1}. When *P. rhodozyma* was cultured in a glucose medium with minimal oxygen supply, there was a drastic reduction in the astaxanthin content and the accumulation of β-carotene as well as the monoketone echinenone. Johnson and Lewis (1979) therefore proposed that the hydroxyl functions in astaxanthin are formed only in the presence of molecular oxygen and that carotenes and echinenones are formed under conditions of low aeration. When anaerobically grown stationary phase yeast cells were aerated, there was no detectable change in their astaxanthin content. The precise pathway for the introduction of oxygen into carotenes to yield xanthophylls has not been clearly elucidated. Britton (1976) hypothesized that the hydroxyl functions at C-3 and C-3′ of the carotenoid skeleton arise from the incorporation of molecular oxygen into carotene hydrocarbons. One of the pathways postulated for astaxanthin synthesis in *P. rhodozyma* appears to begin from β-carotene

with different intermediates. Andrewes *et al.* (1976a) postulated that there is a conversion of β-carotene to echinenone which is then hydroxylated to 3-hydroxyechinenone (3-hydroxy-β,β-caroten-4-one). The echinenone is then oxidized to phoenicoxanthin (3-hydroxy-β,β-caroten-4,4′-dione) which is further hydroxylated at the C-3′ to give astaxanthin. Hydroxylation has been reported to occur late in carotenogenesis and involves mixed function oxidase (MFO) reactions (Britton, 1982). Sandmann and Bramley (1985) reported that *in vitro* biosynthesis of β-cryptoxanthin from β-carotene in *Aphanocapsa* membranes may involve a monooxygenase reaction. They found that hydroxylation was dependent on oxygen and sensitive to potassium cyanide (KCN) and monooxygenase inhibitors. Johnson and An (1991) reported that astaxanthin formation in *P. rhodozyma* was inhibited by metyrapone and piperonyl butoxide, compounds that are known to inhibit mixed function oxidase reactions involving cytochrome P450s. Johnson and An (1991) further suggested that factors that affect oxygen radical formation and desaturation would affect carotenogenesis. Beyer *et al.* (1989) also reported that molecular oxygen is essential for carotene desaturation and cyclization in daffodil chloroplasts. They proposed that oxygen acts as an electron acceptor to keep the photoreceptor in the proper oxidation state and that an oxidoreductase served as a redox mediator between phytoene desaturase and oxygen.

The results of these studies enumerated above indicate that astaxanthin formation depends on the presence of molecular oxygen and that the findings in this study agree very well with those reported by the investigators mentioned above. However, the fact

that aeration rates above 0.5 vvm could not improve the biomass concentration and the astaxanthin content any further means that only a minimum concentration of oxygen is required for astaxanthin synthesis. Excess oxygen does not have any extra stimulatory or inhibitory effects.

Some evidence has come to light explaining, at least in part, that excessive oxygen has no harmful effects on the growth of and pigment synthesis by microorganisms and the general consensus is that carotenoid containing microorganisms are able to prevent oxidative damage because of the ability of carotenoids to scavenge singlet oxygen. For example, Kellogg and Fridovich (1975) and Kunert and Tappel (1983) in a series of experimental systems, both *in vitro* and *in vivo*, reported that carotenoids are able to protect plant tissues against oxidative damage caused by reactive oxygen species. Moore *et al.* (1989) reported that in a hyperoxic environment (80% oxygen) or in a culture medium with 100 µM of duraquinone, a redox-cycling quinone known to generate intracellular oxygen molecule, *Rhodotorula mucilaginosa* was not affected by these oxidative challenges unless carotenogenesis was blocked by the addition of 50 µM diphenylamine. They concluded that carotenoids were able to protect this organism against oxidative injury. Other investigators have also reported that carotenoids may also protect non-photosynthetic microorganisms against oxidative damage caused by reactive oxygen species other than singlet oxygen (Mikell *et al.*, 1986; Gillepsie *et al.*, 1986). It can therefore be concluded that in an environment with high concentrations of dissolved oxygen, carotenoids can serve as effective antioxidants, hence the apparent non-inhibitory

effects of high aeration rates observed in this study. Thus, it could be said that once carotenogenesis has begun, the yeast was protected by the initial quantities of carotenoid produced in the cell system.

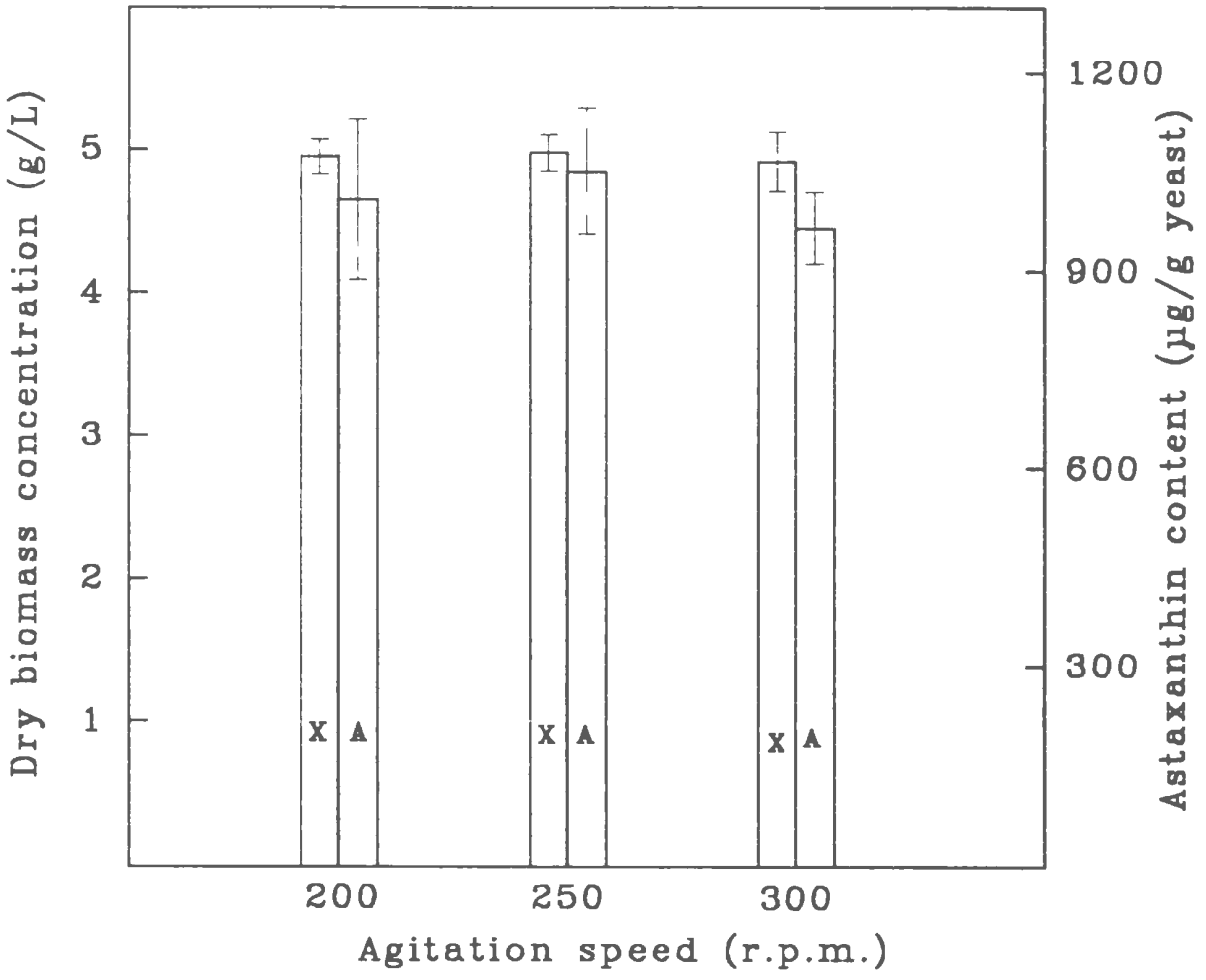

Fig. 4.7. Influence of agitation speed on the growth of and astaxanthin production by *P. rhodozyma* in batch fermentation. X = Dry biomass conc. and A = Astaxanthin content.

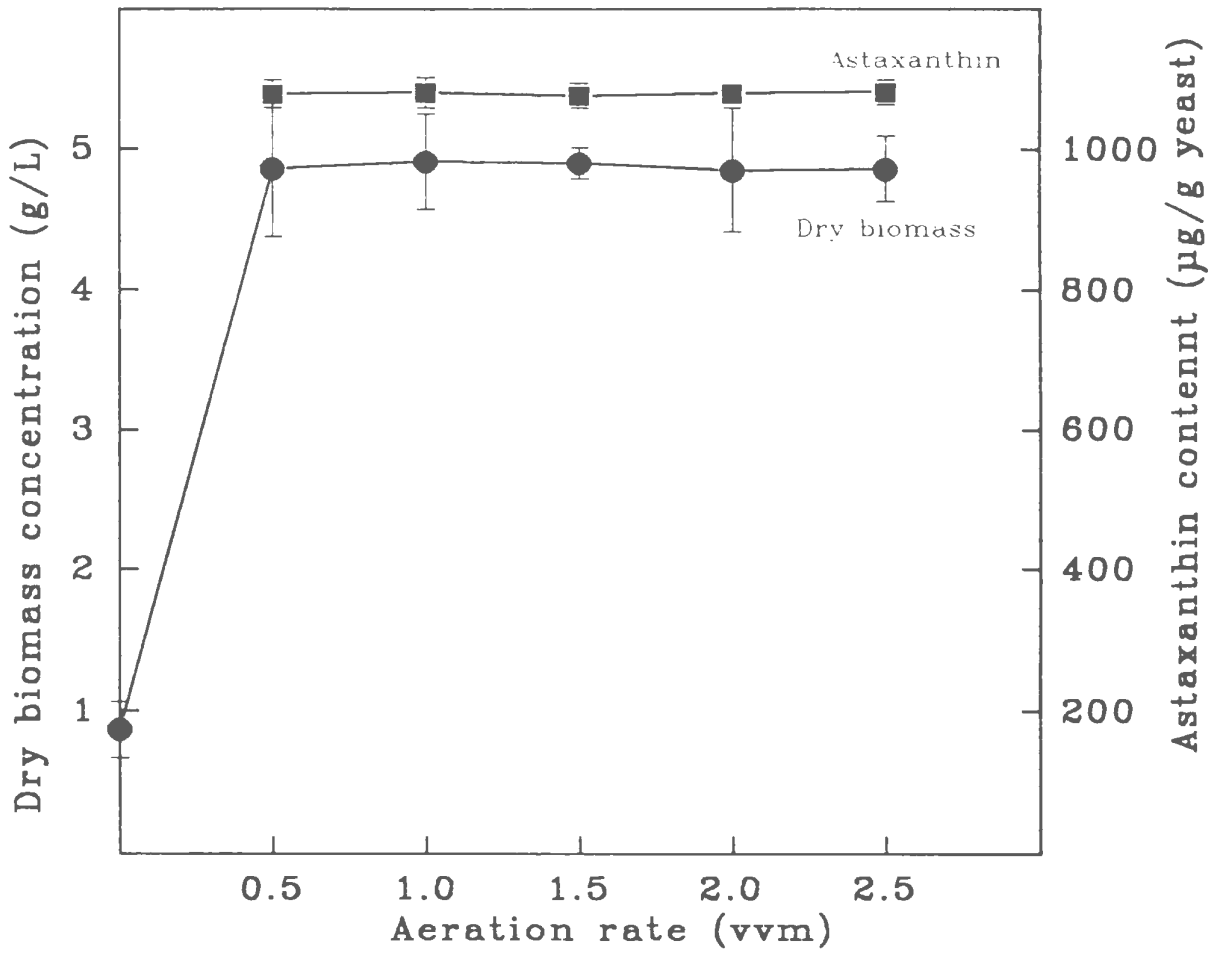

Fig. 4.8 Influence of aeration rate on the growth of and astaxanthin production by *P. rhodozyma*.

4.2.2 Kinetics of the growth of *Phaffia rhodozyma* in peat hydrolysate in batch fermenters

The optimal growth conditions of pH, temperature, agitation speed, aeration rate and inoculum ratio obtained were employed to study the growth characteristics of the yeast in peat hydrolysate. The fermentation was followed over a period of 200 hours and the results are depicted in both Fig. 4.9 and Appendix B.3. It was observed that the growth of the yeast has a number of distinct phases which are described below.

4.2.2.1 The lag phase

It was found out that the growth of *P. rhodozyma* in batch fermenter began after a 10 hour lag period. The lag period is an indication that probably the cells were adjusting to their new environment and synthesizing enzymes required for the new environment. The length of the lag period observed when a medium is inoculated depends on both the changes in nutrient composition experienced by the cells and the age and size of the inoculum. In synthetic glucose medium, *P. rhodozyma* is reported to have a lag period of 10 hours (Johnson and Lewis, 1979). There is not much information available on the growth kinetics of *P. rhodozyma*. However, the fact that only a short lag period was recorded in this work implies that there was proper cultivation and transfer of inoculum which are necessary prerequisites for a successful production of both primary and secondary metabolites by microorganisms.

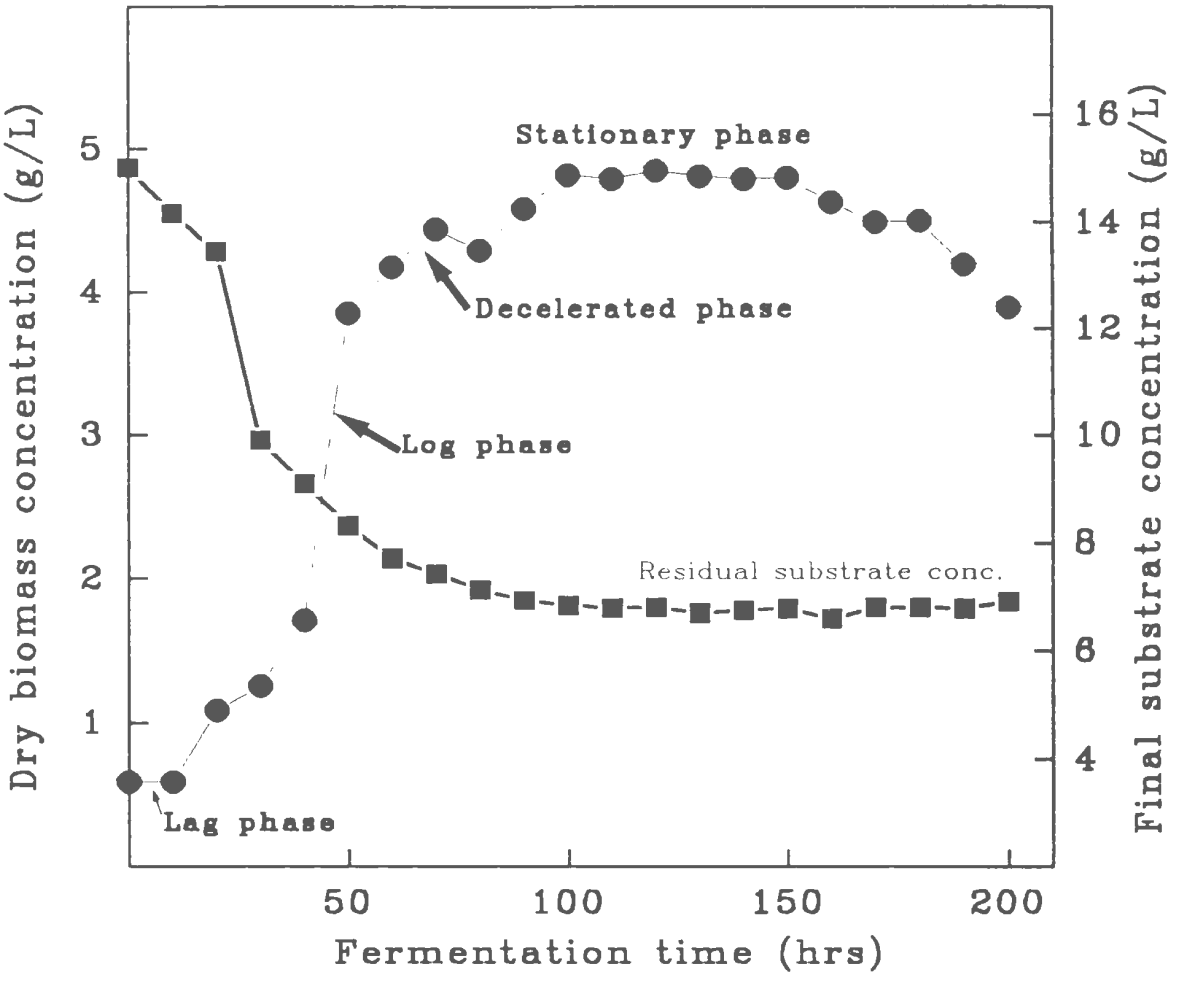

Fig. 4.9 The growth pattern of *P. rhodozyma* in peat hydrolysate.

4.2.2.2 The log (accelerated growth) phase

By the end of the lag phase, the cells have adapted to the new conditions of growth. It was observed that the growth was faster during the log phase which began after 30 hours of inoculation. The highest biomass obtained in the batch culture, 4.86 ± 0.12 $g \cdot L^{-1}$ was reached at the 120th hour. In the shaker flask, the highest biomass was 4.90 ± 0.40 $g \cdot L^{-1}$ and this was also obtained after 120 hours of fermentation. There were however no significant differences between these two values ($P > 0.05$). The nutrients were utilized rapidly during this time. In spite of the fact that there were uptake of nutrients and excretion of metabolic products into the medium, the specific growth rate, μ (*i.e.* the amount of cells produced by a unit amount of cells in a unit time), remained constant during the log phase. The growth rate also remains independent of the substrate concentration as long as excess substrate is present (Crueger and Crueger, 1989). With an excess of all growth-dependent substrates (and in the absence of inhibitors), there was a rapid increase in the biomass concentration. The rate of increase in biomass during the log phase can therefore be correlated with the specific growth rate, μ, and the biomass concentration X ($g \cdot L^{-1}$) by the equation:

$$\frac{dX}{dt} = \mu X \ldots\ldots (a)$$

Upon integration, equation [a] becomes

$$In \frac{X}{X_0} = \mu t \ldots\ldots (b)$$

where X is the biomass concentration ($g.L^{-1}$)

μ is the specific growth rate (h^{-1})

t any time after the lag phase (h)

μ can be more commonly expressed in terms of:

$$\mu = 2.303 \frac{logX_t - logX_0}{t} = \frac{0.693}{t_d}$$

where X_t is the cell biomass concentration at time t

X_0 is the cell biomass concentration at time 0

t_d is the doubling time

A plot of the log biomass concentration against time yields a straight line for the log phase with the slope as the specific growth rate, μ, (*i.e.* $0.693/t_d$). A plot of log biomass concentration against time yields a straight line with the slope as the maximum specific growth rate, μ_{max}. Such a plot is shown in Fig. 4.10a. The relationship between μ, t_d, dry biomass concentration and time is shown in Fig. 4.10b.

From the Fig. 4.10a, it can be inferred that the log phase occurred between 40 and 50 hours after inoculation. A maximum specific growth rate, μ_{max} and generation time (doubling time) of $0.038h^{-1}$ and 18.24 hours respectively were obtained. Compared to the μ_{max} of *P. rhodozyma* grown in glucose media (*i.e.* 0.21 h^{-1}, Johnson and Lewis, 1979),

our culture seems inferior. But when the biomass concentration and the astaxanthin content obtained in this study are compared with those obtained with other substrates (An *et al.*, 1989; Johnson and Lewis, 1979; Haard, 1988; Okagbue and Lewis, 1983a) our culture performed very well. Furthermore, the μ_{max} is dependent on the organism and the conditions of the fermentation. Since an organism needs extra energy to split long chain substrates, the μ_{max} for simple substrates always supercedes that of long chain complex molecules (Crueger and Crueger, 1989). Therefore, it is not surprising that the μ_{max} of *P. rhodozyma* grown in comparatively simpler media is greater than that obtained in this study.

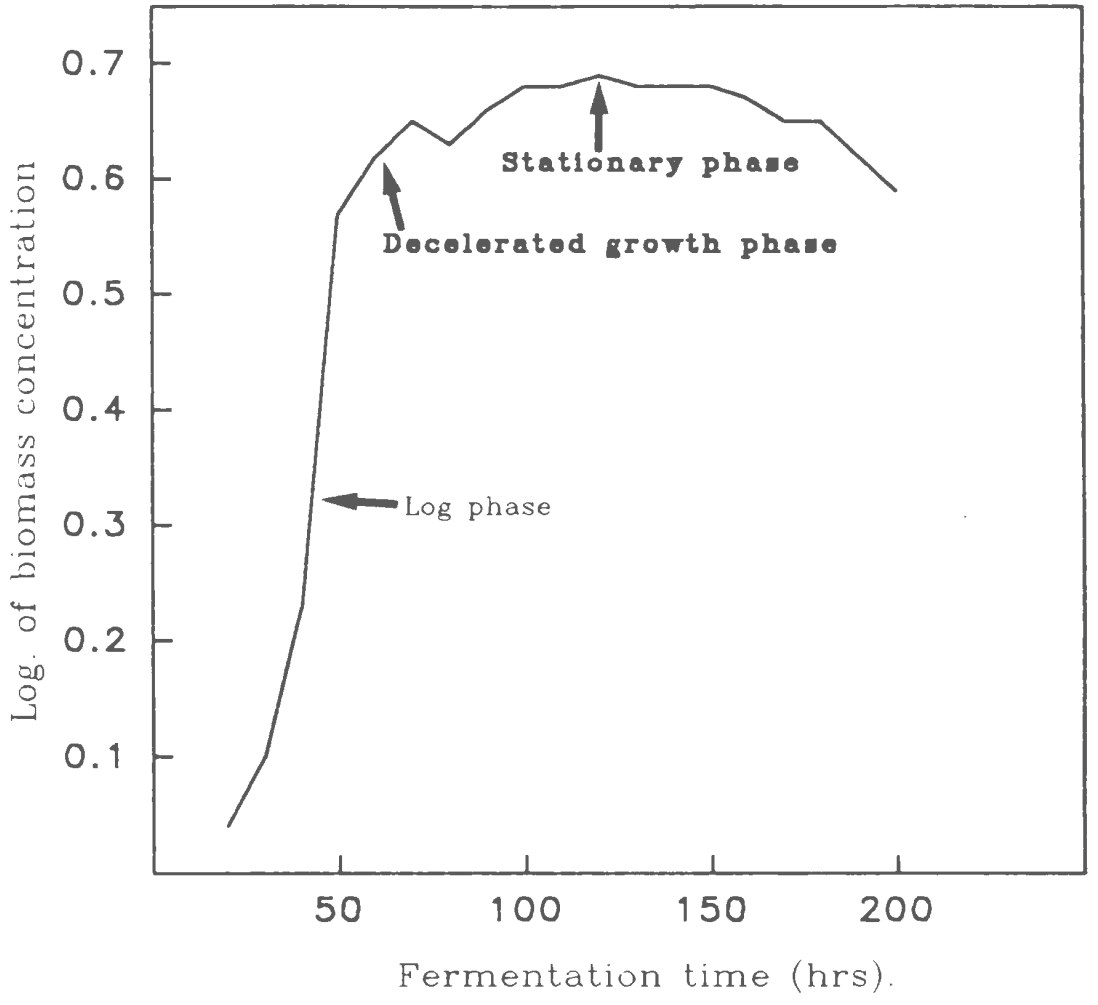

Fig. 4.10a The growth curve of *P. rhodozyma* in peat hydrolysate.

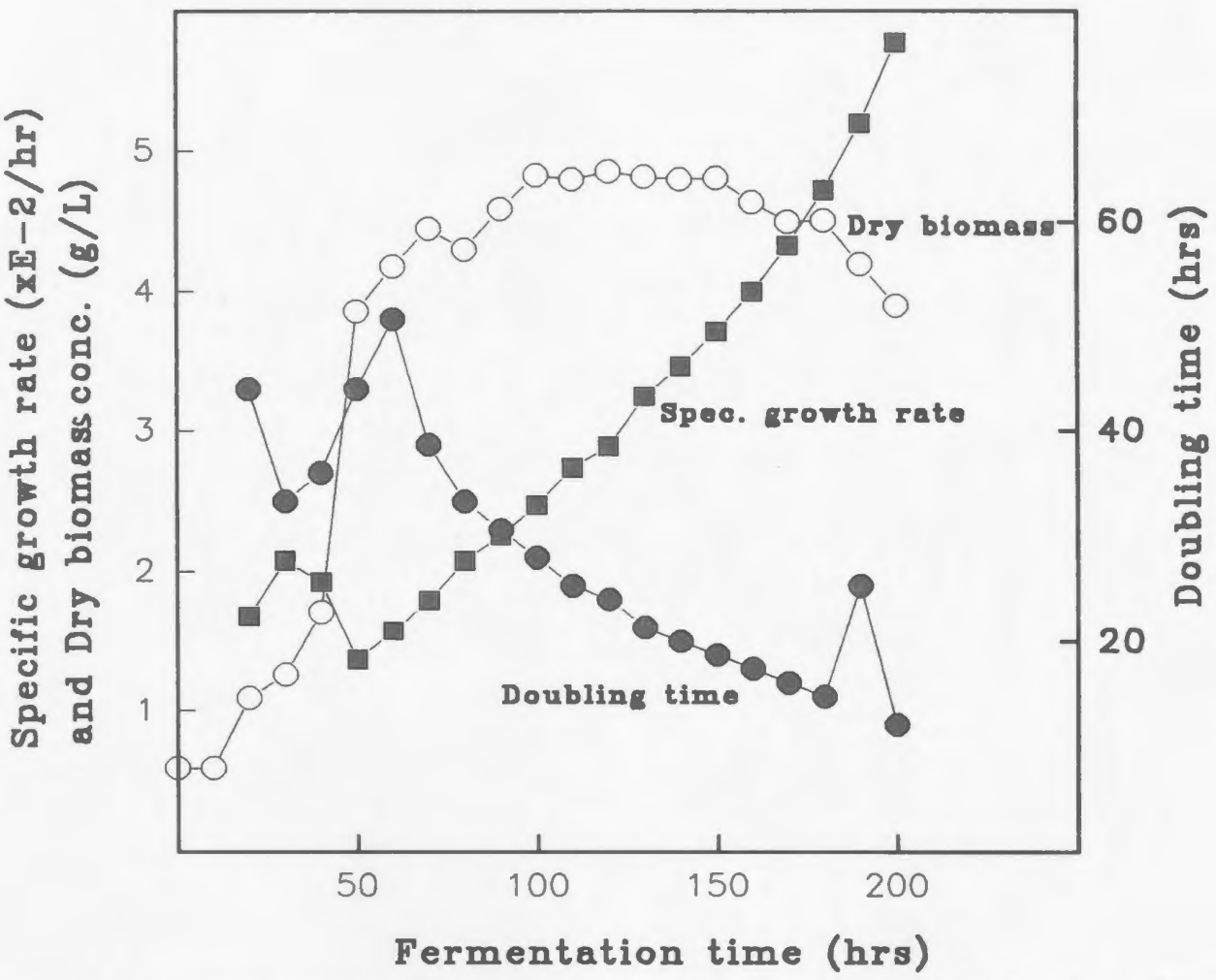

Fig. 4.10b Changes in the growth parameters of *P. rhodozyma* as a function of time.

4.2.2.3 Decline in the growth rate

The decline in growth rate began after the accelerated growth rate had stopped at the 50th hour. This phase in the growth of the yeast was associated with a constant value in the residual total carbohydrate content. Since not all the carbohydrate was utilizable, it is possible that the organisms were deprived of valuable nutrients at this stage. In any chemical reaction, growth rate depends on the concentration of chemical nutrients (concentration of limiting substrates), S, the maximum specific growth rate, μ_{max}, and a substrate-specific constant, K_s (Monod, 1949). In a fresh medium containing glucose as the sole carbon and energy source, and with all other nutrients in excess, the specific growth rate remains constant throughout the log phase; and during the deceleration phase where the substrate concentration becomes non-saturating, tends to be zero (Monod, 1949). The relationship between the specific growth rate, μ, and the substrate concentration, S, is a hyperbolic, saturation curve similar to that describing Michaelis-Menten-type equation (Monod, 1949). The growth rate therefore becomes a function of the substrate concentration (Monod, 1949) and is represented by the equation:

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

where: μ is the specific growth rate (h^{-1})

S is the substrate concentration ($g.L^{-1}$)

K_s is the substrate concentration at which half the maximum specific growth rate

was obtained ($\mu = 0.5\ \mu_{max}$)

By taking reciprocals, the Monod equation can yield the linear expression:

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} \frac{1}{S} = \frac{1}{\mu_{max}}$$

A plot of 1/u against 1/S yields a straight line with a slope K_s/μ_{max}, intercept on the abscissa of $-1/K_s$ and on the ordinate μ_{max}. K_s values are generally reported to be very low, and consequently difficult to determine using normal batch culture fermentation during the log phase. Because of the large biomass which is present by the end of the log phase , the substrate is exhausted quickly so that the period of time during which the substrate concentration is near that of the K_s is very short and the stationary phase is reached abruptly (Crueger and Crueger, 1989). Unfortunately no systematic work has been done on the determination of K_s values for *P. rhodozyma* in different carbon sources so there are no data available for comparison.

4.2.2.4 The stationary phase

A constant dry biomass was reached after 110 hours of fermentation and this continued till the 150th hour. This period is considered as the stationary phase. Johnson and Lewis (1979) found that in glucose medium, a constant dry weight of *P. rhodozyma* was achieved after 80 hours of fermentation. The stationary phase was characterized by no net growth and growth might have been occurring but was balanced by the rate of cell

death or lysis. The cause of the stationary phase can be attributed to several factors and these include the depletion of an essential nutrient or build up toxic materials. From Fig. 4.9, it can be inferred that the stationary phase began when the residual substrate concentration became constant. Even though not all of the substrate was utilized, it is possible that those remaining were not utilizable. It can therefore be concluded that the depletion of the utilizable nutrients was a probable cause of the stationary phase in this study.

4.3 Synthesis of carotenoids by *Phaffia rhodozyma* in fermenter batch culture

In the fermenter batch culture, the determination of the astaxanthin content of *P. rhodozyma* was started only at the 40th hour of fermentation because there were not enough samples for analysis during the early hours of growth. However, visual inspection of the cells shows the distinctive red coloration of *P. rhodozyma* indicating that synthesis of astaxanthin in *P. rhodozyma* began early during the growth period. The concentration of astaxanthin in the yeast increased from 220 ± 13 $\mu g \cdot g^{-1}$ yeast in the early stages of growth to about 952 ± 28 $\mu g \cdot g^{-1}$ yeast during the acceleration growth phase (40th to 50th hour). The astaxanthin content then increased gradually to 1086 ± 15 $\mu g \cdot g^{-1}$ during the next 50 hours (Fig. 4.11 and Appendix B.5), and then remained steady till the 200th hour.

It can be inferred from these results that astaxanthin synthesis in this study was growth associated. This viewpoint was echoed by Johnson and Lewis (1979) who asserted that astaxanthin production in *P. rhodozyma* is growth associated but its formation does not exactly coincide with increase in biomass. It also continues to be synthesised after growth has stopped (Johnson and An, 1991) and, on the exhaustion of glucose, the concentration of xanthophylls in *P. rhodozyma* continued to increase (Johnson and Lewis, 1979). It is therefore likely that *P. rhodozyma* excretes a carbon intermediate during growth, which is later reassimilated and stimulates carotenogenesis (Johnson and An, 1991). In contrast, carotenoid production in *Sporobolomyces roseus* (Bobkova, 1965) and *Rhodotorula glutinis* (Vecher and Kulikova, 1968), has been reported to occur only after the yeast had stopped growing. Similarly, Goodwin (1972, 1959) found that

carotenoid biosynthesis in *Rhodotorula rubra* occurred mainly after growth had stopped; suggesting that nutrient depletion or physiological changes triggered carotenogenesis in the yeast (Johnson and An, 1991). It had been found in *Rhodotorula rubra* that carotenogenesis occurs in three main phases: a period of active synthesis leading to maximal concentration, a period of persistence during which the concentration stays relatively constant, and a period during which the pigments gradually disappear from the yeast (Goodwin, 1959). A similar observation was made in leaves of trees, where the onset of carotenogenesis becomes rapid with the appearance of first leaves, a climax, and then gradual senescence in the autumn (Goodwin, 1959). In fungi grown in surface cultures, carotenogenesis does not occur until growth has stopped, and carotenoid formation is stimulated by excess carbohydrate and limiting nitrogen (Goodwin, 1959). From the results of this study and the results reported by other investigators, it appears that *P. rhodozyma* is different from other carotenogenic fungi in that massive carotenoid formation occurs during the entire growth period and after growth has stopped whereas the others start production only after growth has stopped.

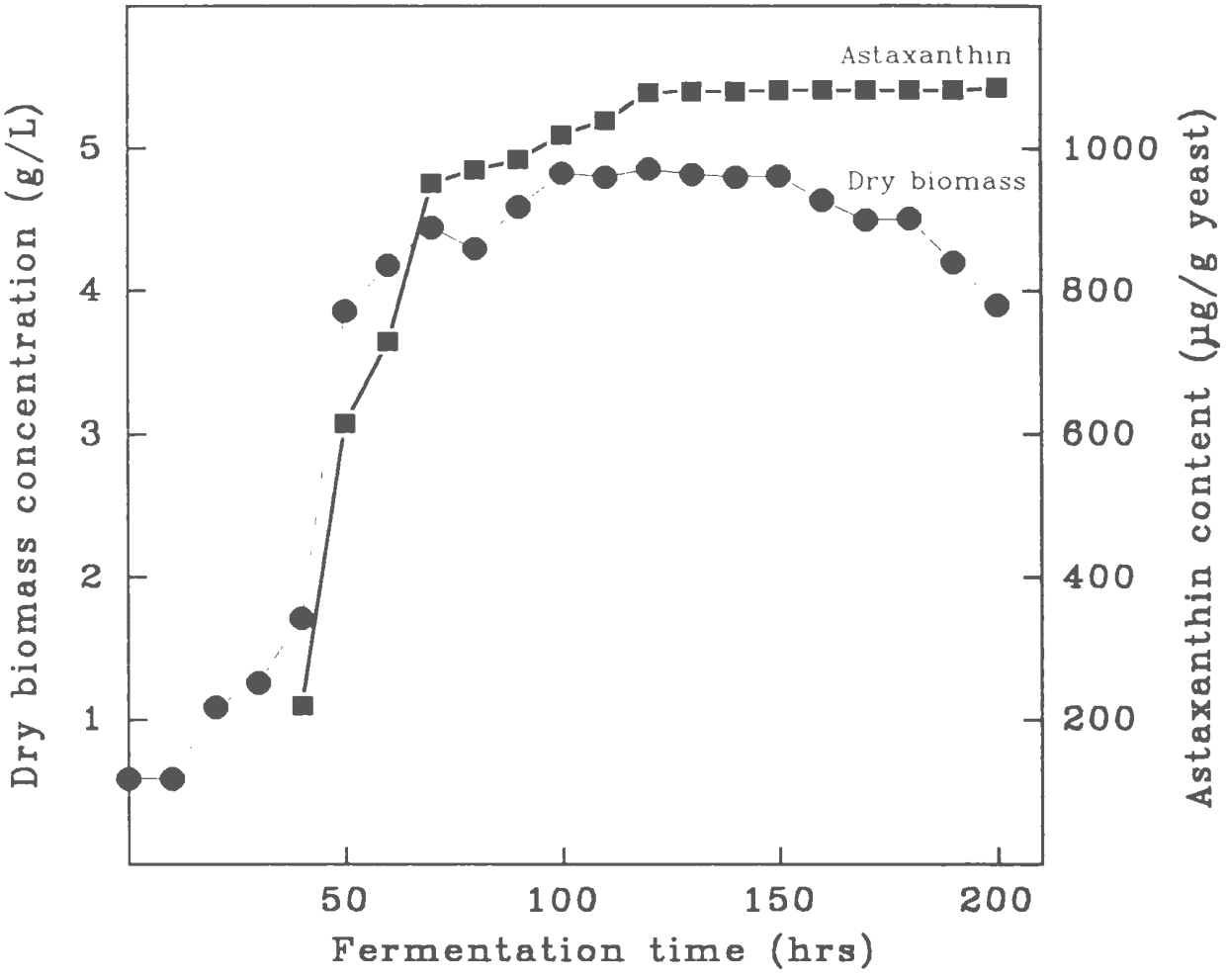

Fig. 4.11 Pattern of carotenoid production by *P. rhodozyma* in peat hydrolysate.

as a result of leaching of cellular materials during washing.

The total carbohydrate content (TCH) was also low (28.90% ± 4.25) when compared with that reported for *P. rhodozyma* by Johnson *et al.* (1980) (40.30%) and Fleichsmann's commercial dry yeast (36.50%). The crude protein content, 47.10 ± 0.94%, was greater than that found for *P. rhodozyma* grown in glucose and Fleichsmann commercial yeast (Table 4.11). The high protein content was however within the maximum range of 45.50-58.00% protein generally reported for various fodder yeast cultivated in different peat hydrolysates (LeDuy, 1981b). Thus it can be concluded that peat hydrolysate has a beneficial effect on protein synthesis in yeasts.

The RNA content of the yeast was found to be 5.20 ± 0.69%. In comparison with the RNA content reported for *P. rhodozyma* by Johnson *et al.* (1980), 8.20%, *Saccharomyces cerevisiae*, 9.20%, (Johnson *et al.*, 1980), and bacteria, 8-16% (Kihlberg, 1972), the nucleic acid content of the yeast in this study was low. This is very important because of the adverse effects of high concentrations of nucleic acid on the health of animals, especially mammals. Nucleic acids in the diet are depolymerized by nucleases in the pancreatic juice and then converted to nucleosides by intestinal enzymes before absorption. The purine bases guanine and adenine are metabolized to uric acid which should be oxidized to allantoin before it can be removed from the body. Most mammals however, do not possess the enzyme uricase that oxidizes uric acid. Increased consumption of nucleic acids therefore can lead to formation of stones in the kidney and the bladder and can also cause precipitation of ureate in the tissues and joints. In contrast

to mammals, fishes and other animals appear to be free from this problem since they have this enzyme and thus could metabolise the uric acid to allantoin which is excretable.

The total lipid content of the yeast in this study was 18.00 ± 5.09%. This value was higher than that reported for Brewer's yeast, 4.03% (Johnson *et al.*, 1980), but comparable to that of *P. rhodozyma* grown in other medium (Johnson et al., 1980).

Table 4.11: The proximate composition of *P. rhodozyma* biomass in comparison with that of other microorganisms (% of total dry weight).

Component	*Phaffia rhodozyma*[1]	*Phaffia rhodozyma*[2]	Fleischmann's dry[3] active yeast
Ash	4.41 ± 0.14	5.60	4.90
Total lipid	18.00 ± 5.09	17.00	0.40
Total carbohydrate	28.90 ± 4.25	40.30	36.50
Protein (N × 6.25)	47.10 ± 0.94	30.10	41.40
RNA	5.20 ± 0.69	8.20	-

[1]This work
[2]Johnson *et al.* (1980)
[3]LeDuy (1981a)

4.4.2 Fatty acid composition of *Phaffia rhodozyma* cell biomass

The composition of the fatty acids of *P. rhodozyma* produced in this study and that of other yeasts are depicted in Table 4.12. *P. rhodozyma* has been reported to have a much higher lipid content than most other yeasts (Hunter and Rose, 1971). The content of the unsaturated fatty acid of the yeast in this study was found to be about 77.05%. This value compares very well with that reported by Johnson *et al.* (1980), but higher than that of *Candida utilis* and Fleichsmann commercial yeast (Table 4.12). The yeast biomass contained three saturated fatty acids (myristic, 3.9%; palmitic, 15.4%; and stearic, acid 4.01%). The pattern of the fatty acid composition of the yeast found in this study was similar to that of *P. rhodozyma* reported by Johnson *et al.* (1980), Fleichsmann's commercial yeast and *Candida utilis* (LeDuy, 1981a). The most important unsaturated fatty acids of the *P. rhodozyma* cells analyzed in this study were oleic (42.10%), linoleic (31.30%) and linolenic (2.65%) which were similar to that reported for *P. rhodozyma* by Johnson *et al.* (1980). In the case of the Fleichsmann's commercial yeast, however, the major unsaturated fatty acids were oleic (45.60%) and palmitoleic (31.0%).

Fish almost lack the ability of *de novo* synthesis of linoleic and linolenic acids and may require exogenous acids as essential fatty acids (Kanazawa *et al.*, 1977). Yu and Sinnhuber (1972) also reported that linolenic acid is important in fish nutrition. *P. rhodozyma* grown in peat hydrolysate can therefore not only provide astaxanthin for the salmonid industry, but can also be a valuable source of linoleic acid. The low content of the linolenic acid however means that the yeast will be a poor source of this fatty acid,

but since the amount of fat in the yeast is so great, the content of the linolenic acid may be of nutritional significance (Johnson *et al.* 1980).

4.4.3 Amino acid composition of the *Phaffia rhodozyma*

The amino acid composition of protein primarily determines its value as a source of nitrogen for growth and maintenance. Valuable information about the potential nutritional value is therefore obtained from an analysis of the amino acid content of a protein. The amino acid spectrum of the *P. rhodozyma* is compared with those of other sources in Table 4.13 and 4.14. It is clear from the table that *P. rhodozyma* in this study has a well-balanced essential amino acid content. The amino acid spectrum is similar to that of *P. rhodozyma* reported by Johnson *et al.* (1980) (Table 4.13), eggs and the Food and Agricultural Organization (FAO) standard (Table 4.14). The amino acid content of most microorganisms is reported to compare very well with the Food and Agricultural Organisation reference protein (FAO, 1973) except that the content of sulfur amino acids is low (Johnson *et al.*, 1980). The *P. rhodozyma* in this study appears to be an exception to this rule in that it has a reasonable amount of the sulfur amino acid methionine. Among the essential amino acids, leucine (41.08 $mg.g^{-1}$), valine (32.32 $mg.g^{-1}$), lysine (58.18 $mg.g^{-1}$), phenylalanine (23.79 $mg.g^{-1}$), threonine (25.71 $mg.g^{-1}$), and isoleucine (26.55 $mg.g^{-1}$) all have favorable concentrations when compared with other sources reported in Tables 4.13 and 4.14. The well-balanced amino acid pattern in the yeast is an indication that it will be a good source of protein for aquaculture.

Table 4.12: The fatty acid spectrum of *Phaffia rhodozyma* cultured in peat hydrolysate (% of total methyl esters).

Name	*Phaffia*[1] *rhodozyma*	*Phaffia*[2] *rhodozyma*	Fleischmann's[3] active dry yeast	*Candida utilis*[3]
Myristic (14:0)	3.90	0.24	0.80	0.60
Palmitic (16:0)	15.40	16.05	3.70	16.30
Palmitoleic (16:1)	1.00	0.45	31.00	4.60
Stearic (18:0)	4.10	6.11	6.50	8.30
Oleic (18:1)	42.10	41.30	45.60	22.20
Linoleic (18:2)	31.10	32.80	1.10	42.20
Linolenic (18:3)	2.65	3.00	0.30	3.60

[1]This work
[2]Johnson *et al.* (1980)
[3]LeDuy (1981a)

Table 4.13 The pattern of amino acid in *P. rhodozyma* grown in peat hydrolysate compared with that of *P. rhodozyma* grown in glucose medium ($mg \cdot g^{-1}$ protein)

Amino acid	Peat Hydrolysate[1]	Glucose Medium[2]
Alanine	38.26±1.70	18.30
Arginine	36.67±1.22	13.90
Aspartic	51.30±1.41	23.00
Glycine	31.18±1.14	12.90
Glutamic	62.81±1.89	26.50
Histidine	14.14±0.42	6.50
Isoleucine	26.55±0.88	9.40
Leucine	41.08±1.23	23.80
Lysine	58.18±1.42	21.40
Methionine	10.02±0.43	3.90
Phenylalanine	23.79±0.74	11.00
Proline	21.71±0.61	9.90
Serine	22.00±0.37	13.60
Threonine	25.71±0.57	11.60
Tyrosine	16.33±0.78	7.90
Valine	32.32±1.16	12.40

[1]This work
[2]Johnson *et al.* (1980)

Table 4.14: The pattern of amino acid in *P. rhodozyma* grown in peat hydrolysate compared with that of eggs and FAO standard ($g \cdot g^{-1}$ protein)

Amino acid	*Phaffia rhodozyma*[1]	FAO Standard[2]	Whole egg[3]
Alanine	3.83	-	-
Arginine	3.70	-	6.10
Aspartic	5.10	-	-
Glycine	3.10	-	-
Glutamic	6.30	-	-
Histidine	1.40	-	2.40
Isoleucine	2.70	4.20	6.30
Leucine	4.10	4.80	8.90
Lysine	5.80	4.20	7.00
Methionine	1.00	2.20	3.40
Phenylalanine	2.40	2.80	5.70
Proline	2.20	-	-
Serine	2.20	-	-
Threonine	2.60	2.80	5.10
Tyrosine	1.60	-	-
Valine	3.20	4.20	7.30

[1]This work
[2]Food and Agricultural Organisation (FAO, 1973)
[3]Khanna and Garicha (1986)

CONCLUSION

It can be concluded that peat hydrolysate has the potential for use as an inexpensive substrate for the submerged production of *P. rhodozyma*. Higher dry biomass and astaxanthin concentrations can be expected from *P. rhodozyma* grown in the acid and non-acid liquid extracts of peat supplemented with only nitrogen, phosphorous and potassium.

Of the six peat hydrolysates employed in this study, the acid hydrolysate PH4-02-185 supported the best biomass and pigment production by the yeast. The concentrations of biomass and astaxanthin obtained in this work are comparable with those reported by other researchers.

The optimal growth parameters obtained in this work are : pH 7, incubation temperature of 18°C, agitation speed of 250 r.p.m. (200 r.p.m. for shaker flask), an aeration rate of 0.5 vvm, inoculum ratio of 5% ($v \cdot v^{-1}$), and a fermentation time of 120 hours. The maximum specific growth rate and the generation time of the yeast in peat hydrolysate were 0.038 h^{-1} and 18.24 h, respectively. The biosynthesis of carotenoids by *P. rhodozyma* was found to occur during the entire growth period and after growth has stopped.

Peat hydrolysate with a TCH of 15 $g \cdot L^{-1}$ was found to support the best growth.

The addition of yeast extract to the peat hydrolysate resulted in significant increases in the biomass and astaxanthin concentrations. Supplementation of the medium with potassium phosphate alone and in combination with yeast extract also resulted in the production of high microbial biomass concentration but astaxanthin production was significantly reduced.

The *P. rhodozyma* cell biomass had a high protein content, a rich amino acid composition, and high lipid content with a larger percentage being unsaturated fatty acids. Thus in addition to being used as a source of carotenoids, *P. rhodozyma* grown in peat hydrolysates can also be a potential source of protein for salmonid aquaculture.

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APPENDIX A: SHAKER FLASK EXPERIMENTS

A.1: Effects of substrate concentration on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185.[1]

Initial Substrate Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
15	$1.34 \pm 0.09^{b,c}$	17.57 ± 2.36^{c}	$7.57 \pm 1.60^{a,b}$
20	1.80 ± 0.07^{c}	$22.59 \pm 3.09^{a,b,c}$	9.00 ± 0.36^{b}
25	2.35 ± 0.05^{b}	24.40 ± 1.01^{b}	9.40 ± 0.10^{a}
30	2.90 ± 0.09^{a}	27.52 ± 0.21^{a}	$9.66 \pm 0.30^{a,b}$

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.2: Effects of substrate concentration on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185[1]

Initial Substrate Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
15	2.67 ± 0.10^{c}	33.41 ± 0.45^{a}	17.80 ± 0.40^{a}
30	$4.19 \pm 0.10^{b,c}$	31.52 ± 0.50^{b}	13.97 ± 0.27^{b}
45	$5.06 \pm 0.18^{a,b}$	21.96 ± 1.34^{c}	11.24 ± 0.40^{c}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.3: Effects of initial pH on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185 [1]

Initial pH	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
4	nd	nd	nd
5	$2.91 \pm 0.15^{a,b}$	27.59 ± 2.23^{a}	$9.69 \pm 0.51^{a,b}$
6	$2.93 \pm 0.19^{a,b}$	28.05 ± 3.70^{a}	$9.76 \pm 0.62^{a,b}$
7	3.13 ± 0.07^{a}	30.48 ± 0.58^{a}	10.43 ± 0.24^{a}
8	2.74 ± 0.09^{b}	26.03 ± 2.31^{a}	9.13 ± 0.31^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different (P>0.05).

nd Not determined: There was no growth at this pH.

A.4: Effect of initial pH on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185[1].

Initial pH	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
4	2.05 ± 0.04^{c}	24.02 ± 2.84^{a}	13.64 ± 0.20^{c}
5	2.65 ± 0.28^{a}	34.29 ± 7.43^{a}	$17.68 \pm 1.87^{a,b,c}$
6	2.65 ± 0.00^{a}	34.63 ± 1.46^{a}	17.64 ± 0.03^{a}
7	2.72 ± 0.09^{a}	36.29 ± 5.18^{a}	18.14 ± 0.62^{a}
8	2.35 ± 0.06^{b}	29.35 ± 1.62^{a}	15.55 ± 0.46^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.5: Effect of incubation temperature on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185[1].

Incubation Temp. (°C)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
16	2.64 ± 0.08^{b}	24.43 ± 0.48^{b}	8.80 ± 0.28^{b}
18	3.46 ± 0.16^{a}	31.90 ± 1.82^{a}	11.31 ± 0.69^{a}
20	$3.10 \pm 0.09^{a,b}$	29.17 ± 0.32^{a}	10.44 ± 0.32^{a}
22	2.89 ± 0.01^{b}	28.42 ± 0.16^{a}	9.62 ± 0.03^{b}
24	2.15 ± 0.48^{b}	22.30 ± 3.93^{b}	7.50 ± 0.16^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.6: Effect of incubation temperature on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185[1].

Incubation Temp. (°C)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
16	2.75 ± 0.21^{a}	44.80 ± 3.08^{a}	18.33 ± 1.41^{a}
18	3.06 ± 0.10^{a}	50.28 ± 3.14^{a}	20.40 ± 0.68^{a}
20	2.99 ± 0.14^{a}	45.10 ± 6.97^{a}	19.91 ± 0.96^{a}
22	2.72 ± 0.09^{a}	36.29 ± 5.18^{b}	18.14 ± 0.62^{b}
24	2.63 ± 0.09^{b}	31.60 ± 2.33^{b}	17.57 ± 0.59^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.7 Effect of fermentation time on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185[1].

Fermentation Time (Days)	Final Substrate Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
2	23.31 ± 0.89^{a}	1.52 ± 0.22^{a}	22.63 ± 0.45^{a}	5.07 ± 0.74^{a}
3	21.81 ± 0.96^{a}	$2.03 \pm 0.35^{a,c}$	24.60 ± 1.36^{a}	$6.74 \pm 1.16^{a,b}$
4	18.37 ± 0.38^{b}	$2.36 \pm 0.12^{b,c}$	$27.20 \pm 2.66^{a,b}$	7.87 ± 0.40^{b}
5	18.37 ± 0.48^{b}	3.59 ± 0.24^{d}	30.85 ± 1.21^{b}	11.97 ± 0.79^{c}
6	17.91 ± 0.07^{b}	$2.68 \pm 0.16^{b,c}$	22.18 ± 1.43^{a}	8.94 ± 0.53^{b}
7	17.47 ± 0.37^{b}	$2.64 \pm 0.13^{b,c}$	21.06 ± 0.75^{a}	8.78 ± 0.45^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.8: Effect of fermentation time on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185[1].

Fermentation Time (Days)	Final Substrate Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
2	9.87 ± 0.18^{b}	1.57 ± 0.03^{d}	30.57 ± 1.65^{b}	10.44 ± 0.23^{d}
3	8.75 ± 0.67^{b}	2.06 ± 0.10^{c}	$33.31 \pm 3.49^{b,c}$	13.73 ± 0.65^{c}
4	$7.97 \pm 0.47^{a,b}$	2.48 ± 0.13^{b}	35.78 ± 1.05^{c}	16.51 ± 0.86^{b}
5	7.88 ± 0.08^{a}	3.37 ± 0.07^{a}	47.39 ± 0.39^{a}	22.49 ± 0.44^{a}
6	6.97 ± 0.39^{a}	2.61 ± 0.16^{b}	32.48 ± 0.63^{d}	17.40 ± 1.04^{b}
7	6.60 ± 0.53^{a}	2.43 ± 0.05^{b}	29.12 ± 2.45^{d}	16.22 ± 0.32^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.9 Effect of inoculum ratio on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185[1].

Inoculum ratio (%)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
1	2.66 ± 0.25^{a}	19.69 ± 2.53^{c}	8.96 ± 0.96^{b}
3	3.56 ± 0.45^{a}	30.20 ± 1.85^{a}	$11.86 \pm 1.50^{a,b}$
5	3.79 ± 0.60^{a}	32.08 ± 0.39^{a}	12.63 ± 2.01^{a}
7	3.47 ± 0.45^{a}	24.81 ± 0.33^{b}	$11.56 \pm 1.51^{a,b}$
9	2.93 ± 0.35^{a}	$23.42 \pm 0.1)^{b,c}$	$9.57 \pm 1.19^{a,b}$

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.10 Effect of inoculum ratio on the growth of *P. rhodozyma* in peat hydrolysate PH4-02-185[1].

Inoculum ratio (%)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
1	3.36 ± 0.10	46.66 ± 0.77	22.38 ± 0.34
3	3.61 ± 0.20	49.40 ± 2.63	23.90 ± 1.33
5	3.97 ± 0.40	52.58 ± 3.12	26.48 ± 2.65
7	3.57 ± 0.30	47.95 ± 2.52	23.78 ± 2.01
9	3.48 ± 0.10	47.10 ± 0.38	23.18 ± 0.27

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.11: Effect of agitation speed on the growth of *P. rhodozyma* in peat hydrolysate PH4-01-185 in shaker flask[1].

Agitation speed (r.p.m.)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
150	2.73 ± 0.38	28.18 ± 1.01	9.11 ± 1.26
200	4.04 ± 0.14	34.82 ± 1.20	13.48 ± 0.47
250	3.61 ± 0.10	32.37 ± 0.69	12.02 ± 0.30
300	3.58 ± 0.49	31.93 ± 2.96	11.92 ± 1.63
350	2.81 ± 0.21	25.29 ± 1.72	9.38 ± 0.69

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

A.12 Effect of agitation speed on the growth of *P. rhodozyma* in peat hydrolysate PH4 02-185 in shaker flask[1].

Agitation speed (r.p.m.)	Dry Biomass Conc. ($g \cdot L^{-1}$)	% Yield	% Efficiency
150	2.93 ± 0.09^{b}	44.48 ± 1.71^{b}	19.56 ± 0.63^{b}
200	4.63 ± 0.13^{a}	56.76 ± 0.89^{a}	30.89 ± 0.83^{a}
250	4.16 ± 0.13^{a}	55.73 ± 0.63^{a}	26.89 ± 1.37^{a}
300	3.57 ± 0.05^{b}	49.26 ± 0.53^{b}	23.78 ± 0.32^{a}
350	3.31 ± 0.08^{b}	45.76 ± 2.25^{b}	22.53 ± 1.07^{b}

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

APPENDIX B: BATCH FERMENTATIONS

B.1: Influence of agitation speed on *P. rhodozyma* growth and astaxanthin production in peat hydrolysate in batch fermentation[1].

Agitation Speed (r.p.m.)	Dry Biomass Conc. ($g \cdot L^{-1}$)	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
200	4.65 ± 0.56^a	1072 ± 26^a
250	4.85 ± 0.49^a	1079 ± 27^a
300	4.45 ± 0.25^a	1065 ± 45^a

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

B.2 Influence of aeration rate on *P. rhodozyma* growth and astaxanthin production in peat hydrolysate in batch fermenters[1].

Aeration Rate (vvm)	Dry Biomass Conc. ($g \cdot L^{-1}$)	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
0	0.87 ± 0.20^a	nd
0.5	4.86 ± 0.49^a	1078 ± 14^a
1.0	4.91 ± 0.34^a	1080 ± 18^a
1.5	4.90 ± 0.11^a	1076 ± 13^a
2.0	4.85 ± 0.44^a	1079 ± 14^a
3.0	4.86 ± 0.23a	1081 ± 13^a

[1]Mean values of three determinations ± standard deviations. Values in the same column with the same superscripts are not statistically different ($P > 0.05$).

B.3 The growth pattern of *P. rhodozyma* in batch fermentation.

Fermentation Time (h)	Final Substrate Conc. ($g \cdot L^{-1}$)	Dry Biomass Conc. ($g \cdot L^{-1}$)
0	15.00 ± 0.00	0.59 ± 0.10
10	14.15 ± 0.15	0.59 ± 0.30
20	13.44 ± 0.78	1.09 ± 0.20
30	9.90 ± 0.85	1.26 ± 0.20
40	9.10 ± 0.95	1.71 ± 0.32
50	8.32 ± 0.64	3.86 ± 0.27
60	7.72 ± 0.64	4.18 ± 0.35
70	7.43 ± 0.59	4.45 ± 0.10
80	7.13 ± 0.43	4.30 ± 0.14
90	6.94 ± 0.32	4.59 ± 0.28
100	6.84 ± 0.10	4.83 ± 0.25
110	6.80 ± 0.36	4.80 ± 0.10
120	6.81 ± 0.26	4.86 ± 0.12
130	6.70 ± 0.70	4.82 ± 0.22
140	6.76 ± 0.44	4.80 ± 0.11
150	6.79 ± 0.18	4.81 ± 0.20
160	6.81 ± 0.46	4.64 ± 0.39
170	6.81 ± 0.43	4.50 ± 0.90
180	6.79 ± 0.39	4.51 ± 1.08
190	6.79 ± 0.32	4.20 ± 0.53
200	6.82 ± 0.25	3.90 ± 1.05

[1]Mean values of three determinations ± standard deviations.

B.4 Changes in the growth parameters of *P. rhodozyma* as a function of time.

Fermentation Time (h)	Dry Biomass Conc. ($g \cdot L^{-1}$)	Specific Growth Rate (μ)	Doubling Time (h)
0	0.59 ± 0.10	-	-
10	0.59 ± 0.30	-	-
20	1.09 ± 0.20	0.031 ± 0.01	22.36 ± 5.71
30	1.26 ± 0.20	0.025 ± 0.01	27.73 ± 3.39
40	1.71 ± 0.32	0.027 ± 0.01	25.67 ± 3.05
50	3.86 ± 0.27	0.038 ± 0.00	18.24 ± 2.55
60	4.18 ± 0.35	0.033 ± 0.00	21.00 ± 1.32
70	4.45 ± 0.10	0.029 ± 0.01	23.90 ± 0.82
80	4.30 ± 0.14	0.025 ± 0.00	27.73 ± 1.11
90	4.59 ± 0.28	0.023 ± 0.00	30.14 ± 1.52
100	4.83 ± 0.25	0.021 ± 0.02	33.00 ± 1.58
110	4.80 ± 0.10	0.019 ± 0.00	36.48 ± 1.05
120	4.86 ± 0.12	0.018 ± 0.00	38.51 ± 1.31
130	4.82 ± 0.22	0.016 ± 0.02	43.32 ± 5.05
140	4.80 ± 0.11	0.015 ± 0.00	46.21 ± 0.85
150	4.81 ± 0.20	0.014 ± 0.01	49.51 ± 1.90
160	4.64 ± 0.39	0.013 ± 0.00	53.32 ± 0.90
170	4.50 ± 0.90	0.012 ± 0.02	57.76 ± 6.55
180	4.51 ± 1.08	0.011 ± 0.01	63.01 ± 3.14
190	4.20 ± 0.53	0.010 ± 0.00	69.32 ± 3.65
200	3.90 ± 1.05	0.009 ± 0.01	77.02 ± 3.64

[1]Mean values of three determinations ± standard deviations.

B.5: Pattern of carotenoid production by *P. rhodozyma* in batch fermentation.

Fermentation Time (h)	Dry Biomass Conc. ($g \cdot L^{-1}$)	Astaxanthin Content ($\mu g \cdot g^{-1}$ yeast)
0	0.59 ± 0.10	nd
10	0.59 ± 0.30	nd
20	1.09 ± 0.20	nd
30	1.26 ± 0.20	nd
40	1.71 ± 0.32	220 ± 13
50	3.86 ± 0.27	615 ± 15
60	4.18 ± 0.35	729 ± 22
70	4.45 ± 0.10	952 ± 28
80	4.30 ± 0.14	971 ± 30
90	4.59 ± 0.28	985 ± 18
100	4.83 ± 0.25	1020 ± 10
110	4.80 ± 0.10	1040 ± 37
120	4.86 ± 0.12	1079 ± 17
130	4.82 ± 0.22	1080 ± 22
140	4.80 ± 0.11	1080 ± 38
150	4.81 ± 0.20	1082 ± 14
160	4.64 ± 0.39	1083 ± 23
170	4.50 ± 0.90	1082 ± 12
180	4.51 ± 1.08	1084 ± 17
190	4.20 ± 0.53	1085 ± 15
200	3.90 ± 1.05	1086 ± 15

[1]Mean values of three determinations ± standard deviations.
nd Not determined : There was not enough cells for astaxanthin analysis.