CONSTRUCTION OF GALACTOSE ASSIMILATING, CAROTENOID PRODUCING YEASTS BY PROTOPLAST FUSION

by

CHRISTINE S. HANSEN

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The University of British Columbia
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ABSTRACT

Protoplasts were prepared from two yeast strains P. rhodozyma (ATCC 24202) and K. fragilis (ATCC 8455). Protoplasts prepared from P. rhodozyma were facilitated by prior growth of the cells in a media containing S-(2-aminoethyl)-L-cysteine. Protoplasts from these two yeast genera were fused either by the use of electrofusion or polyethylene glycol treatment. Stable carotenoid producing cell lines were selected by growth at 30°C on yeast nitrogen base plus galactose.

Selected single fusants display taxonomic characteristics common to both genera with a cellular morphology and a carotenoid composition similar to that of P. rhodozyma.

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DEDICATION

This thesis is dedicated to my parents for their help, patience and understanding, and to my sister, Clo and to Cassie who has helped me more than she knows.

INTRODUCTION

Previous experiments have demonstrated that Phaffia rhodozyma has potential commercial value as a dietary source of astaxanthin for poultry and pen-reared salmonids (Johnson et al. 1979, 1980). Unfortunately, the multilayered, tough cell wall of this organism makes liberation of the carotenoids impractical with regards to commercial applications (Okabue et al. 1984, Johnson et al. 1980). Modification of the cell walls prior to inclusion in animal diets have proven to be inapplicable on a large scale, or destructive to the carotenoids (Okabue et al. 1983).

Kluyveromyces is a genera of yeast that is able to utilize a variety of carbon sources. One such source is whey, a byproduct of the cheese industry. The high biological oxygen demand (BOD) of whey from cheese plants renders waste treatment impractical (Al-shabibi et al. 1984; Farahnak et al. 1985).

Attempts at interspecies and intergeneric yeast protoplast fusions have recently become prominent. In one study the fusion of S. cerevisiae and Z. fermentati was performed to obtain a high ethanol producer that utilized a number of carbon sources (Pina et al. 1986). The objective in most cases is to improve industrial strains with respect to carbon utilization, increased alcohol production or other such parameters.

Since P. rhodozyma produces potentially useful carotenoids and K. fragilis is able to use a variety of economical carbon sources such as whey, the main aim of this study was to obtain an organism by

intergeneric protoplast fusion that combines these characteristics. Protoplast fusion was the method of choice since it has been used extensively for this purpose (Pina et al. 1986; Farahnak et al. 1986).

LITERATURE REVIEW

I. Genetic Manipulation

The drive to improve organisms to better suit their users has been a long standing objective of many researchers (Peberdy, 1980). The genetic manipulation and strain enhancement of plants and animals occurs in an ever increasing application (Sink 1984, Yu et al. 1984). Selection and mating are classical ways of altering the genetic content of an organism, but this approach can be time consuming and not always accurate or practical (Sink 1984). In order to avoid and to simplify constraints, involved in formal cell reproduction, genetic information can be introduced to a cell by fusion of their respective protoplasts (Peberdy 1980).

a) Protoplast Production

The generation of viable protoplasts can be a very complicated and difficult procedure, the success of which varies greatly, depending upon the organism used (Yu et al. 1987, Pina et al. 1986). The isolation of protoplasts requires the digestion of the cell wall, releasing the cellular cytoplasm encased within the plasma membrane. To maintain the integrity of the plasma membrane, which is quite fragile, the entire process must be carried out in a hypertonic environment to provide osmotic stability (Peberdy, 1980).

In the past, researchers were forced to purify their own enzymes for cell wall digestion which was time consuming, and often non-reproducible. Today, enzymes are commercially available not only from the traditional source, such as the snail Helix pomatia, but also

from various microorganisms such as the fungi Aspergillus sp. and Neurospora sp.

The technique involved in the development of protoplasts from fungi poses a particular problem when it comes to digestion of the cell by enzymes. The fungal cell wall is a physical and chemical complex of many different structural components such as chitin and β-glucans which require more than one enzyme for their degradation. Hamlyn et al. (1981) demonstrated that high levels of chitinase and β-glucanase mixed in a "soup" successfully digested cell walls of various species of Aspergillus.

There are also other factors affecting the production of protoplasts such as cell age, growth conditions, pretreatment of cells, duration of digestion and the pH of the protoplast buffer. Morgan et al. (1980) found that for the yeast K. fragilis, desirable protoplasts were obtained from shaking flask cultures that were in late log, early stationary growth phase. Peberdy (1980), on the other hand, found that growth of Streptomyces in glycine, improved protoplast yields. Pre-treatment of the isolated culture with thiol compounds such as mercaptoethanol also enchanced the percentage of protoplasts recovered (Hamlyn et al. 1981). The optimization of the parameters involved in cell wall dissolution of a particular organism has resulted in an increase of undamaged protoplast numbers per unit digestion time.

b) Protoplast Fusion

Several methods have been developed to alter the genetic material in organisms, some of which include: protoplast fusion, hybridization and transformation. The use of fusion bypasses the normal cellular cycles and restrictions commonly placed upon the pooling of genetic

information. Generally, the cell wall and its various surface receptors will dictate compatibility. Cell wall contact is thought to be a triggering device for pre-conjugal activities.

The basic concept involved in the fusion of cells is quite simple. Protoplasts are collected from the desired strains and added in equal parts to a fusogen, causing the membranes to associate. The aggregated protoplasts are then left to incubate, either shaking gently or standing, to enable an association between the two cell membranes. After an alloted time, the fusogen and the fused protoplasts are diluted and transferred to an osmotically stablized agar plate. Here plasmogamy, karyogamy and regeneration of the cell wall are completed.

One of the most commonly used fusogens is polyethylene glycol (PEG). PEG is available in a variety of different molecular weights from 1500 daltons to 8000 daltons and the choice of molecular weight as well as concentration of the solution can have a dramatic effect on fusion (Peberdy, 1980). Generally 4000 daltons is the weight of choice used in final solution concentrations ranging from 30% to 60% (w/v).

Goodey and Beven (1983) carried out an experiment proving that an attachment inducing agent was required for successful fusion. Two different yeast strains of S. cerevisiae were induced to form protoplasts. These were allowed to fuse with and without the presence of PEG. Only those cells subjected to the PEG treatment fused to create "cybrids".

PEG, in itself, does not induce plasmogamy. Rather, it draws the cells together in aggregates creating an association of the plasma membrane. The fusogen is then diluted, allowing plasmogamy to occur.

With this method, fusion frequencies of 15-17% can be obtained with certain cells (Menczel 1984). Aggregation of the protoplasts can be improved by the addition of the divalent cations Ca^{2+} and Mg^{2+} to the fusion medium. The most effective concentration of these divalent metals has been found to be a 0.01 M solution (Peberdy 1980). One criticism of the PEG-divalent metal induced type of fusion is that it occurs as a very random and low frequency event (Hamlyn et al. 1981).

Another fusogen that has recently gained importance is the application of a short pulse electric current to coupled protoplasts (Senda 1982, Bates 1983, Pohl 1982). Cells and other polarizable particles can be seen to spin when subjected to an alternating current (AC) (Mischel et al. 1982). Cells are placed between electrodes and an alternating current is applied. Due to their mutual dipole formation the cells associate or create "pearl-chains" (Appendix I). At the point when cells line up in close association, a short pulse of direct current (DC) is applied causing a reversible membrane breakdown, resulting in point adherations of protoplasts (Bates et al. 1983).

Generally, the apparatus consists of two platinum wires set 0.5-1 mm apart on a flat microscope slide (see Appendix II). These electrodes are then attached to a supply circuit (Pohl 1982). The capacitor, in most cases, is able to deliver between 50 and 1000 volts with the capacity of 0.01 to 1000 µF. Once activated, an electric charge between 1 and 50 mA with a duration of 0.25 to 50 ms runs through a drop of low-conducting, osmotically stablized buffer plus protoplasts (Senda 1982). Once point adherations are made, a further 30 to 60 minutes are required for plasmogamy to occur.

"Electro-fusion" has a number of advantages over the more conventional PEG method. It requires no chemical pretreatment to facilitate fusion, and fusion is rapid and synchronous (Bates et al. 1983). Reports of between 60-80% fusants have been registered (Bates et al. 1983). Some problems that face this method include optimizing equipment design, and the fact that the protoplasts tend to stick to the metal electrodes. The latter difficulty may be overcome by the placement of an inert membrane between the electode and protoplasts.

II. Carotenoids

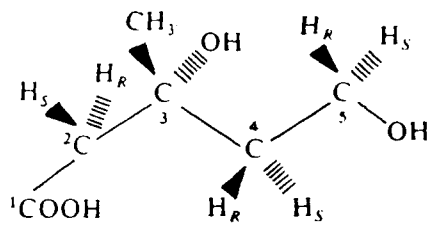
Carotenoids form a group of more than 400 known naturally occurring pigments whose colours range from yellow to red. These pigments are responsible for the brilliant colours seen in many fruits and vegetables, Crustacea sp., fish and eggs. Only algae, microorganisms and plants are capable of synthesizing the carotenoids; animals must obtain them from their food supply.

In addition to functioning as attractants or deterrents, carotenoids can also play a role in physiological functions. Many act as photoprotective agents by absorbing potentially harmful sunlight energy or quenching singlet oxygen molecules (Muller et al. 1980). Carotenoids also function as accessory pigments in photosynthesis and thus are often associated with the chloroplast. In animals, carotenoids play an important role in physiological function. One such carotenoid, β-carotene, is converted to Vitamin A and subsequently stored in the liver. Vitamin A is required for the normal biosynthesis of mucopolysaccharides.

a) General Biosynthesis of Carotenoids

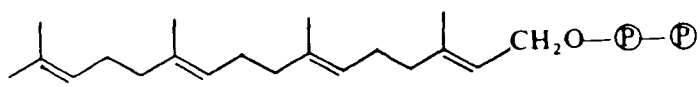
Carotenoids are structured basically from a highly unsaturated isoprene nucleus. Most of those naturally occurring are made up of eight isoprene residues. The biosynthesis of carotenoids is a multistep process that utilizes a number of different cellular enzymes (Goodwin 1979).

Initially, three acetyl-Co A molecules form mevalonic acid (formula 1), which is a six carbon molecule. This is then converted to the five carbon molecule isopentenyl pyrophosphate (IPP). Four IPP molecules link to form the twenty carbon geranylgeranyl pyrophosphate (GGPP) (formula 2).



mevalonic acid

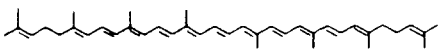
formula 1



geranylgeranyl pyrophosphate

formula 2

Two GGPP molecules link tail to tail to yield the first forty carbon precursor called phytoene, which is desaturated to form lycopene (formula 3).

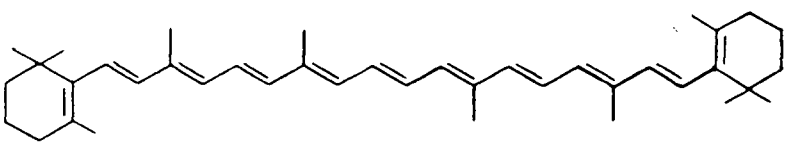


lycopene

formula 3

From this point, there are a number of routes that can be taken depending upon which carotenoid is being produced, such as cyclic carotenoids and xanthophylls (O_2 containing).

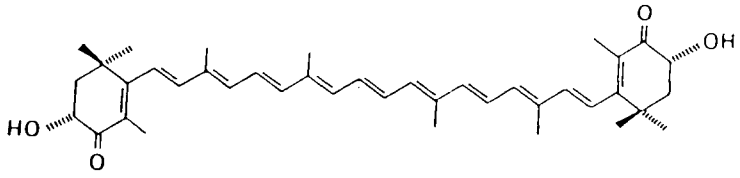
Only those carotenoids containing a β-ione ring are classified as provitamin A. These rings are formed by specialized cyclizing enzymes that work on the ends of the lycopene molecule. β-carotene is an example of a compound with such ends (formula 4). This molecule is capable of producing two molecules of Vitamin A.



β-carotene

formula 4

The carotenoid that is of particular interest to this paper, astaxanthin, is a β-carotene derivative (3,3' dihydroxy-β-β-carotene 4,4'-dione) (formula 5). This particular carotenoid has four optical isomers that can be produced naturally, one of which can be found in lobster and shrimp. When shrimp are ingested by a fish such as salmon, the astaxanthin pigments the flesh to produce the characteristics red-orange colour (Johnson et al. 1980).



astaxanthin

formula 5

b) Carotenoids and Microorganisms

As mentioned previously, microorganisms are capable of synthesizing their own carotenoids, and because of this, have been the object of a number of different avenues of research. One area focused on pertains to the fungus Blakeslea trispora, which produces β-carotene. When the cells were fractionated, researchers found that the carotenoid occurred in two of the cell fractions, the supernatant and the cell wall/mitochondria fraction (Goodwin 1980). This suggests that the carotenoid is held in both the membranes as particulate matter as well as an oil in the cytoplasm.

In the fungus Neurospora crassa (Mitzka-Schabel et al. 1981), cell fractions containing the endoplasmic reticulum membrane, and supernatant had the highest carotenoid levels. These same cell fractions also held the majority of enzymes for carotenogenesis. Another fraction containing carotenoid related enzymes was the plasma membrane. This suggests that there is not one single subcellular site for carotenogenesis in fungi, unlike the higher plants where, following melvolic acid, the carotenoid enzymes are located within the chloroplasts.

Other research by Roncero et al. (1982) looked at the genetics of carotenoid production in the fungus Phycomyces blakesecanus, which is

yellow due to β-carotene. It was found through a series of mutant mating experiments, that two genes (Car B and Car RA) were closely linked and located approximately 10 map units from the centromere. These genes produce two enzymes, phytoene dehydrogenase and lycopene cyclase that are responsible for the last six steps of carotenogenesis. (Similar associations have also been noted in the yeast S cerevisiae in the genes for galactose utilization.)

One non-genetic factor that appears to affect carotenoid production in the microorganism is the carbon source. Studies by Ciegler et al. (1978) demonstrated that growth on various grains incorporated into the fermentation media, influenced the carotenoid production of B. trispora with corn giving the highest yield of carotenoid per gram of dry mycelium weight. Other studies by Johnson and Lewis (1979) on P. rhodozyma demonstrated that carbon sources such as maltose, sucrose, and cellobiose gave a higher yield than glucose.

A second non-genetic factor affecting carotenogenesis is the pH of the growing media. Microorganisms appear to respond to a drastic reduction of pH prior to the production of carotenoids. In organisms such as P. blakeslea and Rhodotorula gracilis, a drop in pH to as low as pH 2 has been noted (Goodwin 1980). If the growing medium is buffered at higher pH, there is no carotene production.

Other stimulants of carotenogenesis include light, and in the case of N. crassa, the photoreceptor may be β-carotene itself (Mitzka-Schnabel et al. 1981). An increase in oxygenation of cultures has been shown to increase carotenoid production in P. rhodozyma (Johnson and Lewis 1979).

c) Determination of Carotenoid Pigments

Due to the very sensitive nature of carotenoids towards light and oxygen, much care must be taken in the extraction and purification of the carotenoids. No one method of extraction can be considered completely satisfactory as a standard technique to meet the potential variety of material to be extracted (Davis 1965). Often the organic extracting solvents used must be miscible with water in order to efficiently remove the carotenoids from the fresh tissue.

Several analytical procedures are used to establish the identity of the carotenoids. Generally it is conceded that visible and infared spectroscopy, nuclear magnetic resonance and mass spectra afford the best means of chemical characterization. Separation via thin layer chromatography is often used in conjunction with some of the afore mentioned techniques to provide a reliable proof of identity (Moss and Weedon, 1976). The visible absorption spectrum is quite important as structural variations will effect the shape and location of the spectrum; for example, oxo-carotenoids do not have the typical three banded spectrum of β-carotene but rather a single symmetrical band (Davies, 1965). Cis-trans isomerization may also be detected at shorter ultra-violet wavelengths.

Mass spectrometry can be considered as a very powerful tool for resolving carotenoids and their structures. Advantages include the requirement for only a small amount of material. Some limitations are also inherent such as artifacts caused by volatile impurities and the rapid loss of water masking the molecular ion peak (Moss and Weedon, 1976). Variations can also occur if differences of ionization, temperature or insertion conditions exist.

III. Phaffia rhodozyma

Phaffia rhodozyma is a red yeast genus in the Deuteromycotina in the form order Blastomycetes. The yeast was originally isolated from deciduous tree exudates (slime fluxes) in Japan and northwestern North America (Miller et al. 1976). This is the only carotenoid producing yeast capable of fermenting sugars (Andrewes et al. 1976). One other uncommon aspect of this yeast is its ability to produce astaxanthin. Astaxanthin production under certain cultural conditions may represent an average of 85% of the total carotenoids found in the cell (Andrewes et al. 1976).

P. rhodozyma is an ellipsodial yeast that appears to lack a sexual stage within its life cycle. It reproduces by budding, which generally occurs at the same site leaving a thick bud scar. The vegetative cell wall is multilayered, containing a high proportion of α 1-3 glucan (Miller et al. 1976). The optimal temperature for growth and astaxanthin production is 22°C (Johnson and Lewis, 1979) but growth can occur at temperatures ranging from 0°C to 27°C. No growth has been recorded above 27°C. This biotin requiring yeast assimilates carbon sources such as D-glucose, maltose, sucrose, cellobiose and soluble starch, and will ferment D-glucose, maltose and sucrose. Under austere conditions, chlamydosphores are produced which germinate by budding, but no promycelium or spore formation has been noted.

a) Carotenoid Production of P. rhodozyma

Investigation by Andrewes, Phaff and Starr (1976) concentrated principally upon the identity of the pigments produced by P. rhodozyma. It was found that the total carotenoid mixture made up 0.003% of the total wet cell weight. Of those carotenoids, astaxanthin comprised 87%

of the total. Other carotenoids present in minor but measurable amounts were: β-carotene, echinenone, phoenicoxanthin and a new carotenoid, 3 hydroxy-3'4'-dihydro-β-ψ-caroten-4-one. With respect to the astaxanthin, both the trans and the chromatographically more polar cis isomers, were isolated from the same cell preparations.

In an attempt to optimize pigment production, Johnson and Lewis (1979) did extensive work with the yeast (see Appendix IV). They found that when the yeast was grown within a batch culture fermentor, astaxanthin was produced mainly during the late exponential growth period. The optimum pH for astaxanthin formation appeared to be 4.5 however, this pigment was produced at all culture pHs tested. Various carbon sources exhibited pronounced effects on both cell growth and astaxanthin content. Cellobiose produced the most pigment with other dissaccharides following close behind (Appendix IV). Oxygen was proven to be an important factor since cultures permitted to ferment displayed β-carotene as the major pigment.

Based on carotenoid content and known biochemical pathways for other organisms, Andrewes et al. (1976) proposed a biosynthetic scheme for the production of astaxanthin in P. rhodozyma (Appendix V). Neurosporene is converted to β-carotene (12) which in turn is converted to echinenone (14). A specific enzymatic reaction then converts the echinenone to hydroxyechinenone. A keto-insertion results in phoenicoxanthin which eventually will form the terminal carotenoid astaxanthin (11). Generally, the cis isomer is the only one isolated from other sources, but it is thought that the precursors and enzymes utilized by the yeast allows it to produce both cis and trans isomers.

b) P. rhodozyma as a Source of Astaxanthin in Salmonid Diets

In trout, salmon and charr, astaxanthin is the major pigment responsible for the typical red coloured flesh (Foss 1984). In the wild, these fish obtain the colour through their diet by ingesting shrimp, shrimp larvae and plankton such as P. Prasinophyceae that contain the pigment. Fish cultivated by aquaculture do not have the luxury of such a diversified diet, thus the pigment must be fed to them as an ingredient in their fish meal. This can be quite costly, especially in lieu of increasingly strict regulations concerning the safety of chemicals as food additives (Johnson et al. 1980).

P. rhodozyma is a natural source of astaxanthin and when fed to salmonids, depending upon carotenoid liberating treatments, it will confer the pigment to fish (Johnson, Villa, and Lewis 1980). As with other yeasts, P. rhodozyma also acts as a potentially excellent source of protein, fats and other nutrients (Appendix III). One drawback to the commerical use of this yeast for feed, is that the fish is unable to digest the cell wall and thus release the pigment (Johnson et al. 1980, Okagbue and Lewis 1983). In-vitro cell lysis treatment could be costly and may have an adverse effect on the stability of the carotenoid and nutritional value.

A second drawback regarding the use of P. rhodozyma in fish feed is the relatively high cost of fermentation. Many of the substrates tested by Okagbue and Lewis (1983) that provided adequate cell yield and astaxanthin content, can be very expensive. This substrate cost plus the additional costs involved in maintaining the fermenter at 22°C would contribute significantly to the selling price of the fish food.

Depending on growth conditions, P. rhodozyma produces between 30 and 800 µg astaxanthin/g yeast. This amount of carotenoid far exceeds that found in crustacean shells which normally yield 10 to 100 µg astaxanthin/g of shell (Johnson et al. 1980).

IV. Kluveromyces fragilis

K. fragilis is a lactose utilizing yeast that has been shown to reduce the chemical oxygen demand (COD) of cheese whey by at least 60% (Al-shabibi and Younis 1984). Interest in this yeast revolves around its enzyme lactase producing ability (Mahoney et al. 1977) and in its ability to ferment whey to produce ethanol and single cell protein (Vienne and von Stockar 1985). Colonies are generally white or cream and cells exhibit multilateral budding as well as forming ascospores. K. fragilis is able to grow over a wide range of temperatures with growth occurring as high as 40°C.

a) Growth and Fermentation on Whey

K. fragilis is able to produce ethanol by the fermentation of cheese whey. As with all microorganisms, culture under different growth conditions can cause differences in end products. Lactase, concentrations increase sharply during log phase of growth and as the cells enter stationary phase, the lactase concentration decreases (Mahoney et al. 1975). In order to increase the lactase production, low aeration is required, however low aeration results in a poor cell yield. When cultured in whey, fortified with salts, this yeast, not recognised as a prolific oil producer, can be stimulated to produce up to 22% oil (Al-shabibi and Younis, 1984).

The optimum pH for the consumption of the media substrate and ethanol yield lies between pH 4 and 5, with the most efficient consumption occurring at a pH of 4.0 (Vienne and von Stockar 1985). In experiments carried out comparing the difference between sterilized and non-sterilized whey permeate; it was concluded that another medium component other than lactose was growth limiting and that this component was affected by heat sterilization (Vienne and von Stocker 1985).

b) Protoplast Fusion in Kluyveromyas sp.

Kluyveromyces sp. have often been used in studies involved in the genetics and biochemistry of yeasts as well as studies related to enhanced alcohol production. These cells, when subjected to enzyme treatment at the appropriate stage of growth, produce protoplasts with relative ease (Farahnak et al. 1986, Morgan et al. 1980). In the majority of cases, the numbers of protoplasts formed within a suspension approaches 100% of the initial cell population (Johannsen et al. 1984).

Morgan et al. (1980) used protoplast fusion between homothallic strains of K. lactis to produce sporulating hybrids. Biochemically, cytologically and genetically, these studies suggest that the majority of fusion products are diploid. The presence of diploidy also demonstrates that nuclear fusion and chromosome exchange does occur in protoplast fusion products. Further support for this position was put forth by Farahnak et al. (1986) when K. lactis was fused with S. cereviceae producing fusants that were capable of both assimilating lactose and producing ethanol in excess of 13% (vol/vol).

MATERIALS AND METHODS

I. Strains

a) *Phaffia rhodozyma* (ATCC 24202)

This organism was maintained on Malt Extract Agar (MEA) (Difco) plates at 22°C until transfer to the pre-protoplast media (AEC-MEA)

b) *Kluyveromyces fragilis* (ATCC 8564)

This organism was maintained on Malt-Yeast-Glucose-Peptone (MYGP) Agar (Difco) at 22°C until transfer to the pre-protoplast media (YNB-galactose) (Difco-BDH).

c) *Rhodosporidium toruloides* (ATCC 10788)

This organism was maintained on MEA plates at 22°C

II. Cultivation of Organisms for Protoplast Production

a) *P. rhodozyma*: One week old colonies were picked from MEA plates and streaked onto MEA + 5 mg/mL. S-(2 aminoethyl)-L-cysteine (AEC) (Sigma) plates and incubated at 22°C for 3 weeks prior to use. This method induced filamentous mutants to form.

b) *K. fragilis*: Cultures 72 hours old were streaked onto yeast nitrogen base + galactose plates (YNB-g). These cultures were grown 48 hours prior to use.

III. a) Protoplast Buffer (Morgan *et al.* 1980) consisted of:

1.0 mL A + 6.0 mL B + 6.0 mL C + 0.2 mL D where:

A = 0.05 M Tris-HCl (pH 7.0) (Difco)

B = 1.2 M KCl (Fisher)

C = 0.02 M $MgSO_4$ .7 H_2O (Fisher)

D = 1 M 2-mercaptoethanol (Swartz/Mann Biotech)

b) Enzymes:

β-glucuronidase from Helix pomatia (Sigma)

Amyloglucosidase from Rhizopus (Sigma)

Chitinase from Streptomyces griseus (Sigma)

c) Fusogens: were prepared as follows:

- Polyethylene Glycol (PEG) (Sigma) (mw) 4000 was added 30% (w/v) to 0.01 m $CaCl_2 \cdot 2\ H_2O$ (Fisher)
- For electrofusion 0.7 m Mannitol (BDH) was used as an osmotic stablizer.

IV. Media

a) Growing media

- Malt Extract Agar (Difco) was used for maintenance and propagation of P. rhodozyma.
- Malt-Yeast-Glucose-Peptone Agar consisting of 3 g malt extract (Difco), 3 g yeast extract (Difco), 10 g D-glucose (Difco), 5 g peptone (Difco), 20 g agar (Difco) per litre with pH adjusted to 5.5 was used to maintain K. fragilis.
- Yeast Nitrogen Base (Difco) + galactose (BDH) was used for maintenance and propagation of K. fragilis and the fusant strains; 1.5% agar was added to the YNB-g (Difco) if plates required.
- Osmotically Stabilized Recovery Media (OSRM) consisting of YNB + galactose + 0.6 m KCl (Fisher) + 1.5% agar was used for the recovery of protoplasts.

- amino acid analogue media contained 2% malt extract (Difco) + 5 mg/ml of either sulfa guanidine, 5-fluoro D,L tryptophan, L tryptophon hydroxamate L-lysine hydroxyamate, s-hydroxylysine HCl or s-(2 aminoethyl) L-cysteine + 2% Bactoagar (Difco).
- Standard Medium consisting of (per litre) $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 1 g; $MgSO_4$ $7H_2O$, 0.5 g; $CaCl_2$ $2H_2O$, 0.1 g; (Fisher) yeast extract (Difco), 2 g; 20 g of a carbon source; 15 g agar (Difco) was added to the Standard Medium if plates required.

b) Media for biochemical tests:

- YCB (Difco) + 5 mm KNO_3 - (Barnett et al. 1983)
- Durham fermentation tubes + glucose; raffinose; maltose or galactose obtained from Microbiology Media Room (Barnett et al. 1983).
- Urea Broth (Difco) was used for urease test. (Barnett et al. 1983)
- YNB (Difco) containing niacin + galactose (BDH) and YNB (Difco) without niacin + galactose (BDH) were used for tests concerning the requirement for niacin. (Barnett et al. 1983)
- YNB (Difco) + glucose (Difco) broth was used for fermentation tests at 22°C and aliquots of this media were also placed on a spot plate + iodine to test for extracellur starch. (Barnett et al. 1983)

V. Protoplast Release, Fusion and Regeneration

The protoplast liberation procedure is a slight modification of that described by Morgan et al. (1980). The cells were grown in their respective media and each was induced to form protoplasts separately. The pretreated Phaffia cells were dispersed in a filter sterilized protoplast buffer, containing 5 mg/mL β-glucuronidase, 2 mg/mL amyloglucosidase and 1 mg/mL chitinase, for breakdown and removal. This mixture was then incubated at

30°C, with gentle shaking for 4 hours. The K. fragilis was treated in a similar fashion in a solution of 5 mg/mL β-glucuronidase for 1 1/2 hours of incubation for cell wall removal.

After their respective incubations, the free protoplasts were washed three times with sterile buffer. After each wash, the protoplasts were centrifuged for 5 min. at 500 x g in a Fisher table top centrifuge at 21°C. Those protoplasts slated for electrofusion were washed a further three times with sterile 0.7 M mannitol.

Protoplasts from the two parental strains were mixed to give 1.0 mL suspensions containing between 10^5 and 10^6 protoplasts/mL. Those suspensions to be fused by PEG were centrifuged for 5 minutes at 500 x g in the Fisher tabletop centrifuge at room temperature and the supernatant was removed. The mixed protoplasts were resuspended in the 30% (w/v) PEG solution and incubated for 30 min. at 30°C. For regeneration, the PEG suspension was diluted with sterile protoplast buffer, plated on OSRM and incubated at 22°C.

Protoplasts from the two parental strains that had been washed in 0.7 M mannitol were mixed in equal parts to form a suspension containing between 10^5 and 10^6 protoplasts/mL. This mixture was then added dropwise to a sterile coverslip that was mounted under the fusion cell. The fusion cell itself consisted of two parallel platinum wires 0.13 mm diameter set 1 mm apart mounted in a polycarbonate plastic frame that could be attached to the stage of the microscope (wild, inverted). These electrodes were attached to wires that were connected to the fusion apparatus (Advanced Engineering Corp.). The capacitor was charged to the desired voltage (which ranged from 1500 v to 2700 v depending upon the experiment). An alternating current of between 40 and 1000 kHz was applied causing the protoplasts to aggregate (Bates et al. 1983). At this point, the electric switch was activated and

after 30 ms the desired voltage of A.C. pulse was applied through the sample. The duration of the pulse was 6 µs. For regeneration, the protoplasts were diluted onto an OSRM plate with 0.7 M mannitol and incubated at 21°C.

VI. Selection of Fusants

Desired fusants were selected on the basis of colour production and their ability to grow on YNB+galactose media. After 7 days, colonies that produced colour ranging from yellow to orange were picked from the OSR media, and transfered to YNB + galactose media and incubated at 30°C. Colonies that grew at 30°C and remained orange were picked and maintained on YNB + galactose plates and liquid media. One presumed fusant, #26, was chosen for its colour intensity, and quick growth to use for further testing.

Presumed fusants were maintained on YNB+galactose plates, incubated at 30°C. When liquid cultures were used, the media was again YNB + galactose. Cultures in liquid medium within 250 mL Erlenmeyer flasks, were incubated at 30°C shaking at 200 rpm. Presumed fusants were also viewed microscopically with a Wild inverted phase contrast microscope.

a) Biochemical tests

Fusant #26, Phaffia rhodozyma, and K. fragilis were subjected to a selected number of biochemical tests in order to sort out the relationship of fusant #26. These tests included: Durham fermentation tubes containing: raffinose, galactose, maltose and glucose; urea broth for urease test; YCB + NO_3 for using NO_3 as nitrogen source; YNB with or without niacin; fermentation tests were also carried out in YNB-glucose, extracellular starch production was also tested using YNB-glucose and iodine indicator. All procedures for these tests were carried out as described by Barnett et al. (1983).

VII. Carotenoid Analyses

Cells grown on either liquid or solid media were collected and frozen at -60°C in a Forma Bio-Freezer prior to crushing. The cell paste was formed into a cylindrical shape within a 5 mL plastic syringe barrel and frozen for at least 24 hours. The frozen cells were then forced by means of a piston through a metal cylinder at -40 to -60°C having a small orifice (1 mm diam) using a Carver Laboratory hydraulic press with a pressure of up to 10,000 lbs/in^2 was used. The crushed, frozen, cells were immediately extracted with cold acetone (BDH).

The pigmented acetone was evaporated using a Buch Rotavapor R110 rotary evaporator. The dried pigment was then resuspended in various solvents depending upon the test (i.e. acetone for absorption spectrum and hexane for column chromatography).

All work involving carotenoids was carried out in subdued light. In all cases, the respective pigments from fusant #26 cultured on both liquid and solid media, P. rhodozyma cultured on solid media, R. toruloides cultured on solid media, β-carotene (Sigma) and astaxanthin (Roche) were used as standards.

a) Thin Layer Chromatography

The pigment samples extracted from fusant #26, P. rhodozyma, and R. toruloides and the pigments β-carotene (Sigma) and all-trans astaxanthin (Roche) were resuspended in acetone (BDH). Aliquots were then spotted onto Keiselgel 60 glass plates. Separation was achieved in a solvent system containing acetone:hexane (30:70). Samples were also co-chromatographed with authentic astaxanthin (data not shown).

b) Column Chromatography

The pigment samples extracted from fusant #26 (grown in liquid & solid media), P. rhodozyma, R. toruloides, and all-trans astaxanthin were dissolved in hexane. These pigments in hexane were then applied to a silica gel 60 (1 cm x 24 cm) column (60-200 mesh) (Baker Analyzed). The pigments were then washed with 30 ml of 100% hexane. Increasing concentrations of acetone:hexane combinations were added to the column starting at 1:24 (v:v) acetone:hexane and finishing with 100% acetone. Eluded fractions under vacuum containing coloured bands were collected and dried in a rotary evaporator.

c) Absorption Specta

The pigments eluted from the column were resuspended in acetone and the total spectrum was scanned on a Varian Cary 210 double beam spectrophotometer. The maximum absorption (λ_{max}) was recorded for each sample as well as the shape of the visible spectrum.

d) Mass Spectrometry

Pigment samples eluted from the silica gel 60 column were collected and dried under vacuum in a rotary evaporator. The samples were resuspended in acetone and were subjected to TLC to determine the number of bands eluted. The samples were then dried with nitrogen gas and the mass spectroscopic analyses were conducted. Low resolution EI was the service used.

e) Colour reactions

Pigment samples and pigment standards were dissolved in acetone. To 2 ml of the mixture, 1 ml of concentrated H_2SO_4 (BDH) (36 M) was added and the immediate colour change was noted (Karrer & Jucker, 1950).

RESULTS

I. Characteristics of the Parent Organisms

a) Phaffia rhodozyma

P. rhodozyma, a carotenoid producing fermentative yeast, produces astaxanthin as its major pigment under most growth conditions with the best carotenoid production occurring under a highly oxygenated liquid medium (Johnson & Lewis 1979). As reported by Miller et al. (1976), the optimum temperature for growth was 22°C with no growth occurring at temperatures above 27°C. The cells grown at temperatures between 23°C and 27°C gave sparse, small colonies with a light pink colour rather than the usual robust orange-red colour of the 22°C colonies. Cells grown at greater than optimum temperatures were smaller than normal, though they did maintain the characteristic "football" shape, and they appeared to have much thicker cell walls.

Growth of P. rhodozyma was observed on a semi-synthetic media (Johnson & Lewis, 1979), with the carbon source being varied. At 22°C, growth was obtained on maltose, cellobiose, dextrose, glucose and sucrose. There was no growth observed on the plates that contained galactose or lactose as the sole carbon source.

When biochemical tests were carried out in order to ensure that this strain of P. rhodozyma behaved as reported in the literature (Table I). All test results were the same as those reported in table I. During microscopic observation, the cells demonstrated polar budding. In older cultures, some large circular chlamydospores were seen.

Table I. Biochemical test results for *P. rhodoxyma*, *K. fragilis*, *R. toruloides* and Fusant #26.

Test	*P. rhodozyma*	*K. fragilis*	*R. toruloides*	Fusant #26
Yeast peptone + glucose	+ (no gas)	+	+	+
Yeast peptone + galactose	-	+	+	+
Yeast peptone + maltose	+	+	+	+
Yeast peptone + raffinose	-	-	-	-
NO_3	-	-	+	-
YCB + NO_3	-	-	+	-
Urease	+	-	+	+
Growth without Niacin	+	-	+	+/- (slowly)
starch formation	+	-	-	-
YNB + glucose fermentation*	+ (gas)	+ (gas)	+	+

* carried out at 22°C

b) Kluyveromyces fragilis

K. fragilis is a yeast capable of fermenting whey and does not produce carotenoids. Cultures were plated on YNB + galactose and YNB + lactose and grown at 30°C. The resulting colonies ranged in colour from white to cream with no obvious morphological differences occurring between media except that those grown on lactose containing medium were less solid when removed with a loop.

When viewed microscopically, the cells grown on lactose tended to be homogenously elongate whereas the cells grown on galactose were more circular. The cell walls in both cases were thin as compared to those of Phaffia.

Some biochemical tests were also done in order to ensure that this strain of K. fragilis behaved as reported in the literature (Table I). This was indeed the case.

II. Protoplast Formation by Parental Strains

a) P. rhodozyma

When P. rhodozyma cell walls were subjected to digestion by enzymes as outlined by Morgan et al. (1980), using both single enzyme solutions and enzyme "soups", there were no protoplasts formed. (Variations in time of digestion did not change the previous negative outcome.) The cells retained their "football" shape and when placed in a hypotonic solution remained intact. When these cells were subjected to a dipole producing electric field no spinning was observed.

Attempts to produce protoplasts from cells grown under different conditions i.e. liquid media, solid media, young (16 hr. old) cultures, old cultures and alternative carbon sources, were not successful. The resistance of the cell wall to digestion is consistent with previous literature

concerning the difficulty in extraction of carotenoids (Okagbue & Lewis 1983, Okagbue & Lewis 1984).

b) K. fragilis

The production of protoplasts from Kluyveromyces sp. has been relatively well documented (Morgan et al. 1980, Farahnak et al. 1985, Johannsen et al. 1984). Cells from galactose containing medium were subjected to digestion by the enzyme B-glucuronidase following the general protocol established by Morgan et al. (1980). At 30°C digestion of cell walls was accomplished in approximately 1.5 hours with more than 90% of the cells converted to protoplasts. This was demonstrated by the osmotic sensitivity of the cells, by their spherical shape in osmotically stablized media, and their ability to spin when subjected to a dipole producing alternating current.

Under microscopic observation the protoplasts appeared to be in good condition. They were turgid, circular and regenerated their cell walls in recovery medium.

III. Amelioration of the cell wall of P. rhodozyma to enzyme digestion

a) Pretreatment of the cells

Cells growing on MEA medium containing 5 mg/mL AEC started to produce "Chlamydophores" (large circular cells). After three weeks these "Chlamydophores" started to "germinate" producing what can be described as "pseudomycelium" and pleomorphic cells (Fig. 1). These cells seemed to have thinner (and in some cases more incomplete) cell walls than Phaffia. At this time these cells were considered as better suited for producing protoplasts than the untreated parent Phaffia.

Table II. Effect of various amino acid analogues on the growth and cell structure of Phaffia rhodozyma*.

Analogue	Growth	Cell characteristics
sulfa guanidine	+++	ovoid and irregular cells, no filaments, thin wall
5-fluoro D,L tryptophan	++	cell debris present no filamentous cells
L tryptophan hydroxyamate	++	cells debris present a few filamentous cells
L-lysine hydroxyamate	++++ (white coloured)	cells granular, thin wall, no filamentous cells
S-hydroxylysine HCl	++++	round and ovoid cells, granula, thin wall
S-(2 aminoethyl) L-cysteine	+++	many filamentous segmented cells, thin wall

* 2 days growth on 2% Malt Extract (Difco) + 5 mg/ml analogue + 2% Bactoagar at 22°C

Other analogues such as sulfaguanadine, 5-fluoro D, L tryptophan, s-hydroxylysine HCl and others were also evaluated for their ability to produce thin walls (Table II). None of those tested were as effective as AEC. Further work using AEC indicates that liquid medium cultures in addition to solid medium cultures were successful in producing thin walled cells. These thin walled cells require an osmotic stablizer such as 0.7 M mannitol in the medium to maintain the cellular integrity during growth (P.M. Townsley, personal communication, 1988).

b) Protoplast formation by AEC treated Phaffia

It was decided that based on microscopic observations that the most suitable cells for protoplast formation were those grown on 5 mg/mL AEC - MEA plates for 3 weeks. An enzyme mixture containing 1 mg/mL chitinase (Sigma), 2 mg/mL amyloglucosidase (Sigma) and 5 mg/mL β-glucuronidase (Sigma) was found to be the most effective for digestion of the cell walls.

After incubation of the Phaffia cells at 30°C for 3.5-4 hours, approximately 50% of the cells were converted to protoplasts as evidenced by osmotic sensitivity and spinning when subjected to alternating current. Due to the large variation in cell morphology, it was difficult to determine which cell type produced the best protoplasts but many were seen to ooze from the "pseudomycelium". When plated on OSRM, most of the protoplasts did recover much more slowly when compared to K. fragilis.

Subsequent investigations indicate that protoplast can also be prepared from cells obtained from liquid medium cultures.

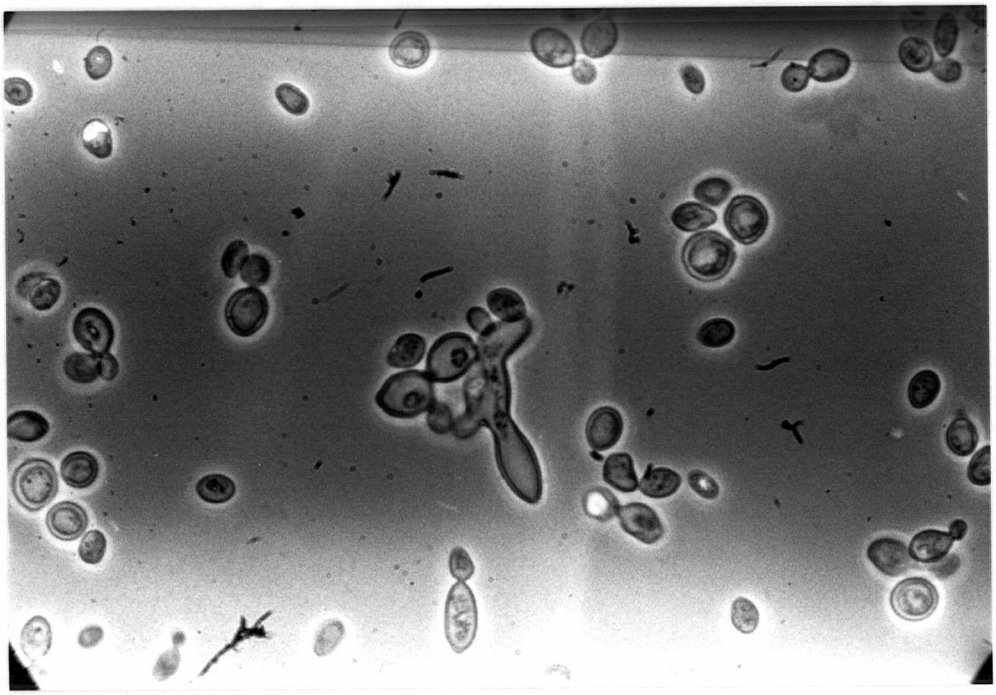


Fig. 1. P. rhodozyma cells grown in the presence of 5 mg/ml AEC in MEA (600X magnfication)

IV. Fusion of K. fragilis and AEC Treated - P.rhodozyma Protoplasts

a) Fusion by Electrofusion

Those cells to be fused by electrofusion, were first subjected to a rotating alternating current inside the fusion cell (Advanced Engineering Corp). During exposure to AC, the spinning protoplasts tended to form pearl chains (single lines of associated cells which often bridge between the parallel platinum electrodes) which were noted by Bates et al. 1983.

Once the pearl chains had formed, fusion was achieved by the application of a 6 µs A.C. pulse ranging from 1500-2700 volts. At voltages exceeding 2700 v many of the cell walls ruptured. Sometimes cells would be pulsed two or three times with a particular voltage without showing adverse effects. Fusion of the plasma membranes is probably immediate (Bates et al. 1983) and after the fusion pulse, the fused cells were not resubjected to the AC rotating current so as not to disrupt the cells. Actual coalescence of the cells was not visually observed due to the fact that water evaporation from the mannitol solution tends to form disrupting crystals within the time required. However some cells were seen that were advanced past the first step of point adherations. Electrodes were then gently flushed with 0.7 M mannitol to wash the associated fused protoplasts onto the OSRM.

b) Fusion by Polyethylene Glycol

Those protoplasts to be fused with PEG were incubated with PEG in a centrifuge tube. When observed under a microscope, the protoplasts were seen to associate but no coalecence was observed on short time observation. The associated protoplasts were diluted with 0.5 ml of protoplast buffer and plated on OSRM.

V. Selection and isolation of fused cells

Since P. rhodozyma neither grows on galactose containing medium nor 30°C, growth under these conditions were considered to be the best inhibiting the growth of Phaffia. Since K. fragilis produces white to cream coloured colonies, the production of orange colour in colonies growing on OSRM containing galactose should represent the desired fusogen. The cells were allowed to recover undisturbed for 7 days and then monitored for coloured colonies.

Under the above conditions, K. fragilis grew very quickly and in some cases overgrew the plate. To avoid further overgrowth, attempts were made to plate no more than 2.5×10^2 cfu/plate. After approximately 10 days small colonies appeared that did show some orange hue. These were left a few days longer until a deeper orange colour appeared. Colonies that ranged in colour from yellow to pink and orange were picked and plated on YNB + galactose and placed in the 30°C incubator. Approximately 45 colonies were selected and treated in this manner. The low number of colonies of this type suggests a low frequency of successful fusions, approximately $1/10^6$.

As the fusant colonies grew, a number of common morphologies were recorded (Table III). The most common colonies appeared as small, convex, shiny with smooth edges. The final selected colonies were taken from coloured colonies that had undergone either of the fusion techniques.

Those presumed fusants that either did not grow rapidly or failed to produce appreciably orange pigments, were culled. The cell lines that were established included #3, 10, 22, 23, 26, 35 and 40. Culture #26 was selected as the most desirable for further study since the cells grew rapidly and possessed the most pigment intensity.

Table III. Colony morphology of some presumed fusants on YNB - Galactose + KCL media.

Colony type	Colony morphology	Representative fusants
1	reddish brown, large (3 mm diam.), convex, shiny with smooth edges fast growing	#1
2	pink/orange, small (1 mm diam.), convex, smooth edges, slow growing, shiny	#3-9, 20, 21
3	pink, very small (.5 mm diam.), dull, convex, smooth edges	#11, 12
4	orange, large (4 mm diam.), convex, jagged edges	#13-15
5	light orange, small (1 mm diam), shiny, convex, smooth edges	#23, 26, 30, 35 & 10

Visual observations with a microscope, indicated that fusant cells were round or slightly oblong demonstrating multilateral budding (Fig. 2). There were no "football" shaped cells or "chamydophores" observed in the fusant as was found in parental cultures (Fig. 3 & 4).

In the early stages of growth on solid media, there were some variations in colour intensity of fusants. During transfers, the darkest colonies were selected and as repeated transfers were made, the colour variation was less frequent. There were no other apparent variations with respect to growth rate optimal temperature or carbon source utilization demonstrated by the fusant.

Fusant cell lines in liquid galactose containing media grew rapidly producing pigment within 72 hrs (Fig. 5). Cells viewed using a microscope, were found to be mostly circular in shape.

Biochemical tests were also done on #26 to determine which parent it most resembled (Table I).

VI. Identification of Pigment Produced

Thin layer chromatography, spectrophotometry, and mass spectrum analysis were used in an attempt to identify the fusant's pigment. Pigments in #26 cells grown in liquid and solid media were analyzed. Pigments from *P. rhodozyma*, *Rhodosporidium toruloides*, and authentic crystalline astaxanthin (Roche) were also examined for a comparison.

a) Thin Layer Chromatography

TLC was used as a technique for identification and isolation. The solvent system used was 70:30 hexane:acetone according to the methods of Andrewes & Starr, 1976; Andrewes, Starr, & Phaff, 1976.

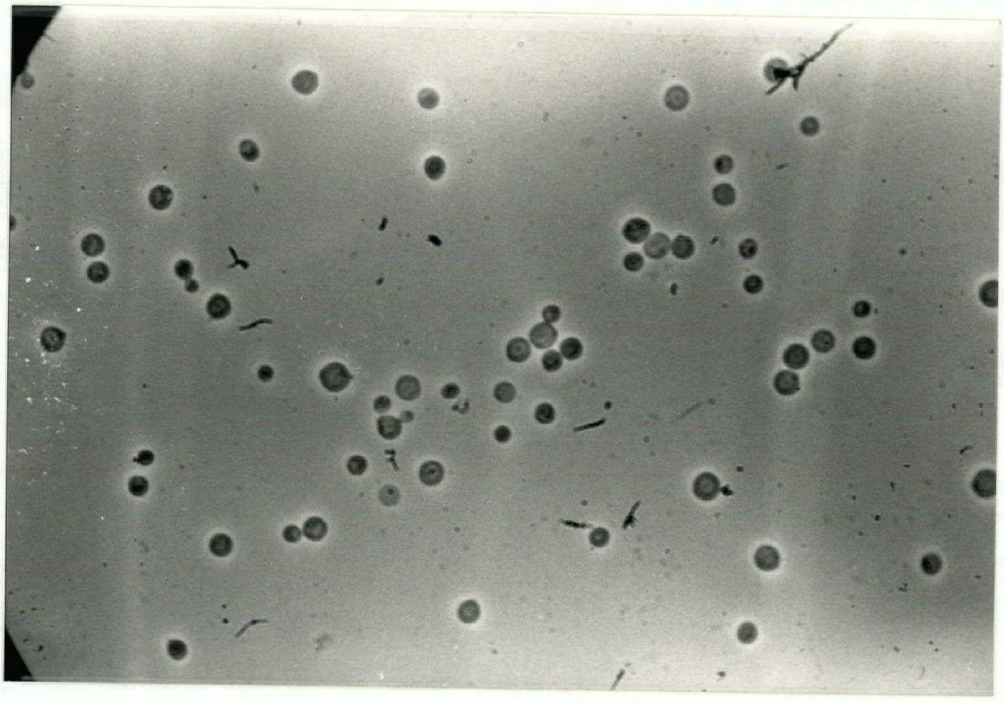


Fig. 2A Fusant #26 grown on YNB-galactose plates at 30°C (600X magnification)

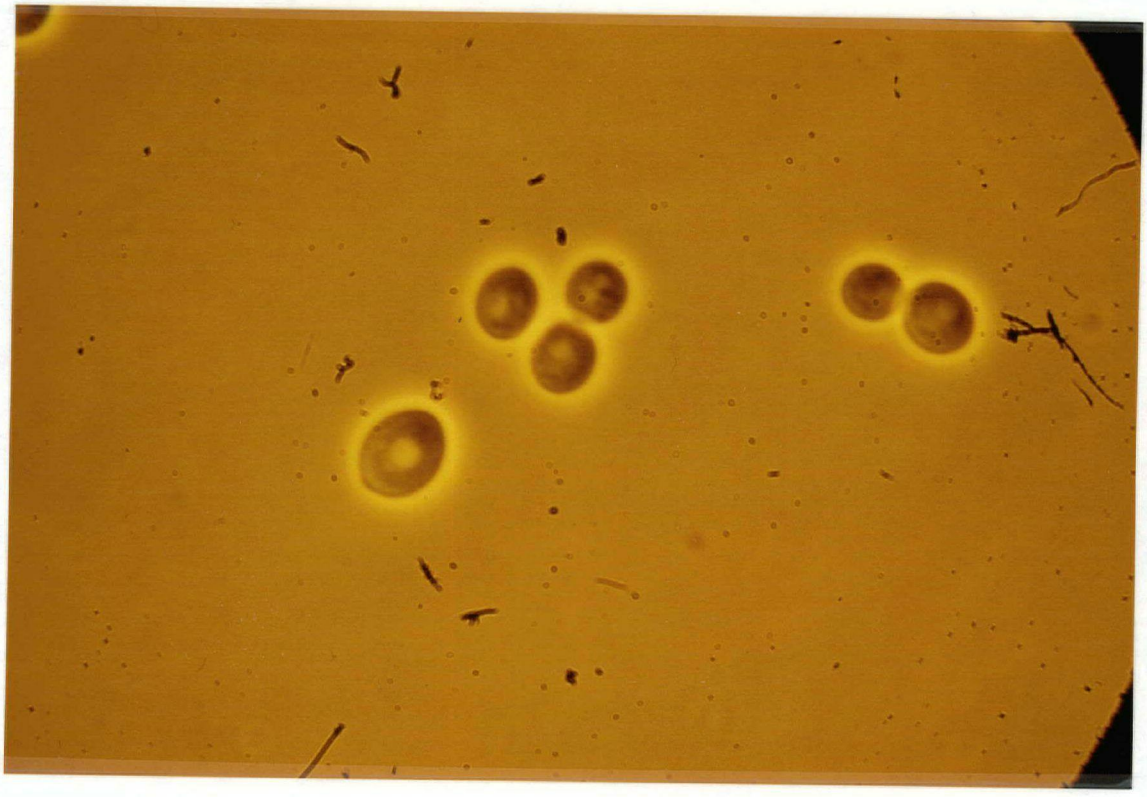


Fig. 2B Fusant #26 grown in YNB-glucose broth at 22°C (1500X magnification)

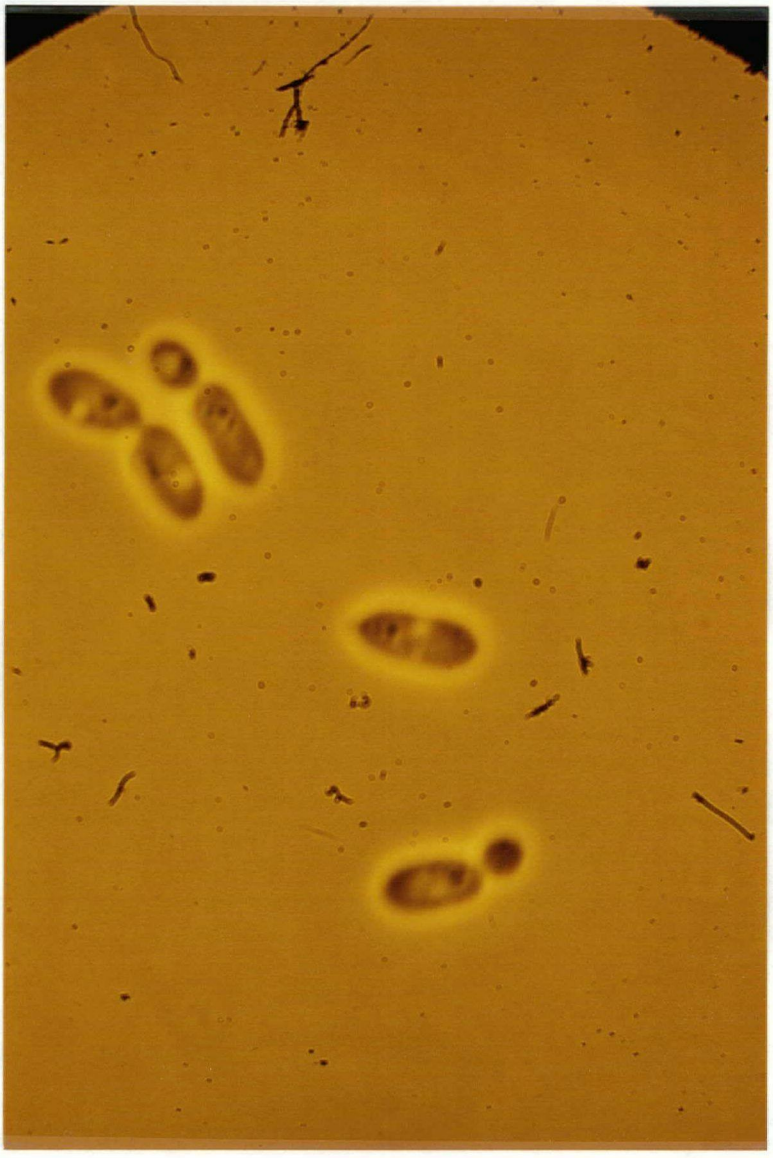


Fig. 3. R. toruloides grown on MEA at 22°C

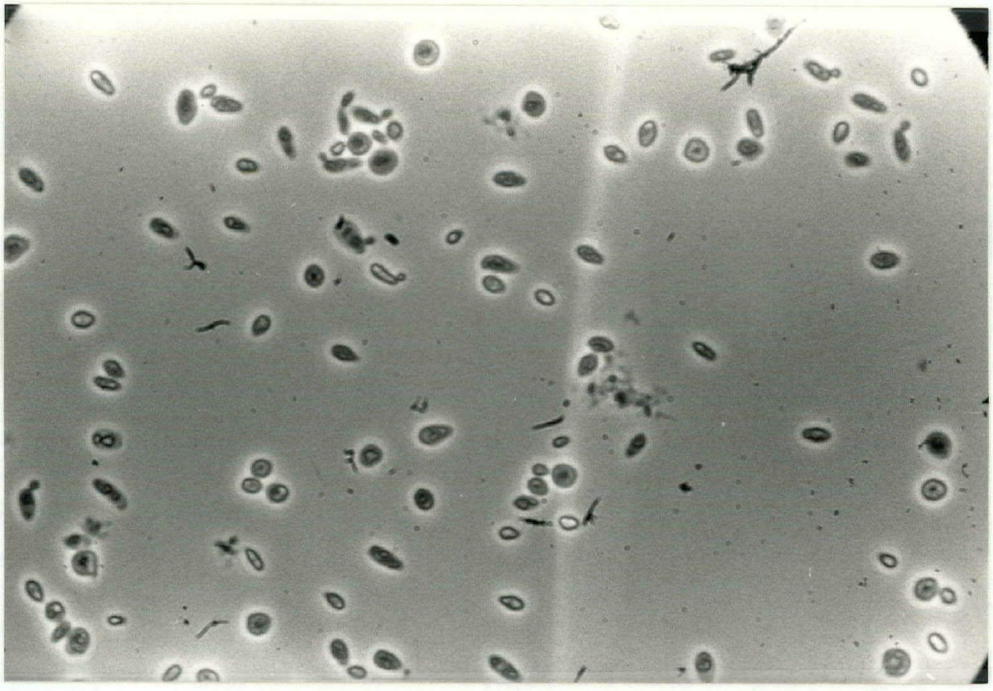


Fig. 4A *K. fragilis* grown on YNB-galactose plates at 30°C (600X magnification)

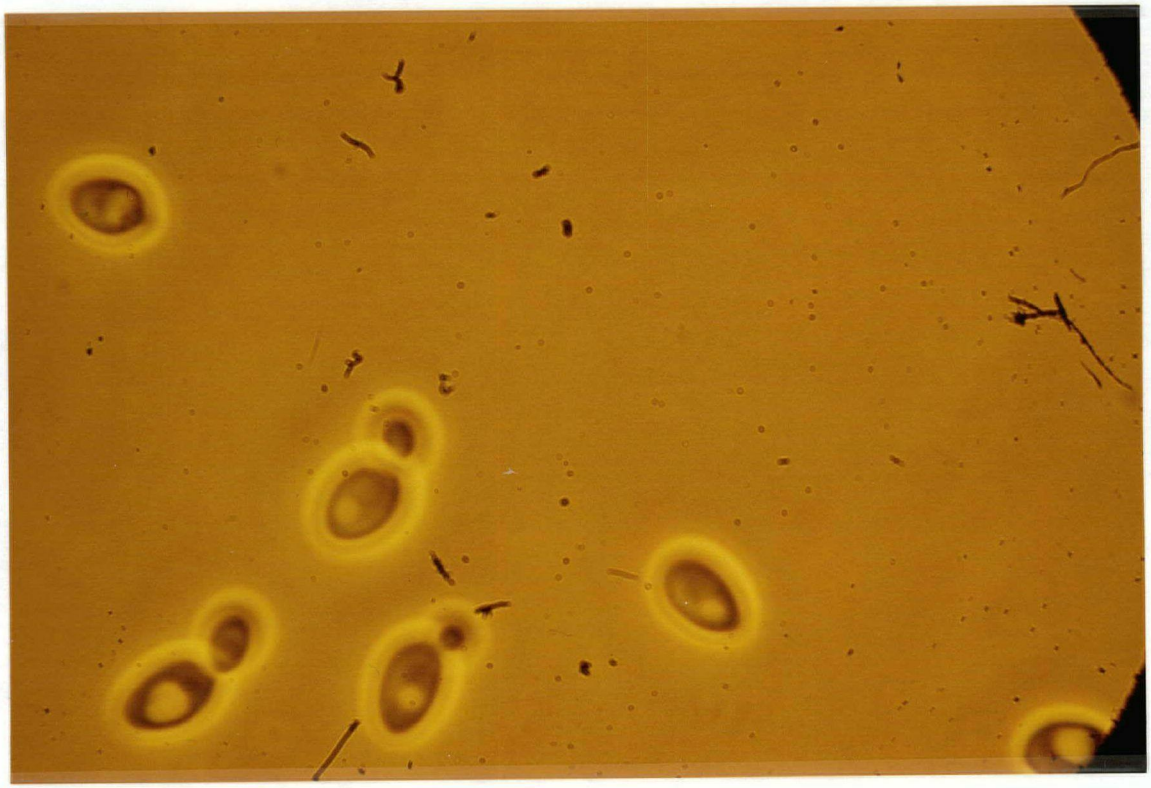


Fig. 4B *K. fragilis* grown on YNB-glucose plates at 22°C (1500X magnification)

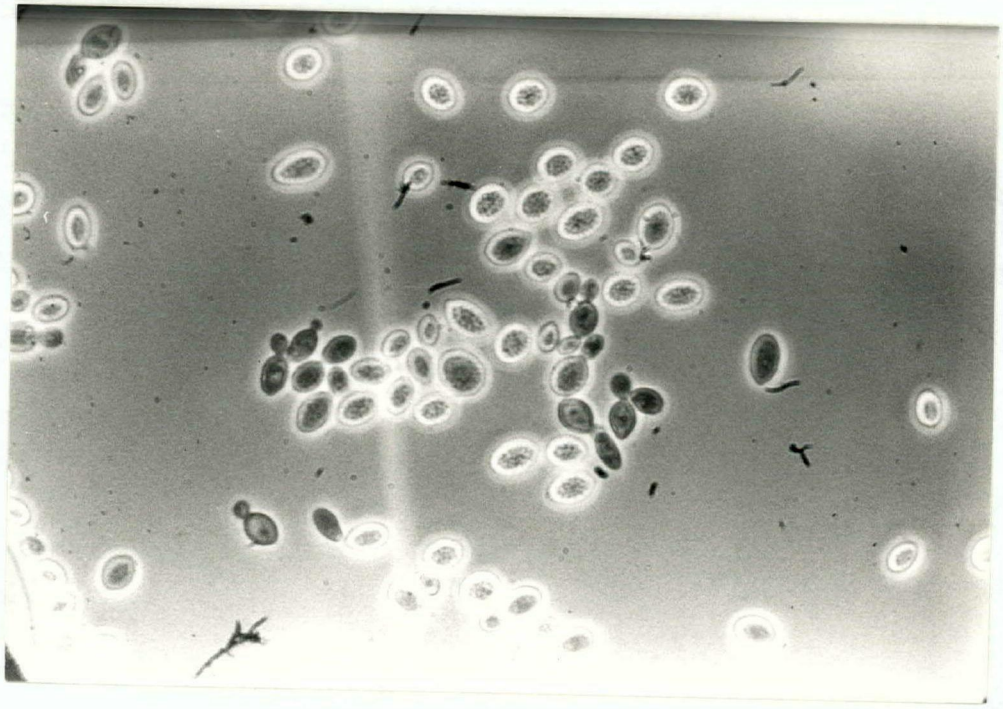


Fig. 5A *P. rhodozyma* grown on MEA plates at 22°C (600X magnification)

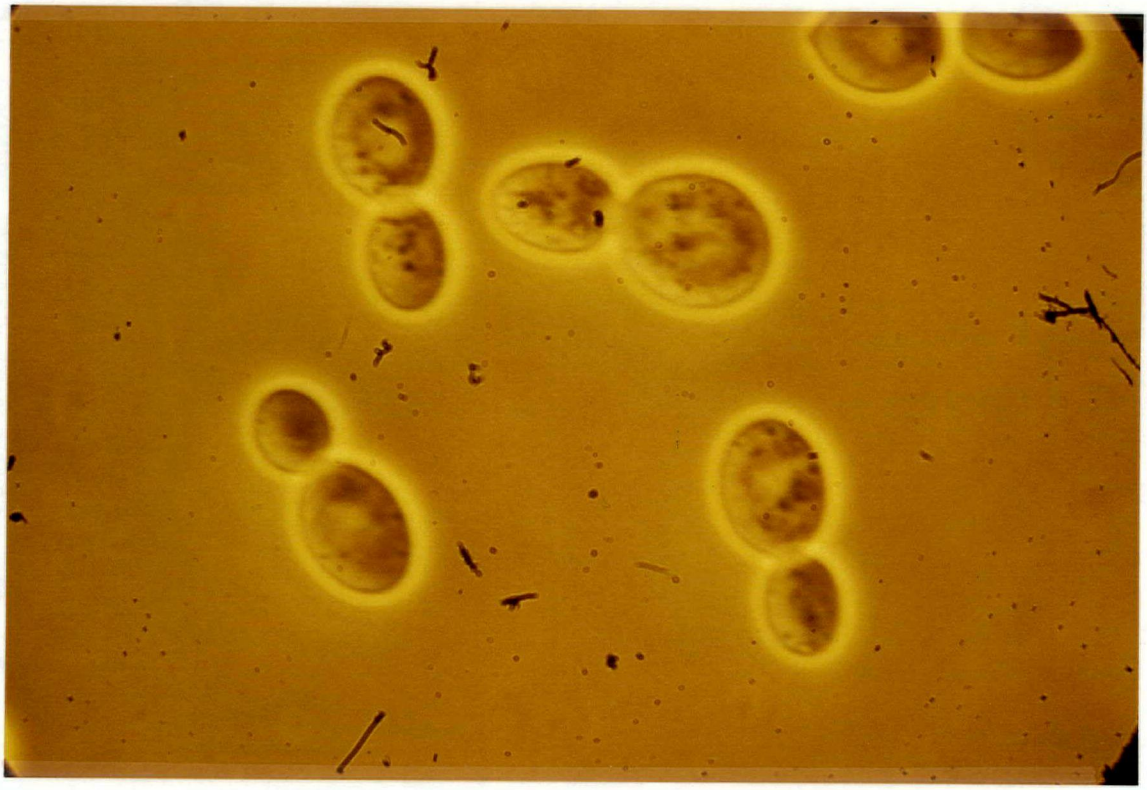


Fig. 5B *P. rhodozyma* grown on YNB-glucose broth at 22°C (1500X magnification)

Table IV. Pigments isolated from various cells and their respective TLC and absorption data.

Pigment	Rf	(λmax) Visible Absorption (nm)
Astaxanthin (Roche)	.24	470
Fusant #26 (plate)	.24	471
Fusant #26 (liquid)		
A (acetone fraction)	.30	470
B (hexane fraction)	.67	473
β-carotene (Sigma)	.72	447
R. toruloides (plate)		
A (hexane:acetone)	.25	487
B (acetone fraction)	.64	483
Phaffia rhodoxyma (plate)	.24	470

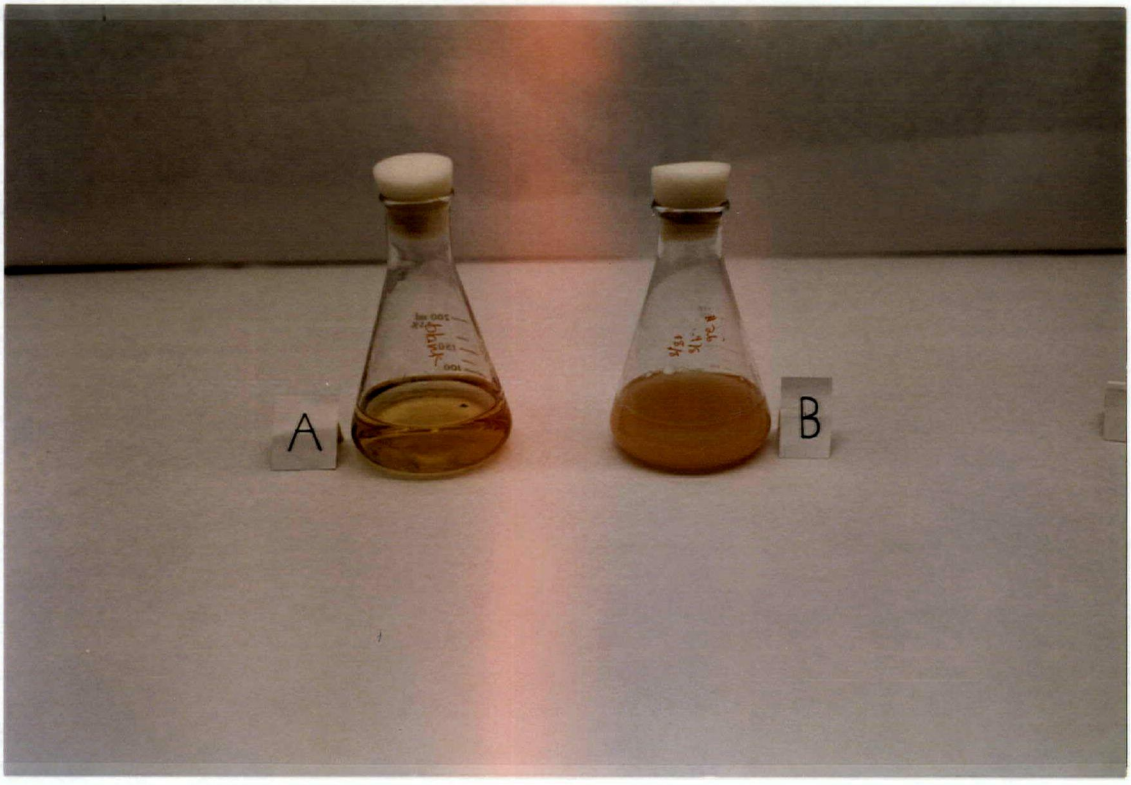


Fig. 6 250 ml flask culture of Fusant #26 grown on Standard Media + galactose at 30°C - 72 hours old. (A - blank; B - Fusant #26)

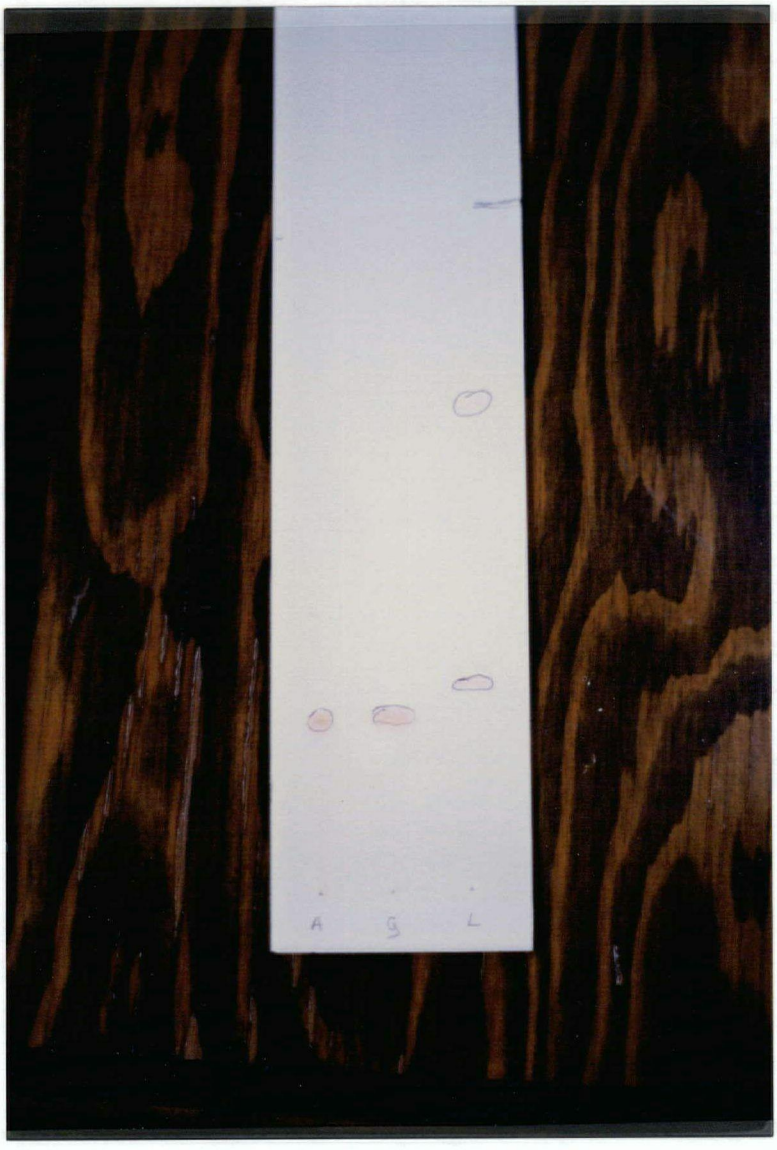


Fig. 7 TLC of all-trans astaxanthin (A), Pigments from Fusant #26 grown on solid media (G) and Pigments from Fusant #26 growin in media (L).

a) 1. Cultures from Solid Media

The carotenoid pigments taken from fusants grown on solid media were extracted into acetone and spotted on silica gel plates and compared to astaxanthin (Roche) and to P. rhodozyma extracts. When run in the afore mentioned solvents, all three pigments from the three sources had identical Rf values of 0.24 (Fig. 6). Co-chromatography of the fusant pigments with astaxanthin resulted in a single band (data not shown). The pigments also had a similar orange colour.

2. Cultures from Liquid Media

The pigments taken from cultures grown on liquid media were spotted on silica gel plates and as above compared to astaxanthin and P. rhodozyma (plates) extracts. These pigments separated into several bands, the first being red in colour with an Rf of .30 and the second being orange with an Rf of 0.67. Neither of these pigments would co-chromatograph with astaxanthin, or P. rhodozyma pigments.

b) Silica Gel Column Chromatography

When pigments from cultures of Phaffia and the fusant #26 from galactose containing solid media were placed onto the silica gel column there was no movement of the pigment bands with the hexane wash. As the increasing, volume ratios of acetone were added to the hexane starting with 1:24 acetone:hexane the pigment started to move as a single band through the column. Recovery of the band was achieved with 100% acetone (Table VI).

When pigments from fusant #26 grown in liquid galactose containing medium were chromatographed, fraction (B) was eluted using 1:12 acetone:hexane while the second fraction (A) could only be completely removed using 100% acetone.

c) Spectrophotometry

1. Cultures grown on Solid Media

Pigments isolated from fusant #26 grown on solid media purified on a silica gel column were dissolved in acetone and scanned over the visible range. These cells produced a spectra that contained a single peak with λ_{max} at 471 nm. This single broad peak corresponds with that of the astaxanthin standard and pigments isolated from Phaffia solid culture that had λ_{max} at 470 nm (Fig. 8). This differs from the β-carotene standard (Roche) (λ_{max} 447 nm) (Fig. 9) that has 3 bands and Rhodosporidium toruloides (Fig. 10) λ_{max} 487 nm for the fraction eluded with acetone and 483 nm for the pigment eluded with hexane (Table IV).

2. Cultures grown in Liquid Media

The pigments isolated from the liquid culture of fusant #26 were eluded from the silica gel column in 2 fractions, the first eluded with hexane (B) and the second with acetone (A). These pigments have a spectra with pigment A having λ_{max} at 470 nm but displaying a large shoulder at 535 nm and pigment B having λ_{max} at 473 nm having three inflections more typical of β-carotene. These pigments differ from those isolated from Rhodosporidium toruloides (Fig. 10).

d) Mass Spectrometry

Carotenoid pigments from fusant #26 cultured in liquid & solid media, P. rhodozyma and R. toruloides cultured in solid media were separated on a silica gel (BDH) column. When each fraction was then checked by thin layer chromatography, it produced a single band. The Mass Spectra observed are shown in Figs. 11-14.

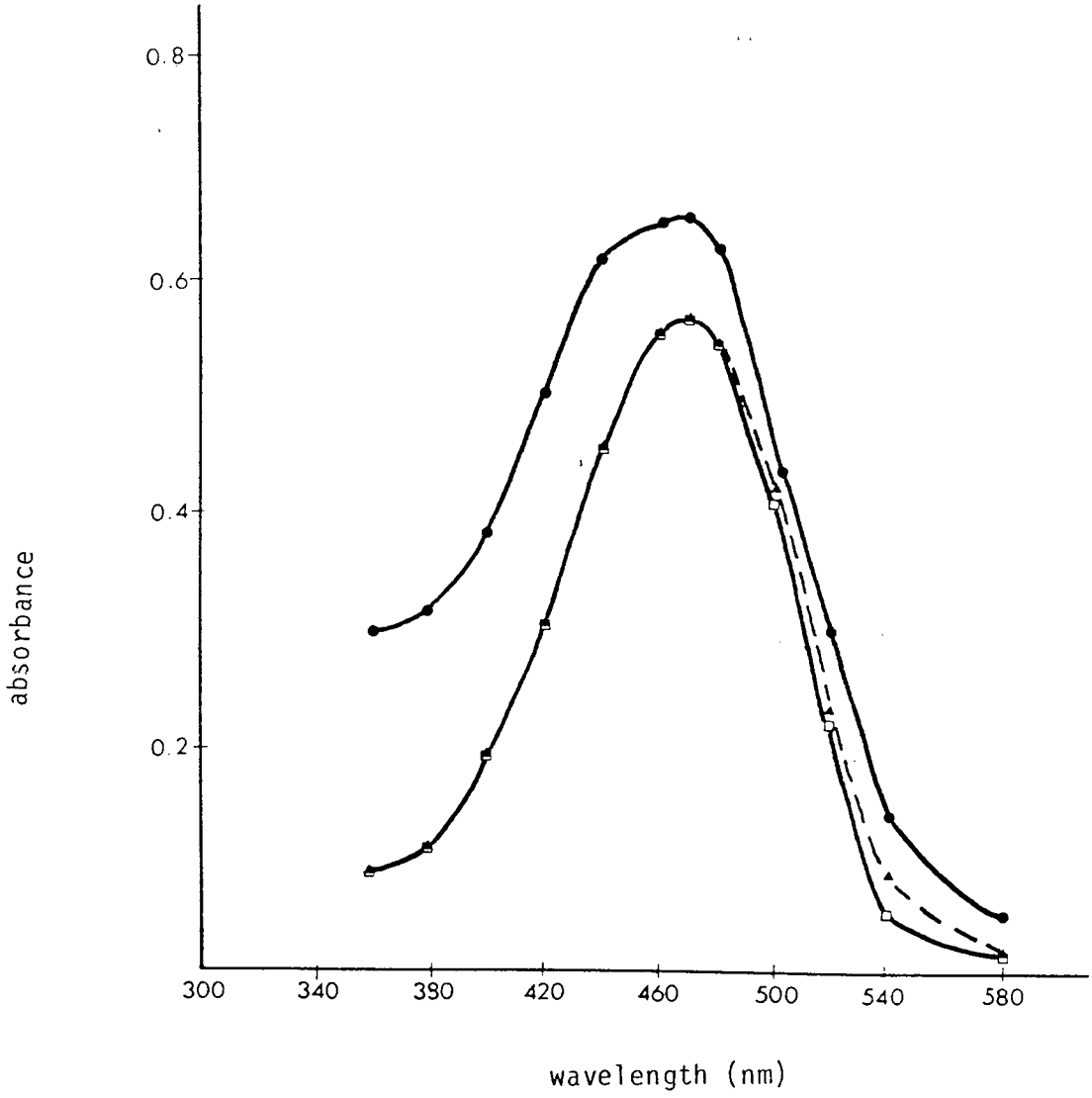


Fig. 8 Visible Absorption Spectra of pigments from P. rhodozyma, all-trans astaxanthin and Fusant #26 grown on solid media.

□-astaxanthin ; ●-P. rhodozyma ; ▲-fusant 26

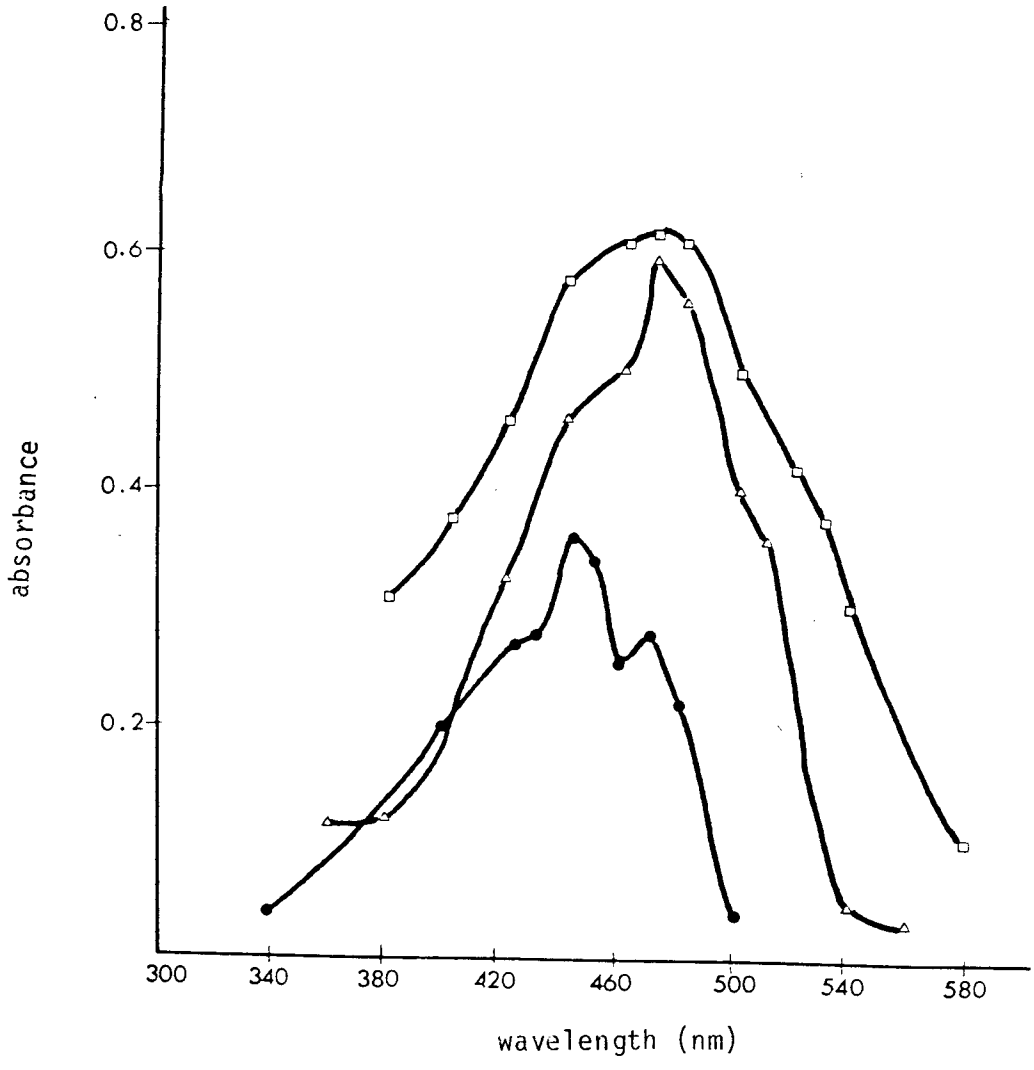


Fig. 9 Visible Absorption Spectra of β-carotene and pigments from the 2 fractions of Fusant #26 grown on liquid media.

● - β-carotene ; □ - fraction A; △ - fraction B

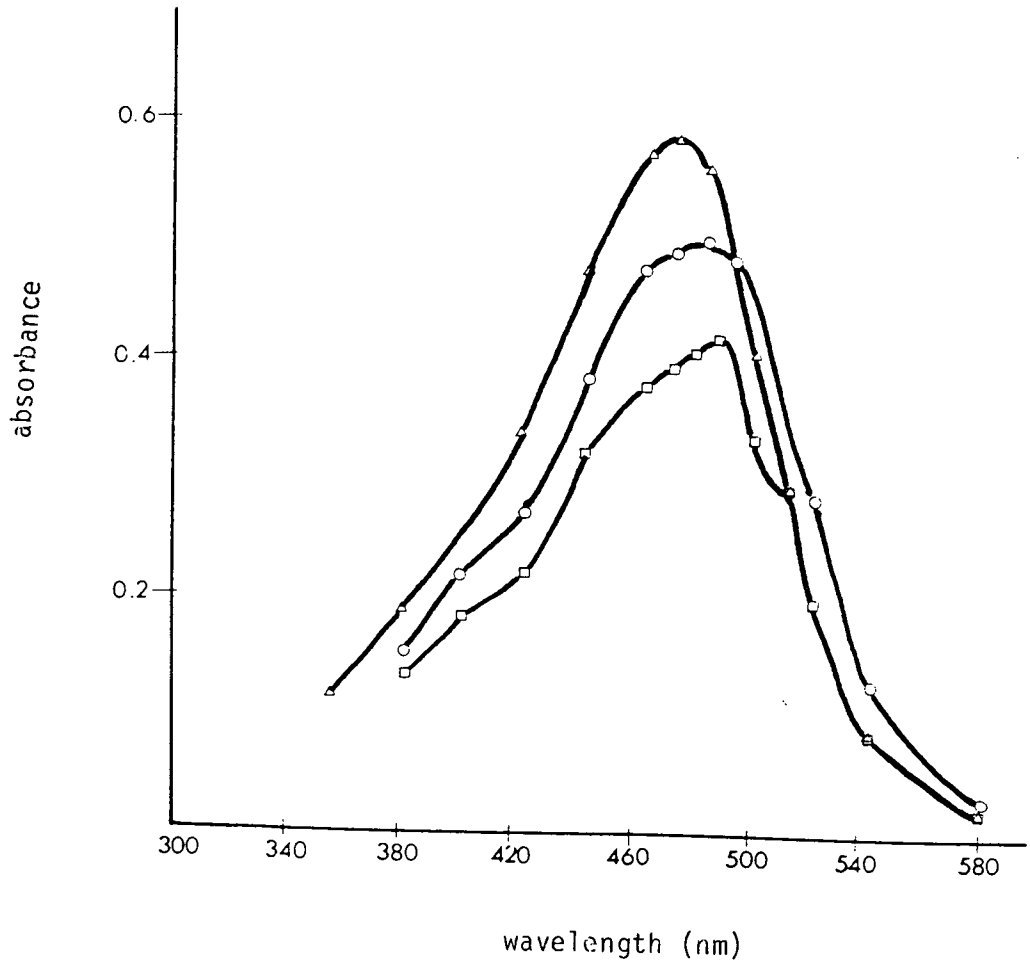


Fig. 10 Visible Absorption Spectra of pigments isolated from Fusant #26 (solid) and R. toruloides.
△-**fusant 26** ; ○-**fraction A** ; □-**fraction B**

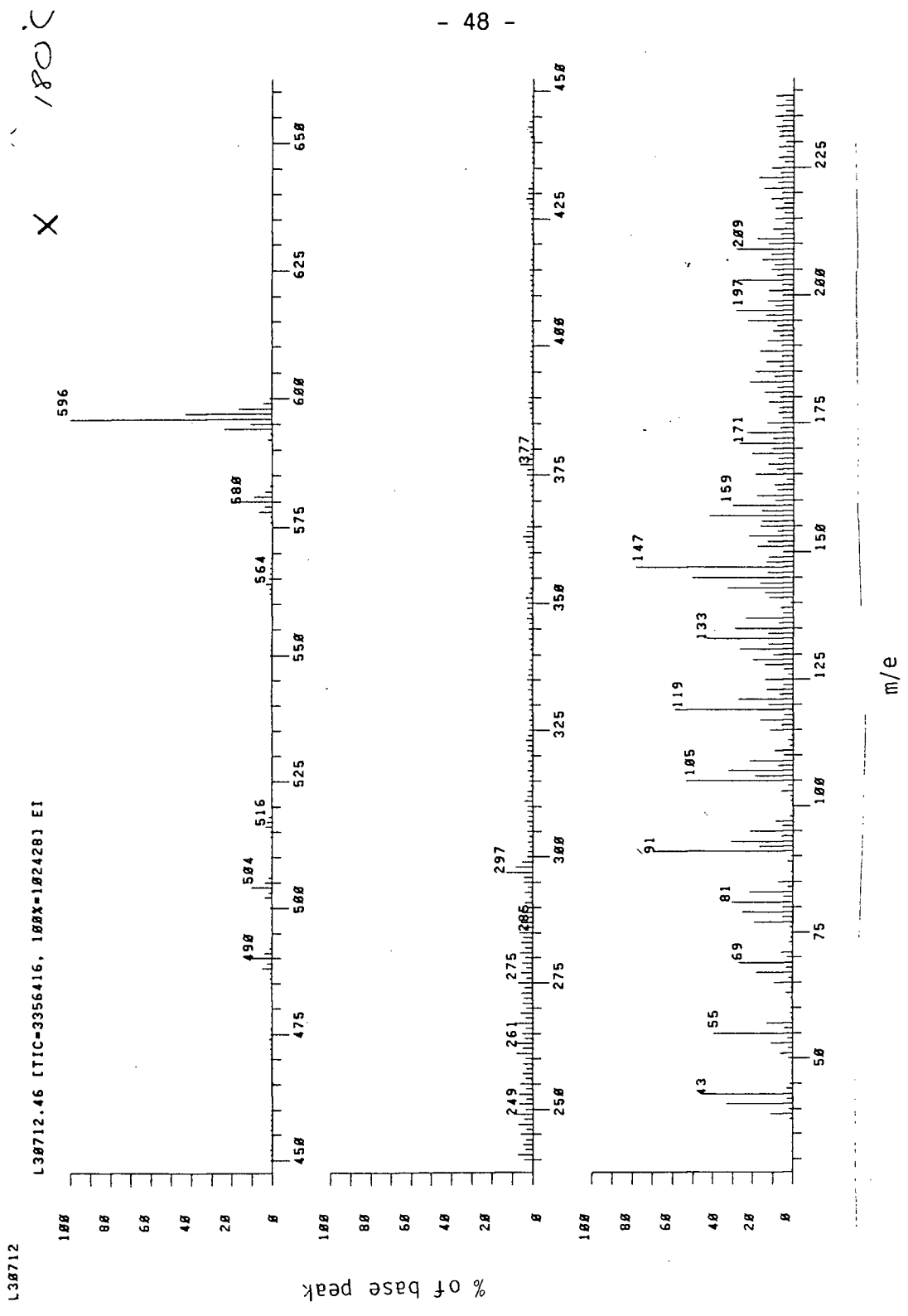


Fig. 11 Mass Spectrum of Astaxanthin.

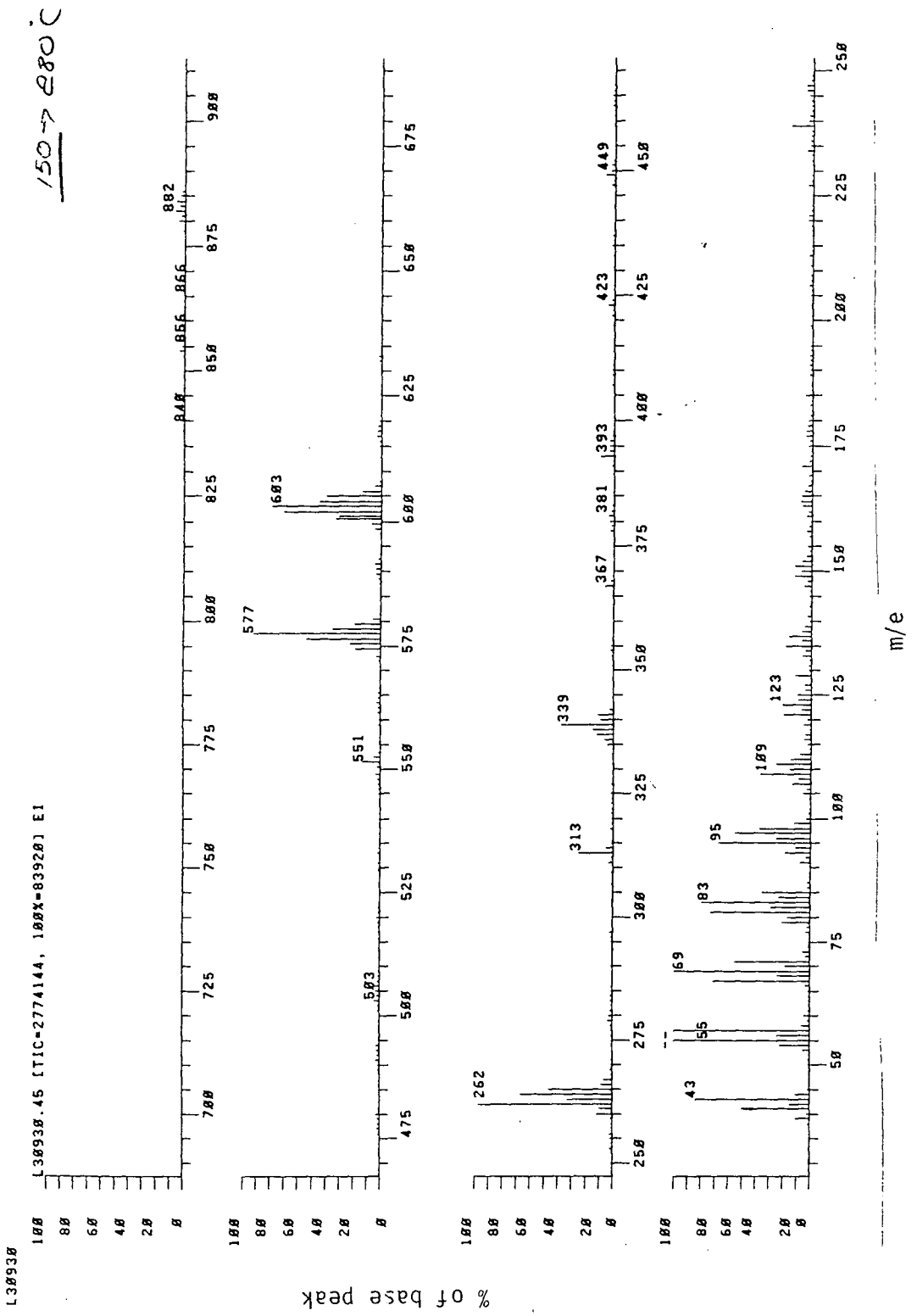


Fig. 12 Mass Spectrum of pigments isolated from P. rhodozyma grown on MEA at 22°C.

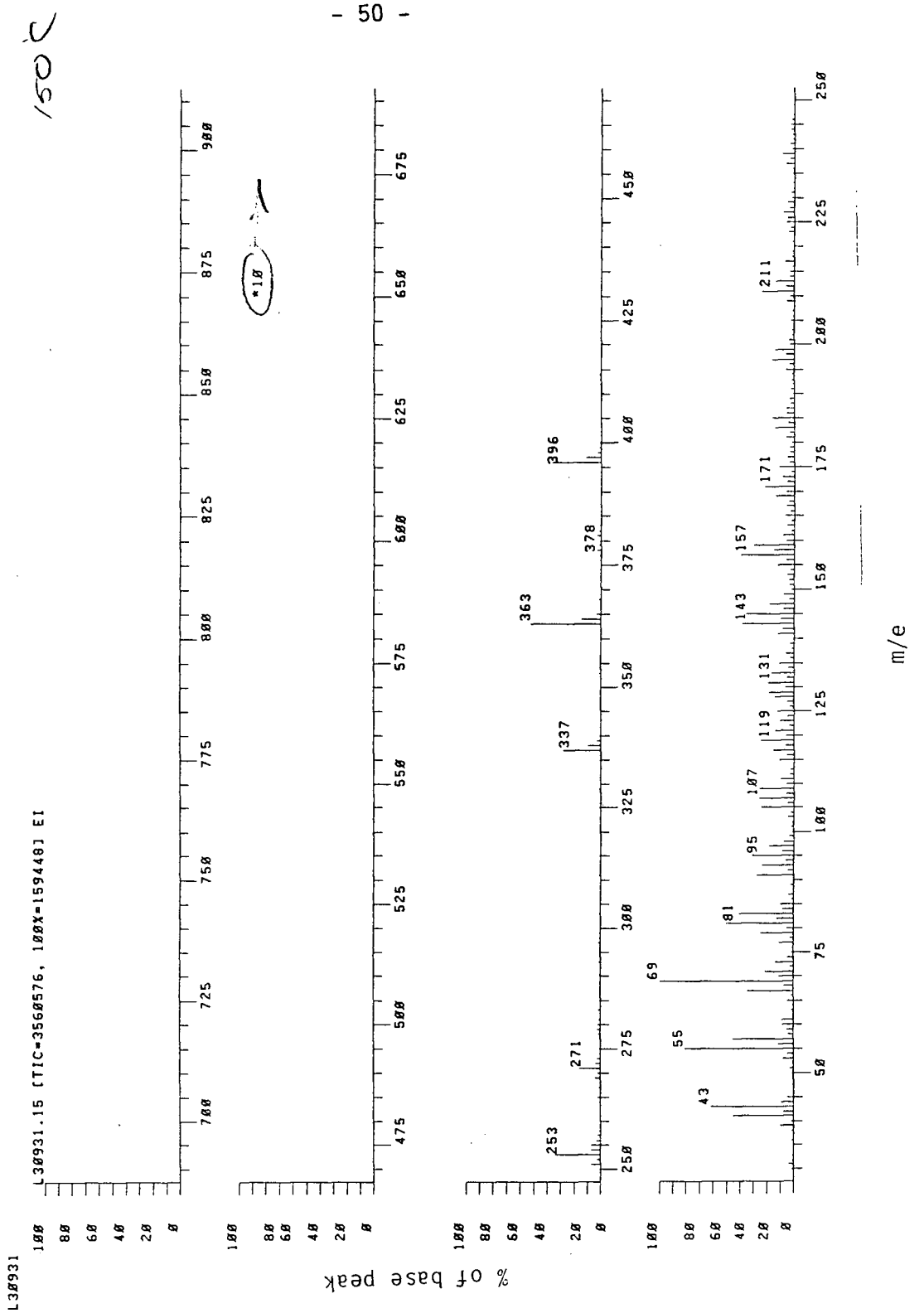


Fig. 13 Mass Spectrum of pigments isolated from Fusant #26 grown on galactose plates - probe temperature 150°C -

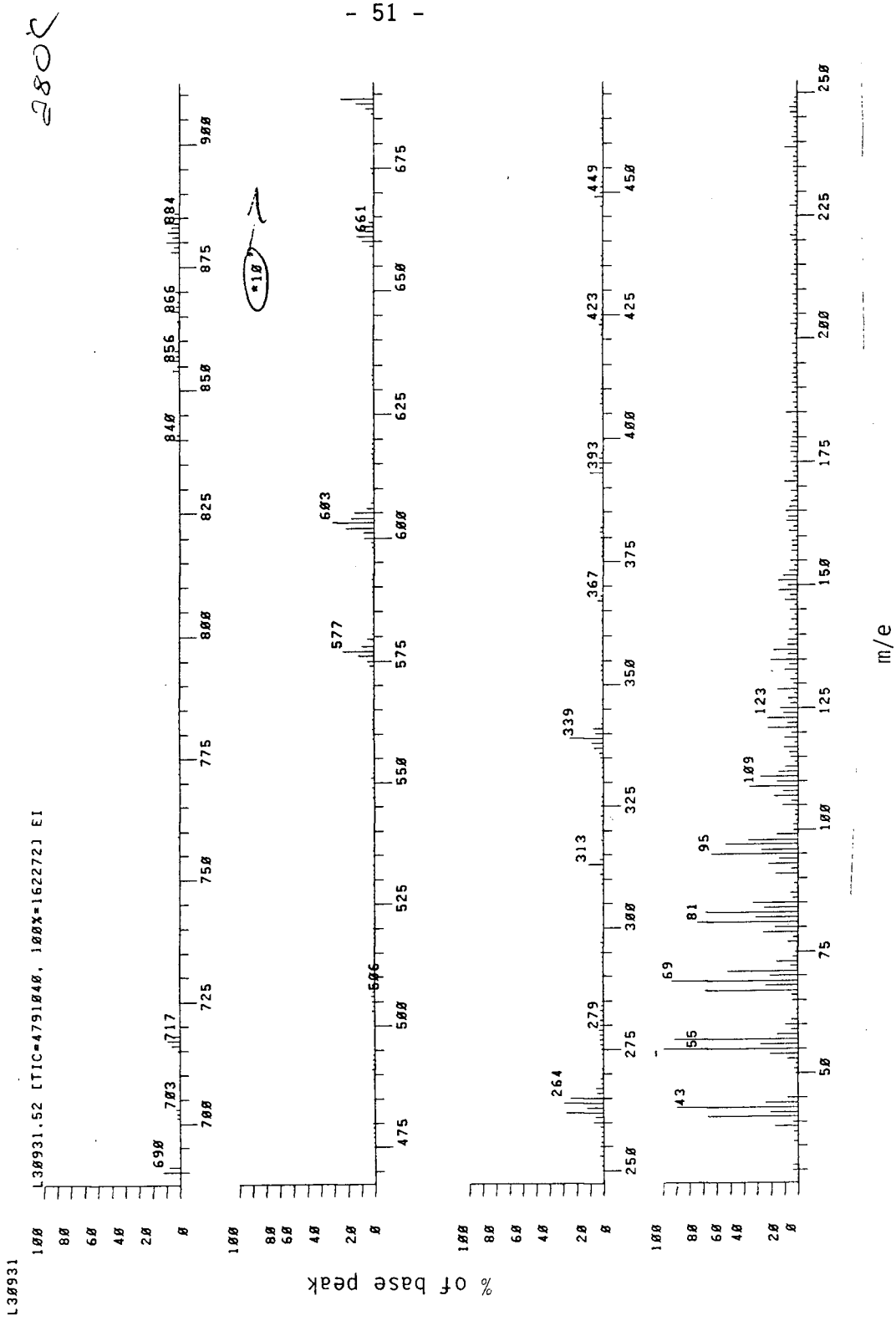


Fig. 14 Mass Spectrum of pigments isolated from Fusant #26 grown on galactose plates - probe temperature 280°C -

One obvious conclusion from mass spectrometry is that none of the spectra obtained from the cell extracts corresponds to that of the all-trans Astaxanthin (Roche) (Fig. 11). The all-trans astaxanthin does give a characteristic mass peak at 596, however the additional but peaks considered to be characteristic such as M-154, M-167 and M-207 are missing and two of the peaks that are present M-219 and M-233 do not have expected intensities.

The pigment from Phaffia (Fig. 12) and the pigment from fusant #26 grown on galactose plates (Fig. 14) are very similar even with respect to their high molecular weight material, and in fact could be considered to be the same (Eigendorff, personal communication).

e) Colour reactions

Astaxanthin and pigments from both liquid and plate cultures of fusant #26 produced a dark blue colour; and β-carotene a light blue colour upon the addition of concentrated sulphuric acid. Pigments from R. toruloides turned immediately colourless (Table V).

Table V. Colour change test of various carotenoids upon the addition of concentrated sulphuric acid to acetone solutions.

Carotenoid	Colour of soln. before H_2SO_4	Colour of soln. after H_2SO_4 added
astaxanthin	dark orange/red	dark blue
β-carotene	yellow	light blue
fusant #26 plate	orange	medium-dark blue
P. rhodozyma	orange	medium-dark blue
Rhodosporidium sp. (acetone:hexane fraction)	pink/orange	colourless
(acetone fraction)	pink	colourless

Table VI. Elution characteristics of pigments isolated from *P. rhodozyma* Fusant #26, and *R. toruloides* on a silica gel 60 column.

Carotenoid	Elution solvent
astaxanthin	acetone
β-carotene	hexane
fusant #26 (plate)	acetone
P. rhodozyma	acetone
Fusant #26 (liquid)	
fraction B	hexane:acetone (12:1)
fraction A	acetone
R. toruloides	
fraction A	hexane:acetone (14:1)
fraction B	hexane:acetone (1:9)

DISCUSSION

The chief aim of this study was to isolate a strain of yeast having characteristics favouring the production of carotenoids from galactose containing medium, while also possessing a thin wall to promote extraction of the pigments. To this goal, P. rhodozyma was chosen for its ability to produce astaxanthin, and K. fragilis for its ability to grow on galactose containing medium.

Enzymatic removal of the cell wall of P. rhodozyma grown under normal conditions met with very little success as expected. The literature suggests that there are multiple layers in the cell wall (Miller et al. 1976) and this factor, as well as the α 1-3 glucans make it resistant to most lytic enzymes. When morphological differences were forced to occur due to the effect of the AEC, areas of the cell wall that were thinner microscopically, were then able to be attacked by the lytic enzymes. Often these susceptible cells resembled chlamydophores, and it appeared that the "germination tube" of these structures that were attacked. When these cells germinated in the presence of AEC the resulting pseudomycelium and pleomorphic cells (Fig. 1) also showed a lack of resistance to enzymatic digestion of the cell wall suggesting that the cells were severely affected by the AEC. This particular cell type remains while the cells grow in AEC but, upon return to MEA the characteristic wall and shape of P. rhodozyma returns.

The other amino acid analogues tested (Table II) did not produce such desirable cells. It is known that many cultures that are resistant to analogues such as AEC are able to accumulate L-lysine due to the change in regulation of aspartokinase (Toskata et al. 1978). It is

possible that in Phaffia components that make up the cell wall are likewise affected.

As compared to other just species grown on media without amino acid analogues, the number of protoplasts recovered from P. rhodozyma cells grown on AEC was quite low. A number of inherent factors could be to blame for this. The release of the protoplasts were not synchronous and in fact occurred over a long period of time possibly due to inconsistencies in the cell wall structure or composition. Because of this, the concentration of the cell wall digestive enzyme mixture was high, and thus protoplasts formed early in the digestion period were most likely destroyed. Not all of the cells exposed to the AEC developed thin walls and odd shapes, thus a percentage of the population likely may not be affected at all by the enzymes. Protoplasts formed later in the digestion period were chosen because a proportionally greater number were released at the later stages.

On the other hand, the recovery of protoplasts from K. fragilis was simple. The young cells were converted to protoplasts within 1 1/2 hours denoting one of the positive qualities to be transfered to a fusant. The high protoplast forming efficiencies observed in this study correlated with those reported by Morgan et al. (1980) and Johannsen et al. (1984).

Two different approaches for the fusion of the yeast protoplasts were attempted. The conventional PEG method and the dipole electroporation method of Rivera et al. (1983). Electrofusion has been reported to have an enhanced fusion efficiency when compared to the conventional PEG method (Bates et al. 1986). In the end, the

electrofusion was very successful producing a number of the selected presumed fusants including fusant #26.

When one considers the actual number of presumed fusants selected as compared to the number of protoplasts, the number seems quite low (see Table III). One must remember that the fusants selected were chosen for their specific properties of growth on galactose and pigment formation. Other fusions that may not have produced the stable desired characteristics are also likely to have occurred. Low numbers of presumed fusants may also be attributed to the suspected poor viability of the Phaffia protoplasts which may not have been able to regenerate their cell walls.

A reduced number of presumed fusants may also be attributed to the voltages used to induce fusion. The voltage selected and applied at the electrodes of the dipole cell fuser was 2700 volts. The distance between the electrodes depending on the experiment ranged from 1 to 2 mm. This high voltage may have affected the regeneration ability of the protoplasts.

PEG in contrast to electrofusion is a much slower method for fusion. The PEG itself does not cause fusion to occur, rather it draws the cells into close association and upon dilution of the chemical, fusion may occur (Peberdy, 1980). This drawing together of cells was accomplished more rapidly and successfully by AC rotating field with the added benefit of inducing fusion by the electric pulse. Some presumed fusants were obtained using PEG such as fusant #22. These fusants did possess qualities such as cell morphology, galactose assimilation and pigmentation, similar to those obtained by electrofusion (data not

shown). This suggests that in these species of yeasts both methods can be used with some success. The advantages associated with the electrofusion include the synchrony of fusion making selection easier, and the reduced handling of the fragile protoplasts, as well as avoiding the potentially harmful effects of PEG addition.

Fusants were selected with the projected goal in mind. The parent Phaffia was discouraged from growth by growing the presumed fusants at 30°C, 3°C above the reported growth temperature limit (Miller et al. 1976), and on YNB-galactose since the parental strains cannot assimilate galactose. The parent K. fragilis on the other hand could be selected against by virtue of colony colour as growth was excellent both at 30°C and on galactose. Other selective media were also used successfully to achieve differentiation of the fusant from the parent culture e.g. niacin requirement of K. fragilis.

The presumed fusant colonies often started out relatively small with very little colour though morphology varied slightly (Table III). Often K. fragilis would overgrow the plate (hence the need to control the growth rate of the K. fragilis parent. The accumulation of carotenoid pigments is considered to be a product of secondary metabolism and usually occurs in late stages of growth. On repeated selection and transfer of coloured colonies, stable cell lines of the fusants were obtained. There is evidence for Phaffia being the recipient of genetic material from K. fragilis as shown in Table 1.

The presumed fusants have a cellular morphology that is quite different than that of Phaffia (Fig. 2 & 4). These differences include

the circular shape of the fusant on YNB-galactose, the thinner cell wall as well as the ability to bud multilaterally. Bright spots within the cytoplasm are also evident in galactose media and may be intracellular lipids similar to those produced by K. fragilis. The morphology and biochemical test results of the red yeast R. toruloides are uniquely different from that of the parent Phaffia or the resultant carotenoid producing fusant #26 (Figs. 2, 3 & 4).

The biochemical tests that were done were chosen for their ability to differentiate between the parental strains as well as offering some differences from possible carotenoid-containing yeast contaminants such as R. toruloides. The presumed fusants appear to demonstrate characteristics that can be considered typical to each parental type (Table I). This is not unexpected since fusion was a random procedure offering no control exerted on the recombination of chromosomes.

The data obtained from the biochemical tests, Table I suggests that is is difficult to determine which parental strain is the donor and which is the recipient. Viewing evidence such as carotenoid production, urease activity and cell growth without niacin, one may venture to suggest that Phaffia is the recipient. Characteristics such as galactose assimilation and heat tolerance were transferred possibly from K. fragilis.

One characteristic common to both P. rhodozyma and K. fragilis is their inability to use nitrate as a source of nitrogen. Fusant #26 is also unable to utilize nitrate. This differs from R. toruloides a common red yeast which is able to use nitrate. Cells grown in various media will also change their morphology; for example, when the presumed

fusants are grown in galactose they are circular unlike either parent or R. toruloides. These very same cells when grown in glucose broth, develop a shape that appears to be the same as P. rhodozyma grown under similar conditions only smaller (see Figs. 2B & 5B). In glucose broth K. fragilis is pear shaped and R. toruloides is elongate (see Figs. 3 & 4B). This again supports the idea of Phaffia being the recipient.

The literature suggests that 85% of the carotenoids formed by P. rhodoyzma under most ambient growth conditions are astaxanthin (Andrewes et al. 1976, Johnson & Lewis 1979). Because of this, authentic all-trans astaxanthin was chosen to be the carotenoid to use for comparisons.

Carotenoid analyses from TLC, column chromatography, chemical reactions, absorption spectra and mass spectrophotometry indicate that the pigments obtained from P. rhodozyma and fusant #26 grown on solid media are the same (Figs. 7, 8, 12, 14 and Tables IV, V). These pigments in turn, tend to correlate closely with the all-trans astaxanthin (Roche) with the exception of the mass spectrometry data. The results also indicate that the pigments from fusant #26 (solid media) and P. rhodozyma are not the same as those isolated from the common red yeast R. toruloides (Fig. 10 and Tables IV, V).

Oxo-carotenoids have a characteristic absorbtion spectra, as do the β-carotenes. Due to the high levels of unsaturation within the molecule these peaks also tend to be broad (Davies 1965). The pigments isolated from P. rhodozyma and fusant #26 grown on solid media have this broad characteristic single peak of the xanthophylls (Fig. 8). The shape of the absorption curve plus their λ_{max} correlates closely with authentic

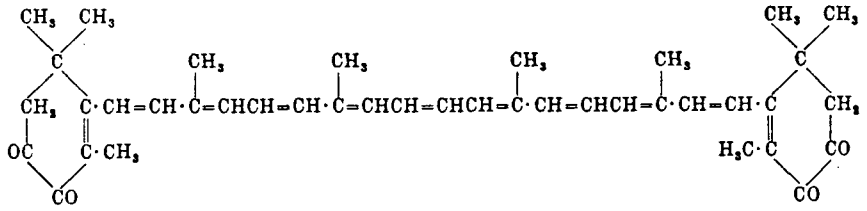
astaxanthin; in fact, the spectra from fusant #26 can almost be superimposed on that from astaxanthin (Fig. 8). Other xanthophyllic-like structures can also be observed in the fraction (A) from the fusant #26's liquid culture as well as fraction (B) from R. toruloides.

The liquid cultures of fusant #26 were unable to produce pigments that resembles those of P. rhodozyma grown on solid media. However, on liquid medium under limited oxygen supply Phaffia alters its carotenoid synthesis, producing β-carotene in excess. Thus the carotenoids produced by these cultures appears to be a function of the culture conditions. Johnson & Lewis (1979) suggest that under fermentative or austere conditions an accumulation of astaxanthin precursors may occur in addition to a decrease in astaxanthin levels. It is possible that a similar event is occurring in these liquid cultures of fusant #26 due to insufficient aeration or other such undiscovered limitations. The two pigments obtained may be astaxanthin precursors; further work along this line is to be done in the future.

The anomaly of the mass spectrometry data can be considered to be not less than confusing. One highly irregular result was the lack of the mass peak at 596 in the pigment isolated from P. rhodozyma which according to the literature (Andrewes et al. 1976; Johnson & Lewis 1979), should be present due to high percentages of astaxanthin reported in the cells (Fig. 12). (These cells were grown and the pigment isolated under apparently identical conditions as reported by Andrewes et al. (1976).) Even the authentic astaxanthin used gave less than encouraging results since three of the characteristic peaks were missing (see Fig. 11).

The results from the mass spectral data show that when the samples were run at two different temperatures, there are quite large differences between these runs. This denotes one of the inherent problems of mass spectrometry as suggested by Moss & Weedon (1976). Irregularities may have occurred due to sample impurities, probe temperatures, insertion conditions or ionization (Moss & Weedon 1976). Again, though one cannot conclude that the carotenoid is astaxanthin, from the mass spectra data, the other tests do suggest that pigments from P. rhodozyma and fusant #26 (solid media) are similar.

Carotenoids can also be distinguished by colour reactions of pigments when subjected to strong acids and bases (Karrer & Jucker 1950). In the presence of air astaxanthin is converted to its oxidized derivative, astacene (formula 6). Astacene upon the addition of



astacene

Formula 6

concentrated sulphuric acid will turn a deep blue (Karrer & Jucker 1950). Results obtained from Table V indicate this occurrance. Colour differences between dark blue and medium blue may be attributed to concentration. Again a reaction difference can be noted when fusant #26 is compared to R. toruloides.

CONCLUSIONS

During the course of this study, an intergeneric cross of *K. fragilis* and *P. rhodozyma* was carried out. The resulting presumed fusants exhibit characteristics common to both parental types such as growth on galactose, heat tolerance and carotenoid pigment production. Morphologically the presumed fusants are most like *P. rhodozyma* suggesting that *P. rhodozyma* is the recipient strain.

Though the carotenoid pigments isolated from fusant #26 and *P. rhodozyma* cannot be called astaxanthin due to the anomally of the mass spectrometry data, they are similar to each other according to all other tests. TLC, visible spectra, column chromatography and colour reactions indicate that the pigment could possibly be astaxanthin.

The presumed fusant cell line #26 has a microscopically thin cell wall that is easy to rupture thus liberating the cellular carotenoids promptly.

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APPENDIX I

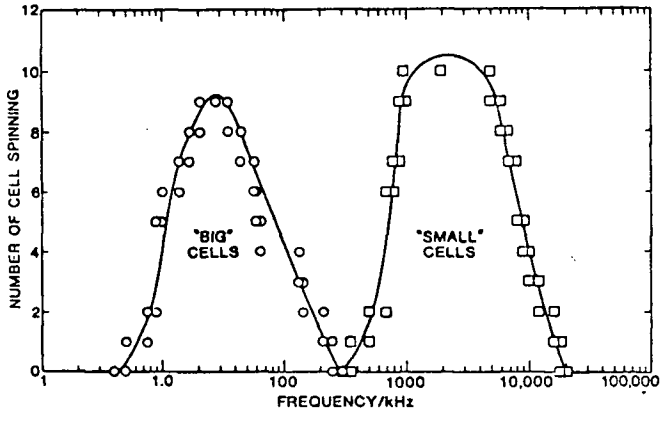

Fig. 17 Spin ranges of individual unbudded yeast cells.

from Pohl et al., 1982

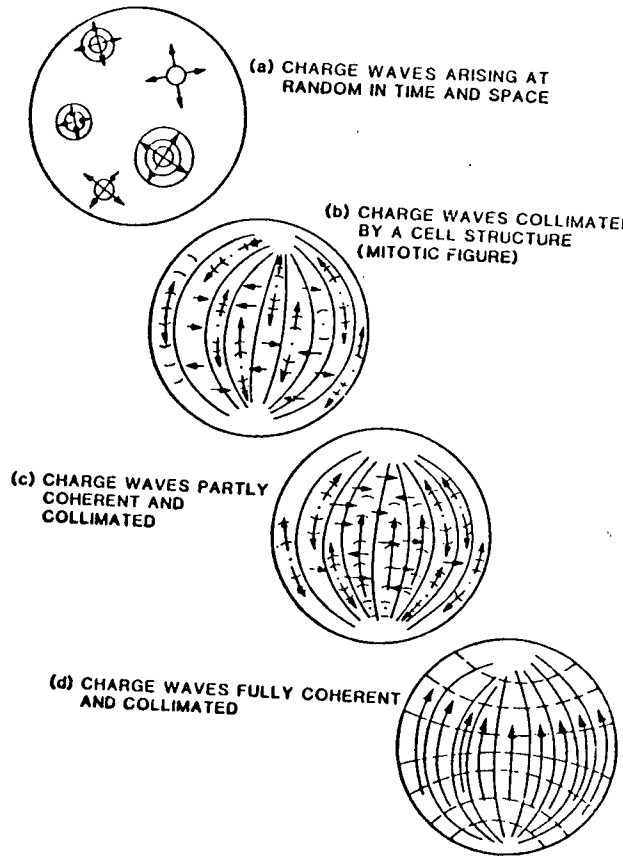

Fig. 18. A model for the dipole moments in a yeast cells.

from Pohl et al., 1982

APPENDIX II

The fusion apparatus used in these experiments

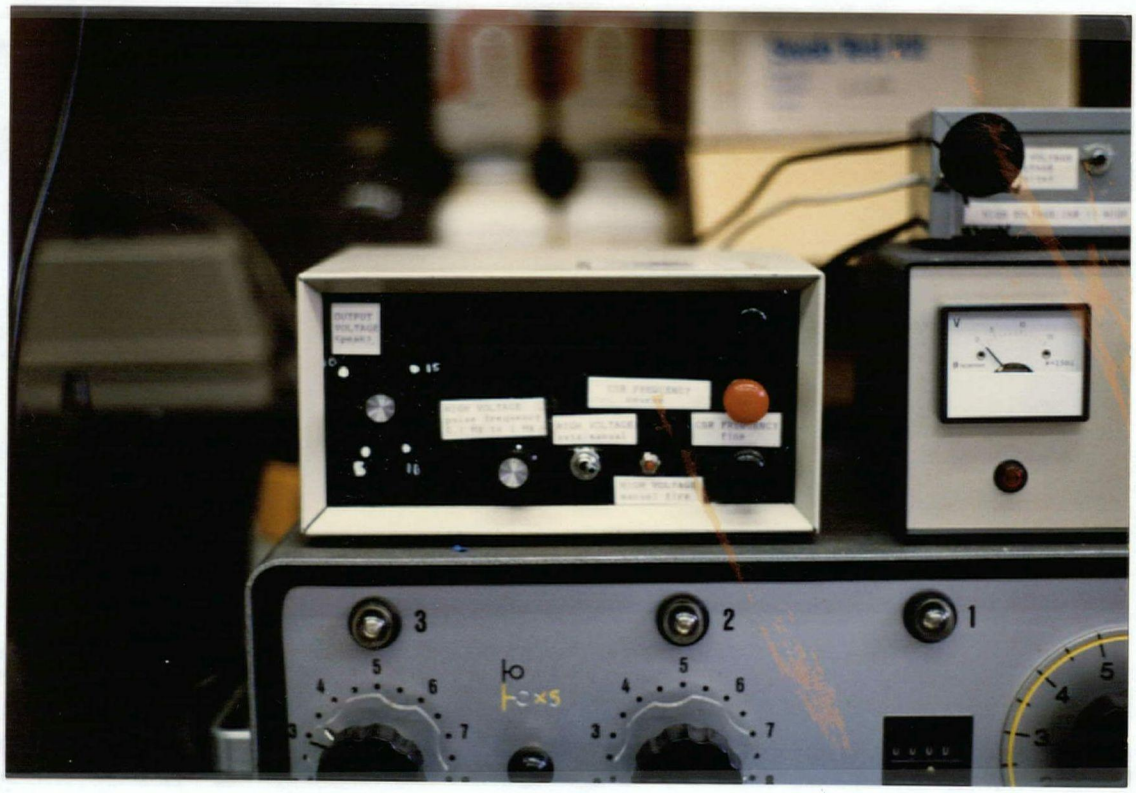

Fig. 19. Fusion apparatus

APPENDIX III

The growth medium was 50 ml 0·1 M-phthalate-buffered yeast nitrogen base medium (see Methods), containing 0·6% (w/v) Bacto-peptone and 200 mg carbon (supplied as the various sugars). The values represent the mean of two determinations. Carbon sources were sterilized separately from the basal medium. No growth occurred in the basal medium without the addition of a carbon source.

Carbon source	Growth rate, μ (h^{-1})	Yeast yield (mg ml^{-1})	Yeast yield [mg (mg carbon)$^{-1}$]*	Astaxanthin yield (μg ml^{-1})	Astaxanthin yield [μg (g yeast)$^{-1}$]
D-Maltose	0·14	3·63	0·91	1·86	512
D-Cellobiose	0·10	3·48	0·87	2·27	652
Sucrose	0·19	3·72	0·93	1·89	508
Succinate	0·09	2·66	0·67	1·33	500
D-Mannitol	0·16	3·68	0·92	1·80	489
D-Xylose	0·04	1·21	0·30	0·58	479
L-Arabinose	0·05	3·30	0·83	1·25	379
Glucono-δ-lactone	0·10	1·48	0·37	0·80	541
D-Glucose	0·20	3·85	0·96	1·62	421
D-Glucose†	0·21	6·46	0·81	1·11	171

* Assuming all carbon utilized. † 800 mg carbon [4% (w/v) glucose].

Table VII. Effect of carbon source on growth and pigmentation of P. rhodozyma in Shake Flask Culture.

from Johnson & Lewis, 1979.

APPENDIX IV

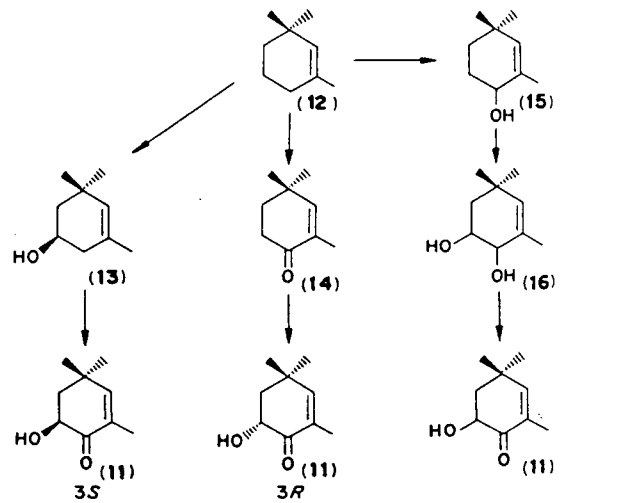

Fig. 21. Possible biochemical pathways from the β-end group (12) to the astaxanthin end group (II).

from Andrewes et al., 1976.

APPENDIX V

Constituents	Content (%)	
	Phaffia rhodozyma	Brewer's yeast
Ash	5.6	6.5
Total carbohydrate	40.3	33.4
Total nitrogen	4.82	8.71
Protein (N x 6.25)	30.1	54.4
Protein (Folin reagent)	25.0	—
RNA	8.2	9.2
Total lipid	17.0	4.03
Astaxanthin	.06	0

Table VIII Proximate composition of Phaffia rhodozyma and brewers yeast (dwb).

from Johnson et al., 1980.