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A new strain of *Rhodotorula rubra* isolated from yogurt

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SUMMARY

A new strain of *Rhodotorula rubra* has been isolated from yogurt which shows promise as a source for pigment and protein feed for aquacultured animals. The pigment was extracted by rupturing the cells with the French press followed by extraction with acetone and purification of the acetone extract using petroleum ether and cold 10% NaCl. The absorption spectrum indicated that the pigment was a carotenoid, the chemistry of which was examined using nuclear magnetic resonance, mass spectroscopy and resonance Raman spectroscopy. A reverse-phase HPLC equipped with octadecylsilylated (ODS) silica column showed nearly 80-times more pigment production under similar cultural conditions than *Phaffia rhodozyma*. The isolate grows optimally at 20 °C when grown on a variety of media. Its morphology has been studied using transmission electron microscopy, scanning electron microscopy and phase contrast microscopy. From the results of the API system, the isolate was identified as *Rhodotorula rubra*.

INTRODUCTION

Due to the depletion of traditional fishing resources, aquaculture is developing worldwide to cope with the demands of additional protein foods. An important factor affecting the consumer acceptance of the cultivated fish is the colour of the fish [10,14]. Since the use of synthetic feed colorants is fast declining due to the strict regulatory agencies and reluctance by the consumers of their use, the possibility of using the red yeast, *Phaffia rhodozyma*, as a substitute for salmonid and crustacean diets is being tested [6,7]. This yeast produces a carotenoid, astaxanthin, as its major pigment [1,4,9]. This could become an attractive alternative to the synthetic carotenoids.

However, three major drawbacks which come in its way of being commercialized are a rigid cell wall which limits the pigment extractability, a slow growth rate and poor digestibility of whole *Phaffia* cells by the fish [7]. The present study was thus undertaken to exploit the potentials of a new yeast strain isolated from yogurt which has a faster growth rate and from which a much higher amount of the carotenoid is readily extractable.

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MATERIALS AND METHODS

Organism

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

Culture conditions

Shake flasks of 2-l capacity containing 800 ml of yeast extract/malt extract medium (YM broth, Difco) were incubated at 20 °C in a 'Psychrotherm' controlled environment incubator (New Brunswick, NJ, USA) in the presence of light and with a shaking rate of 150 rpm for 5 days.

Pigment extraction and purification

The pigment was extracted by rupturing the cells in a French press at an internal cell pressure of 32 000 psi. The ruptured cells were extracted with acetone four to five times. The pigment then was extracted from the acetone extract into petroleum ether (60–80, Analar) and cold 10% NaCl (10:90) [5]. The ether hyperphase was then removed and the pigment concentrated by rotavapor distillation and stored at −20 °C.

Absorption spectrum

The absorption spectrum of the pigment was taken in acetone using Shimadzu ultraviolet-visible recording spectrophotometer UV-160.

HPLC chromatography

A reverse-phase high performance liquid chromatography with an octadecylsilylated (ODS) silica column (Brownlee, 260 × 4.6 mm; Applied Biosystems) was performed for the separation and analysis of pigments using a Beckman model 157 detector with varying wavelength of 345–510 nm. A binary solvent system was used in the set-up: solvent A (80% methanol; 15% Milli-Q water; 5% ion pairing solution containing 7.5 g ammonium acetate in 100 ml water) and solvent B (methanol/acetone, 70:30). A linear solvent gradient was run over 6 min where the solvent flow was changed from solvent A to solvent B gradually.

To 500 μl of the purified acetone extract, 150 μl of the ion pairing solution was added and a 100 μl of the mixture was run on HPLC for 30 min (Fig. 1).

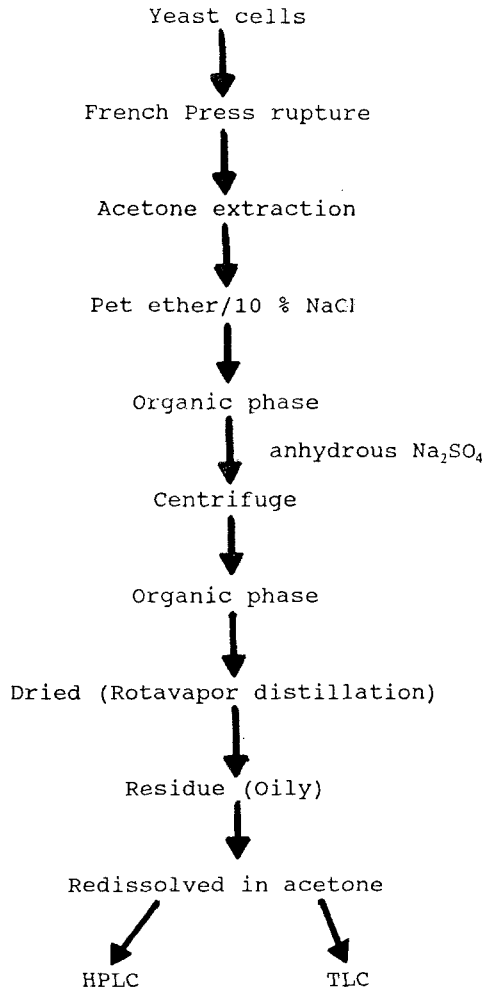

Fig. 1. Scheme of pigment extraction.

TABLE 1

Thin-layer chromatography of the purified pigment

Solvent system	R_F value	
	astaxanthin	sample
Benzene/dioxane/acetic acid (60:36:04)	1.0	1.0
Acetone/petroleum ether (20:80)	0.53	0.54
	0.3	0.31
	0.15	–

TLC

For the TLC of the purified pigment, a standard astaxanthin (molecular formula, $C_{40}H_{52}O_4$; molecular weight 596.82, λ_{max}, 475 nm (acetone)) procured from Hoffmann-LaRoche (Basle, Switzerland) was co-chromatographed on silica gel G plates and developed with two solvent systems: (a) benzene/dioxane/acetic acid

TABLE 2

API 20C test for identification of the isolate

Substrate	Result
Negative control	–
Glucose[a]	++++
Glycerol	++
2-Keto-D-gluconate	–
L-Arabinose	+++
Xylose	+++
Adonitol (ribitol)	++++
Xylitol	+++
Galactose	++++
Inositol	–
Sorbitol (glucitol)	+++
Methyl-D-glucoside	–
N-Acetyl-D-glucosamine	–
Cellobiose	–
Lactose	–
Maltose	++++
Sucrose	++++
Trehalose	++++
Melezitose	++++
Raffinose	+++

[a] Positive control.

–, negative assimilation, growth equal to negative control.

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

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

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

API profile: 6672073.

Correlation index: *Rhodotorula rubra*, 1:75; *Rhodotorula glutinis*, 1:485; *Candida guilliermondii*, 1:1000000.

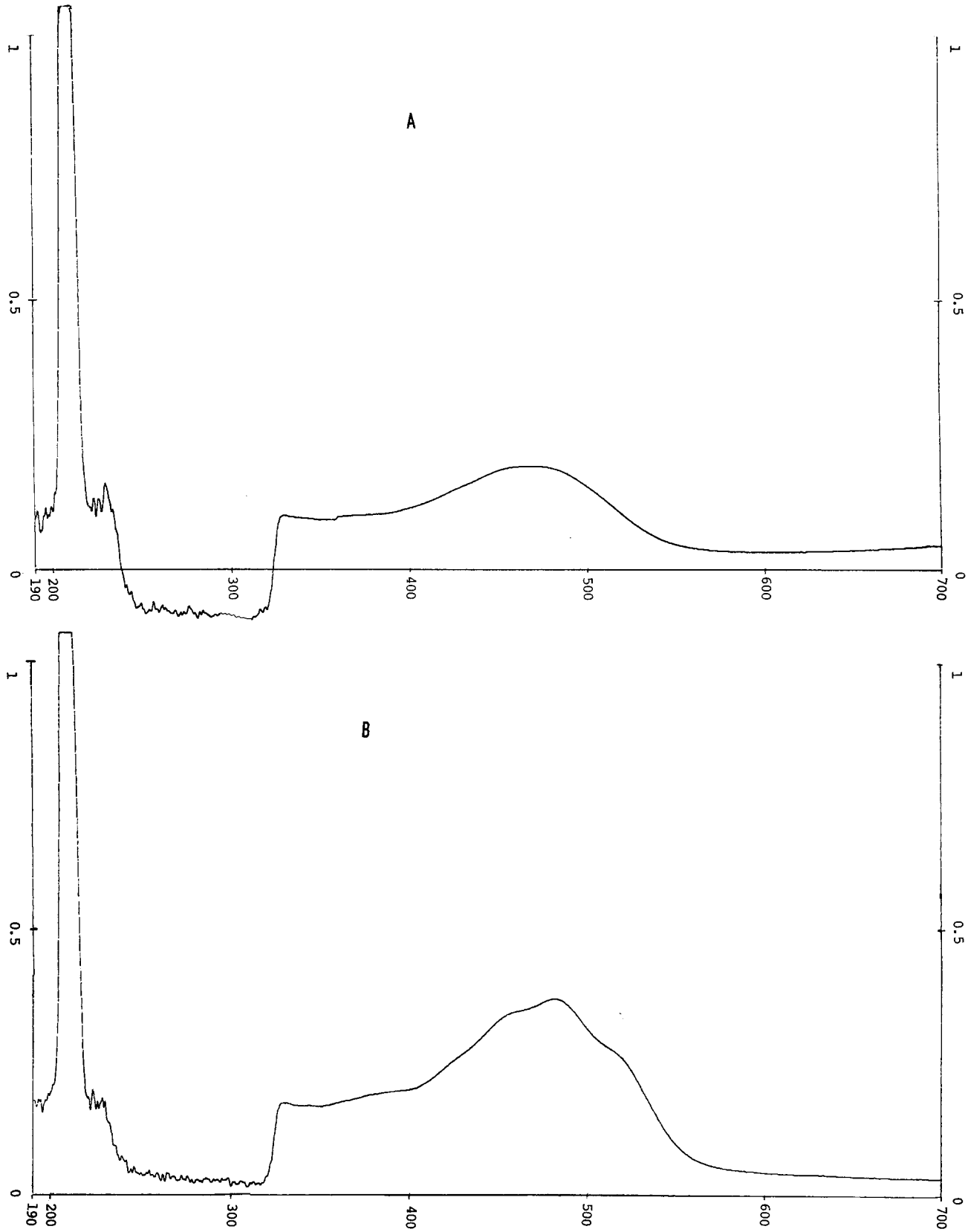

Fig. 2. Absorption spectrum in acetone. (A) Pigment from *Phaffia rhodozyma*. (B) Pigment from new isolate.

(60:36:04) for 90 min; (b) acetone/petroleum ether (20:80) for 60 min. Development was followed by staining with iodine and the R_F values were calculated.

Chemistry of the pigment

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

Morphology of the isolate

The morphology of the isolate was studied using a Zeiss EM 9A Transmission electron microscope and a Hitachi S 570 Scanning electron microscope as well as a Zeiss 100 × Phase Planachromat.

Growth on different media

The new isolate and *P. rhodozyma* were compared for their growth parameters on different media such as YM, malt agar, Sabouraud's dextrose agar (SDA), potato dex-

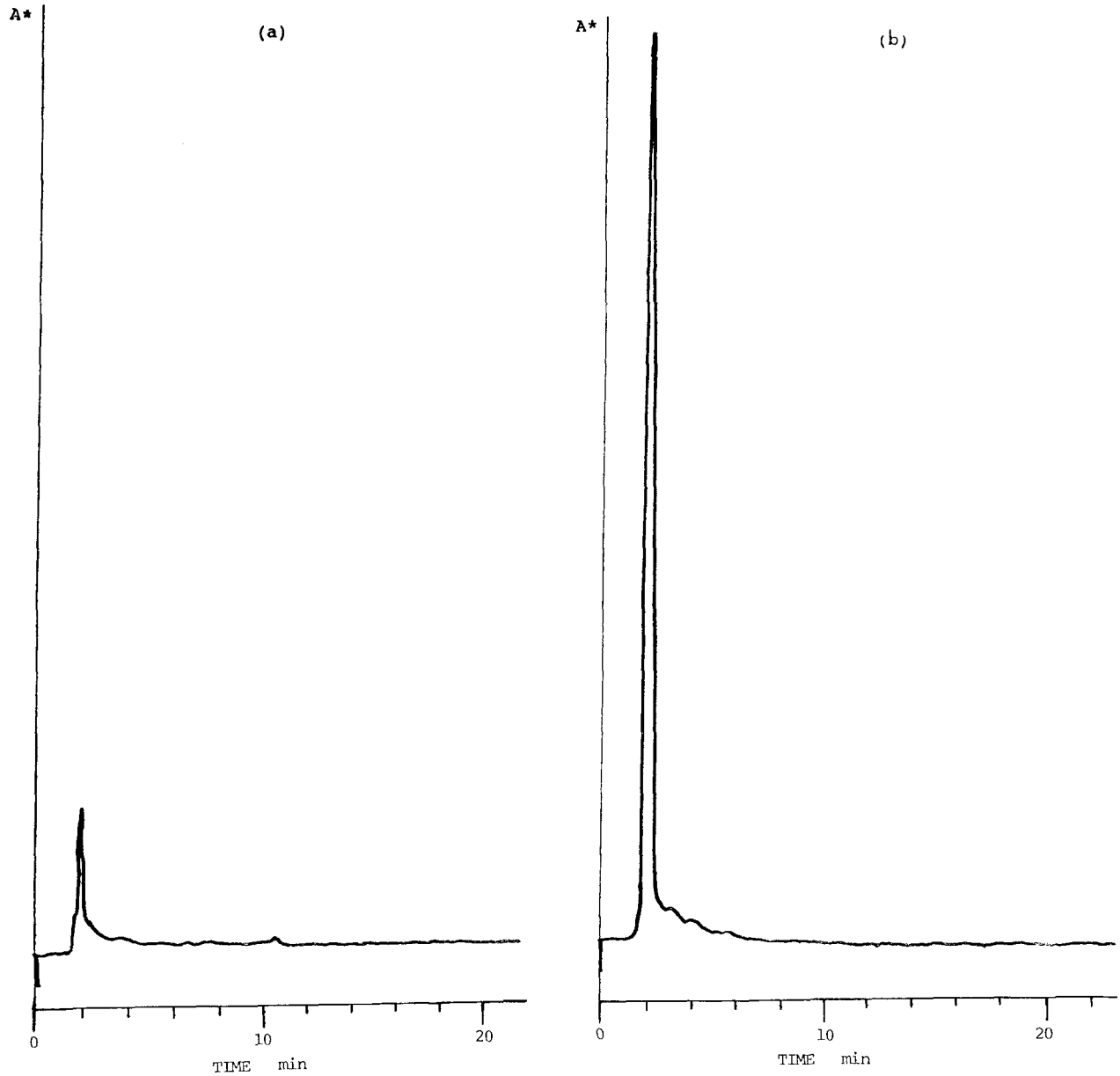

Fig. 3. Chromatographic behaviour of (a) pigment from *Phaffia rhodozyma* and (b) diluted pigment (1:10) from the new isolate. Sample: 100 µl acetone extract containing 23% ion pairing solution. A*, absorption between 345–510 nm.

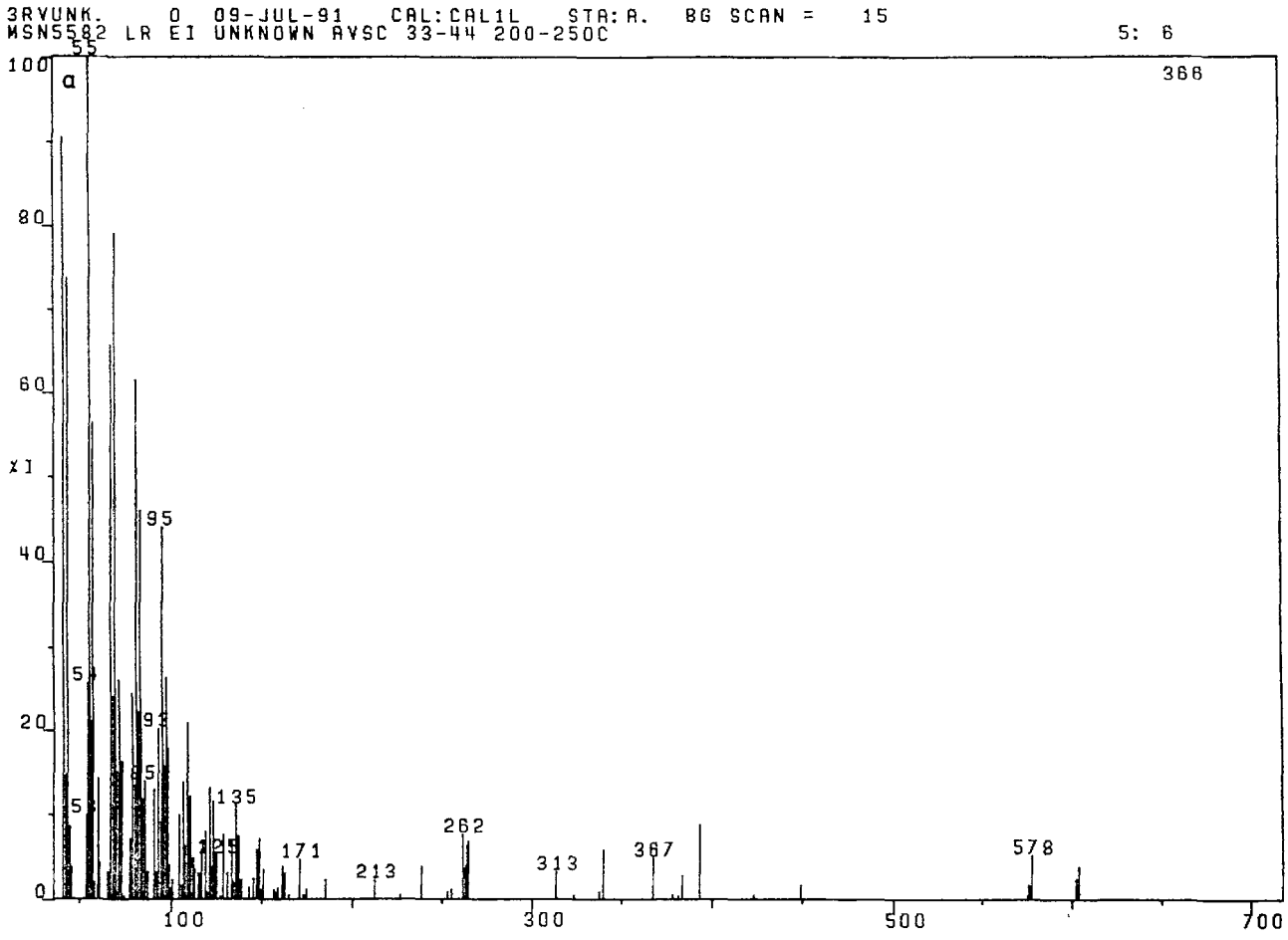

Fig. 4. (a) The mass spectrum of the purified pigment. (b) The mass spectrum of the standard astaxanthin.

trose agar (PDA), Czapek dox and tryptic soy agar (TSA). All these media were supplied by Difco.

Assimilation of carbohydrates

Using the API 20C clinical yeast system (API analytical products, Plainview, NY), a ready-to-use micromethod, 19 assimilation tests were performed and the biochemical reactions were recorded after 72 h of incubation at 20 °C.

Growth characteristics

The growth experiments were performed in YM medium, and the generation time, yield and cell size were investigated.

RESULTS

Absorption spectrum

The absorption spectrum indicated that the pigment belonged to the family of carotenoids (Fig. 2).

HPLC

When equivalent amounts of pigment from the sample and the standard were analyzed, the HPLC profile demonstrated nearly 80-times more pigment in the sample (Fig. 3) compared to the known standard astaxanthin.

TLC

The thin-layer chromatography revealed R_F values very similar to those obtained for astaxanthin under two different solvent systems (Table 1).

Mass spectroscopy

The mass spectrum analysis indicated that the pigment from the unknown yeast has a molecular weight of about 604, while astaxanthin has a molecular weight of 596 (Fig. 4a and b, respectively).

Morphology and ultrastructure of the isolate

The scanning electron micrographs indicated circular and ellipsoidal cell shapes of this isolate and *P. rhodozyma*, respectively (Figs. 5 and 6). From the micrographs it is

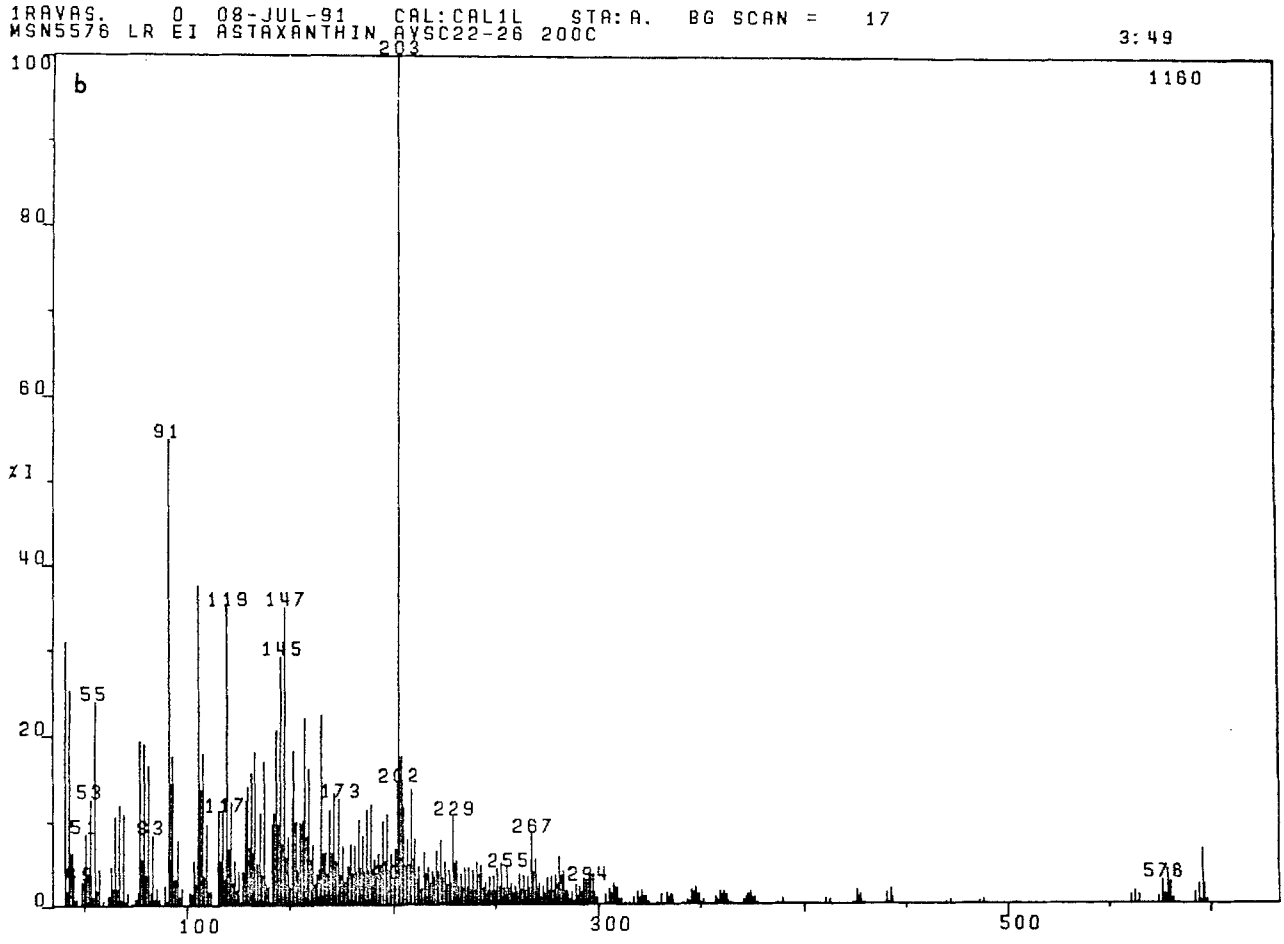

Fig. 4. *Con't.*

apparent that the unknown yeast is considerably smaller in size then *P. rhodozyma*.

The transmission electron micrographs apparently did not reveal a significant difference in the cell wall thickness of the two red yeasts.

Growth on different media

Although *P. rhodozyma* grew on Czapek dox, YM and TSA with slight pigmentation on the latter medium, it failed to grown on SDA, malt agar and showed scant growth on PDA. The new isolate grew readily on all media but had little pigmentation on Czapek dox.

Assimilation of carbohydrates

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

The isolate, thereafter, has been named as *Rhodotorula rubra* TP1.

Nutritional and growth characteristics

The nutritional and growth characteristics of the two red yeasts are outlined in Tables 3 and 4, respectively.

DISCUSSION

Astaxanthin in the red yeast *Phaffia rhodozyma* [11] was recognised as a possible pigment source for cultured fish, shell-fish and poultry. However, its commercialization has not been possible so far as this organism is slow growing resulting in reduced biomass yields compared to the new isolate. Its reduced growth on several common yeast culture media suggests its fastidious nutritional requirements. Also, the cost of the media used is relatively

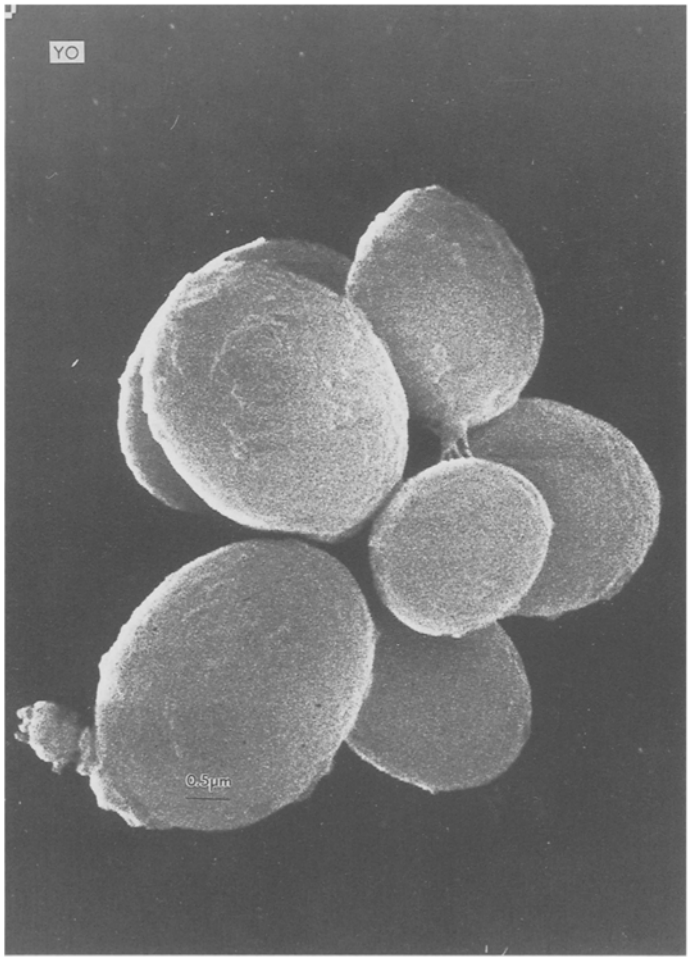

Fig. 5. A scanning electron micrograph showing a circular cell shape.

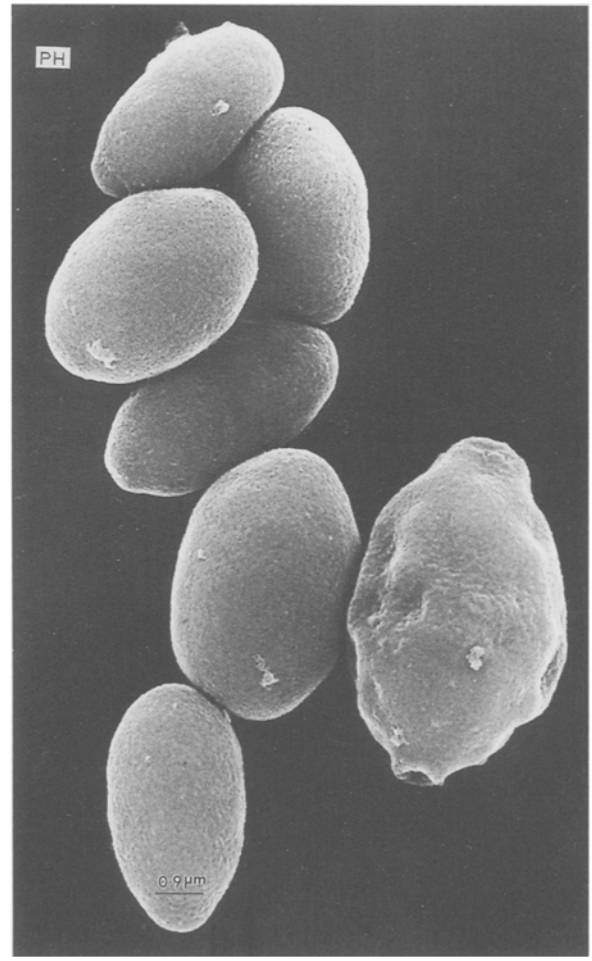

Fig. 6. A scanning electron micrograph of *Phaffia rhodozyma* indicating an ellipsoidal cell shape.

high. Most of the studies have shown the use of yeast nitrogen base with added sugars as the suitable medium for propagating this red yeast. In a recent study by Okagbue and Lewis [12], the potential of alfalfa residual juice as a substrate for the growth of *P. rhodozyma* was examined. However, limited success was achieved as the juice suppressed astaxanthin formation even though appreciable growth of the yeast was obtained. Attempts to use other waste products, especially industrial waste products have not been reported for *Phaffia*. On the other hand, the new isolate, *R. rubra*, because of its versatility with respect to nutrient requirements, may be grown at commercial scale on industrial wastes. Presently, attempts to grow it on food-processing wastes and industrial by-products are underway.

The biggest factor affecting the possible future commercialisation of *P. rhodozyma* is the extractability of its astaxanthin. Several methods have been tried to enhance the pigment extraction including mechanical breakage [16] and chemical (acid or alkaline) hydrolysis, both of which have been found to be laborious and cumbersome. The latter method also denatured the carotenoids [3]. The enzymatic digestion of the yeast cell walls was proposed by Phaff [15] for the extraction of yeast protein employing microbial lytic enzymes. Thus, the breaking of *P. rhodozyma* cells by enzymatic digestion initially appeared an attractive method for the extraction of the carotenoids. But due to technical difficulties, it was not successful. Lytic enzymes have also been tested by Johnson et al. [8] and Okagbue and Lewis [13].

Amongst all the known methods of astaxanthin extraction, no single method has been found to be most suitable for breaking the seemingly stubborn cell wall of *Phaffia rhodozyma*. The enhanced pigment production observed in *R. rubra* may be explained by several factors: (a) easily breakable cells from which the pigment is readily released

TABLE 3

Nutritional and biochemical properties of the new isolate compared with *R. rubra* ATCC 9449

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

+, rapid assimilation.
w, weak assimilation.

TABLE 4

Growth characteristics of the two red yeasts

Growth parameters	New isolate	*P. rhodozyma*
Cell size	diameter: 3 ± 1 μm	length: 7.5 ± 1.5 μm width: 6.0 ± 1 μm
Generation time (h)	10 ± 1	43 ± 1
Cell concentration (g dry weight/l)	24 ± 1	10 ± 1
Growth yield (g dry weight/g substrate utilized)	0.48	0.20
Mean growth rate constant (K)	0.1	0.02

during extraction; (b) higher pigment production in *R. rubra* compared to *P. rhodozyma*; (c) greater biomass yield from *R. rubra* when grown under similar conditions; (d) higher extinction coefficient of the pigment in *R. rubra*. Although evidence thus far obtained points in favour of higher pigment production per unit volume in the case of the new isolate, one or more factors may contribute to the observed results.

The absolute configuration of the *Phaffia* astaxanthin (3R,3′R) is unique because it is opposite to the normal 3S,3′S found in other organisms including fish [2]. This may possibly play a role in reduced absorption of the pigment in the fish muscle. The exact configuration of the *R. rubra* pigment is yet to be determined.

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

Presently, studies are in progress to unravel the detailed chemistry of the pigment. The feeding studies on fish are necessary to determine whether the isolate could be used as source of protein, vitamin, minerals and carotenoid in aquaculture diets.

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