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# Use of alfalfa residual juice as a substrate for propagation of the red yeast *Phaffia rhodozyma*

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**Summary.** Alfalfa residual juice (ARJ) supported good growth of the yeast *Phaffia rhodozyma* but formation of astaxanthin was inhibited. Supplementary nutrients did not reverse the inhibition, indicating that the the juice probably contained some inhibitor of astaxanthin biosynthesis. Six strains of *P. rhodozyma* were tested and found to be susceptible to the inhibitory effects of the juice. Concentrations of ARJ above 1.25% (v/v) were inhibitory to pigmentation of the yeast. Above approximately 3.7%, total inhibition of astaxanthin formation was observed but some chromogenic components of the juice were adsorbed on *Phaffia* cells and appeared as artefacts in astaxanthin analyses. *Phaffia* biomass produced in ARJ showed greater susceptibility to autolysis than that produced in a peptone-glucose-salts medium. Supplementation of ARJ with glucose enhanced yield of cell mass and minimised the autolytic phenomenon, and is potentially useful for producing *Phaffia* biomass for use as a source of single cell protein.

Unsupplemented brewer's malt wort and molasses, separately and in a suitable combination, were compared with ARJ and were found suitable for growth and pigmentation of *P. rhodozyma*.

## Introduction

*Phaffia rhodozyma* is a carotenoid-producing yeast. Astaxanthin, its principal pigment, has proven colorigenic potential for the flesh of salmon (Johnson et al. 1977, 1980b) and for the egg-yolk and flesh of poultry (Johnson et al. 1980a) when suitably-treated yeast is incorporated into appropriate animal diets. Since consumer acceptance of the products is enhanced by the pigmenting property of *P. rhodozyma,* its propagation on commercially-feasible substrates is of interest in the poultry and cultivated salmonid industries.

The use of food-processing wastes as substrates for cultivation of microorganisms of industrial interest is a common practice (Cousin 1980). It has the advantage of producing useful biomass and helps to alleviate problems of waste disposal. Alfalfa residual juice (ARJ) or alfalfa solubles is a relatively new waste product derived from a commercial process which extracts protein from the leaves of alfalfa plant. Its possible application as a microbial fermentation medium has been suggested (Kohler and de Fremery 1975), and it has been successfully utilised for cultivation of an unidentified species of *Candida* (Parades-Lopez and Camargo 1973), and *C. utilis* (Mudgett et al. 1980).

In the present study, we have examined the potential of ARJ as a substrate for growth and pigmentation of *P. rhodozyma.* Limited success was achieved. The juice supported appreciable growth of the yeast but the production of astaxanthin was inhibited.

## Material and methods

*Organisms.* Six strains of *P. rhodozyma* (University of California, Davis (UCD) Department of Food Science and Technology Culture Collection numbers 67-202, 67-203, 67-210, 67-385, 67-484, and 68-653C) were obtained on slants of malt extract agar. They were transferred to YM (Difco) agar slants, incubated for 48 h at 20° C, stored at 4° C and sub-cultured every two months. 67-210 was used in all experiments unless otherwise indicated. *Saccharomyces cerevisiae* 71-141 (Guiness Stout yeast) was obtained from a slant of wort agar in the Brewing Laboratory of UCD.

*Substrates and culture conditions.* ARJ was kindly supplied by Dr. G. Kohler (retired), U.S.D.A. Western Regional Research Laboratory, Berkeley, California, USA and kept at 4° C. Before each

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experiment, an aliquot was diluted ten-fold to give approximately 2% sugar in 0.02 M phthalate buffer pH 5.6; the approximate final concentration of sugar was deduced from the value reported for undiluted ARJ (Kohler and Fremery 1975). Diluted juice was clarified twice by centrifugation (Servall automatic refrigerated centrifuge at 13,000 rpm for 1 h). In some experiments, brewer's malt wort (prepared in the brewing laboratory, UCD), was used for cultivation of *P. rhodozyma*. It was diluted with distilled water to 2% sugar (measured as soluble solids with a refractometer). Molasses (Red Star, Universal Foods Corp., Milwaukee, Wisconsin) was also used as the growth substrate and was diluted twenty-fold and clarified by centrifugation.

All the complex media were dispensed in 500-ml baffled side-arm flasks (Bellco Glass Inc., Vineland, N.J.) in 100-ml amounts and autoclaved at 121° C for 15 min. Supplementary nutrients used in some experiments were autoclaved separately. Inoculated flasks were shaken at 200 rpm (Orbital shaker, New Brunswick, N.J.) and incubated at 20° C for 72–105 h.

*Preparation of inoculum.* Loopfuls of *P. rhodozyma* taken from a slant were used to inoculate 50-ml amounts of medium (in 250–300-ml Erlenmeyer flasks) containing as final concentrations 0.5% $KH_2PO_4$, 0.02% $MgSO_4 \cdot 7\,H_2O$, yeast extract, peptone, and glucose (separately autoclaved in distilled water) each 0.5% in 0.02 M phthalate buffer pH 5.6. The flasks were incubated at 20° C for 28 h with shaking at 200 rpm. Yeast cells were collected aseptically by centrifugation at 800 rpm for 10 min and washed twice with sterile distilled water. They were resuspended to a turbidity of 300 Klett units using a Klett-Summerson photoelectric colorimeter (model 800-3, Klett Manufacturing Co., N.Y., U.S.A.) equipped with a red filter. Two milliliter of each suspension was used to inoculate the complex media (ARJ, molasses, brewer's wort).

*Biomass production.* Yeast cell mass was expressed as the dry weight (mg/ml). Culture samples, taken at suitable times, were centrifuged and washed twice with cold distilled water. Cells were then resuspended in sufficient water to transfer to pre-dried, cooled and tared aluminium pans and dried in a vacuum oven at 70° C for 18 h.

*Extraction and estimation of astaxanthin.* The method adopted was a modification of a published procedure (Nakayama et al. 1954). Cell pellet was treated with 2 N HCl and subjected to a mild heat treatment in a boiling water bath for 1–2 min, followed by rapid cooling, then acetone extraction. Concentrations of astaxanthin in acetone extracts were estimated by measuring the absorbances at $\lambda_{max} = 478$ nm. The specific absorption coefficient A1% 1600 (Andrewes et al. 1976) and the formula provided by Davies (1976) were used in calculations of astaxanthin concentrations.

## Results

### *Pretreatment of ARJ*

Since ARJ was highly viscous and contained approximately 22% sugar (Kohler and Fremery 1975), it was essential to dilute it to a suitable consistency and sugar concentration. In addition, there was a need to clarify it to remove plant debris and other undesirable solids.

Use of phosphate buffer pH 5.6 as diluent (0.02 M–0.10 M, final molarity), followed by suction filtration through Whatman No. 2 filter paper in presence of diatomaceous earth (DE) were not satisfactory. Phosphate appeared to cause formation of a red-brown precipitate when the medium was pasteurised (at 145° C for 12 min) or autoclaved (at 121° C for 15 min). In addition phosphate seemed to enhance adsorption of some chromogenic components of ARJ on to *Phaffia rhodozyma* cells; the resulting green pigment on the pellet could not be satisfactorily removed by washing thrice with water and once with acetone. The use of DE apparently caused the pH of the medium to rise to 7.8–8.02 within 48 h of growth of the yeast; this was probably caused by the presence of some alkali in DE.

0.02 molar phthalate buffer pH 5.6 was a satisfactory diluent for ARJ, and centrifugation of the diluted juice twice at 13,000 rpm for 1 h was sufficient to remove plant debris and other unwanted solids. Autoclaving was preferable to pasteurisation because the latter did not completely eliminate bacterial contaminants which often grew considerably in the culture of *P. rhodozyma*.

### *Growth of P. rhodozyma in ARJ*

Figure 1 shows the growth curve of *P. rhodozyma* in ARJ diluted to contain 2.0% sugar in 0.02 M phthalate buffer pH 5.6. Another curve showing the

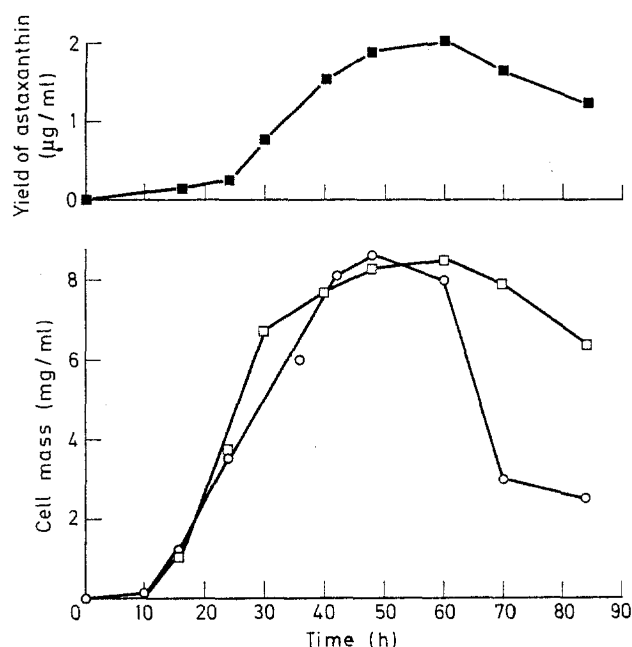


**Fig. 1.** Growth of *Phaffia rhodozyma* in alfalfa residual juice and in a semi-synthetic medium (control). The semi-synthetic medium contained (in 0.02 M phthalate buffer pH 5.6) $MgSO_4$. 0.02%, $KH_2PO_4$; yeast extract, peptone each 0.5%, glucose 2.00%. ○ alfalfa residual juice (ARJ); □ control; ■ astaxanthin production in control

growth of the yeast in an identically buffered semi-synthetic medium (SSM) containing 2.0% glucose and other nutrients, is also shown. The two media supported nearly equivalent rates of growth. Their maximum yields of cell mass were also comparable, though they were attained after slightly different periods of time. After the attainment of maximum growth, yeast mass appeared to decline more rapidly in the ARJ than in the SSM; this is probably due to different rates of autolysis in the two media.

In contrast to the observed patterns of cell growth, production of astaxanthin in the two media was strikingly different. In the control, formation of the carotenoid was closely associated with cell growth and a maximum yield of approximately 2.0 μg/ml was attained at about 60 h (Fig. 1). Unfortunately, the ARJ appeared to inhibit astaxanthin formation; cells produced throughout the 84 h fermentation period lacked the characteristic salmon-pink colour of *P. rhodozyma.* Hence they were not assayed for astaxanthin.

### *Effect of supplementary nutrients on growth and astaxanthin formation by P. rhodozyma in ARJ*

In order to determine whether certain nutrients possibly essential for astaxanthin formation were present in ARJ in limited amounts, or had limited bio-availability for absorption by *P. rhodozyma,* attempts were made to enrich the juice. Table 1 shows that various supplementary nutrients, added separately and in a combination, were unable to promote astaxanthin formation despite a prolonged fermentation period of 105 h. $KH_2PO_4$, alone or in combination with other supplements, appeared to enhance the yield of cell mass, but the pellets were dark-green in colour; this phenomenon was recorded earlier when phosphate buffer was used as diluent. Of the other supplements, glucose seemed to improve cell mass recovery at 105 h; the cell-pellet also showed some yellow-brown color in visual comparison with those of other supplements. Unfortunately, more detailed studies on the effect of glucose supplementation showed that none of various levels ranging from 0.5–3.0% promoted astaxanthin formation. Visual inspection of washed cell pellets derived from samples taken at intervals did not reveal any development of the pink color of *P. rhodozyma* at any stage of growth in media supplemented with the various levels of glucose; hence no analyses for astaxanthin were undertaken. It was observed, however (Fig. 2), that the growth rate and maximum cell mass rose as the level of supplementary glucose increased to about 2%. At 1.5% and 2.0% supplementary glucose, yields of cell mass were maximal at 60 h and roughly equal. Considerable autolysis occurred after attainment of maximum growth, but was apparently minimised by the glucose supplementation.

### *Effect of ARJ on strains of P. rhodozyma*

Five strains of *P. rhodozyma,* other than the type strain 67-210, were grown in ARJ to determine if any of them was resistant to the inhibitory effect of the juice on astaxanthin formation. Each strain was also grown in the usual SSM to serve as a control. As

**Table 1.** Effect of various supplements on the ability of ARJ to support growth and astaxanthin formation by *P. rhodozyma*

| Additive[a] | Biomass (mg/ml) | | | Astaxanthin concentration (μg/ml) | Other observations on washed cell pellet |
|---|---|---|---|---|---|
| | 40 h | 70 h | 105 h | | |
| None | 7.5 | 3.2 | 2.8 | –[b] | |
| Glucose (0.5%) | 7.2 | 4.2 | 6.1 | – | Some yellow-brown pigmentation apparent |
| $KH_2PO_4$ (0.5%) | 9.2 | 11.0 | 12.0 | – | Pellet dark-green |
| $MgSO_4$ (0.02%) | 7.2 | 4.0 | 5.5 | – | |
| Yeast extract (0.5%) | 7.8 | 4.2 | 3.9 | – | |
| Peptone (0.5%) | 7.5 | 4.0 | 3.3 | – | |
| $MgSO_4$ (0.02%); glucose, $KH_2PO_4$, yeast extract, peptone: each (0.5%) | 6.5 | 12.2 | 12.5 | – | Pellet dark-green |
| Control[c] | 7.2 | 7.8 | 6.9 | 1.30 | |

[a] All concentrations were finals in 100 ml of medium (ARJ). All constituents were prepared as stock solutions and added aseptically to autoclaved diluted ARJ. When all were added in combination, glucose was autoclaved separately from other additives
[b] Cell pellet was not sufficiently pigmented to warrant analyses for astaxanthin at 105 h
[c] A semi-defined medium similar to Fig. 1

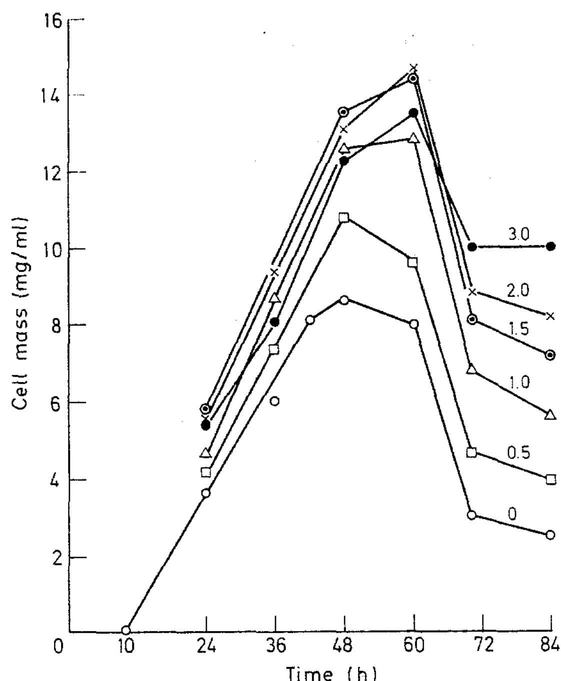


**Fig. 2.** Influence of various supplementary levels of glucose on growth of *P. rhodozyma* in alfalfa residual juice (ARJ). Numbers 0–3.0 represent concentrations (%) of supplementary glucose in 100 ml of medium (ARJ). The glucose supplements were added from sterile stock solutions

**Table 2.** Growth of some strains of *P. rhodozyma* in alfalfa residual juice (ARJ)

| Strain | Growth conditions | Biomass (dry) (mg/ml) | Astaxanthin (μg/ml) | Astaxanthin (μg/g dry weight) |
|---|---|---|---|---|
| 67-202 | Control[a] | 10.03 | 2.0 | 199.30 |
| | Expt.[b] | 5.88 | –[c] | NA |
| 67-203 | Control | 7.70 | 1.59 | 206.49 |
| | Expt. | 3.78 | – | NA |
| 67-210 | Control | 7.08 | 1.78 | 251.41 |
| | Expt. | 3.62 | – | NA |
| 67-385 | Control | 9.55 | 2.06 | 215.71 |
| | Expt. | 5.05 | – | NA |
| 67-484 | Control | 8.18 | 1.38 | 168.70 |
| | Expt. | 6.23 | – | NA |
| 68-653C | Control | 10.56 | 1.75 | 165.72 |
| | Expt. | 6.73 | – | NA |

100 ml of medium was used in both control and experimental flasks. All assays were carried out after 72 h of culture. Data are averages of duplicates

[a] In the controls, the strains were grown in phthalate (0.02 M) buffered semi-synthetic medium (SSM) pH 5.6 containing 0.02% $MgSO_4$, and $KH_2PO_4$, yeast extract, peptone, glucose, each 0.5%

[b] In experimental cultures, medium was phthalate-buffered alfalfa residual juice diluted 10-fold. No supplements were used

[c] Cell pellets characteristically lacked pink colour; hence no analysis for astaxanthin were made

NA = not applicable

expected (Table 2), good yields of astaxanthin and cell mass were achieved by all the tested strains in their control cultures. In contrast, none of them produced astaxanthin in ARJ. Their low yields of cell mass in the juice relative to the controls were probably due to autolysis which was shown in previous experiments (with 67-210) to occur relatively rapidly in ARJ after about 60 h of growth.

### *Effect of various concentrations of ARJ on astaxanthin formation by P. rhodozyma*

Figure 3 shows the effect on *Phaffia* of incorporating various concentrations of ARJ (ranging from 0–12.5% v/v final concentrations) into different batches of the semisynthetic medium (SSM) known to promote good yields of astaxanthin. Control cultures in which ARJ was omitted from the SSM, were also employed. Experimental cultures yielded greater cell mass than the controls; the higher the juice concentration in the medium, the greater the cell mass produced. In contrast, yields of astaxanthin were very high in the controls, remained fairly stable up to 1.25% (v/v) concentration of ARJ, and then declined sharply up to 3.7%. Above this level, quantities of pigment detected as astaxanthin were unexpectedly large even though the colours of the cell pellets and of the pigment dissolved in acetone (greenish-yellow) suggested total inhibition of astaxanthin formation. It appeared that, at the high levels of ARJ (above 3.7%) tested, some chromogens from the juice were adsorbed on the surfaces of *Phaffia* cells and could not be removed by washing with distilled water prior to determination as astaxanthin. This possibility was tested when pigments dissolved in acetone after direct treatment of washed pellets obtained from all the flasks (containing various levels of ARJ) were assayed as astaxanthin. The results (Fig. 3) showed that the dissolved pigments were greenish-yellow and increased in quantity with increasing concentrations of juice in the medium. Thus, there was total inhibition of astaxanthin formation above about 3.7% (v/v) concentration of the juice, but chromogens could adsorb on *P. rhodozyma* cells and show as artefacts in astaxanthin analyses.

Further experiments were performed to confirm the ability of the yeast cell surface to adsorb pigments from ARJ. In the first one, pellets were obtained again from flasks containing 0%, 2.5%, and 11.25% (v/v) ARJ. They were treated with acetone with or without prior mild acid treatment in a manner used in determinations of total and extractable astaxanthin (Okagbue 1982) and electronic absorption spectra of the pigments were determined. Figure 4 shows that, as expected, no pigment was extractable by direct

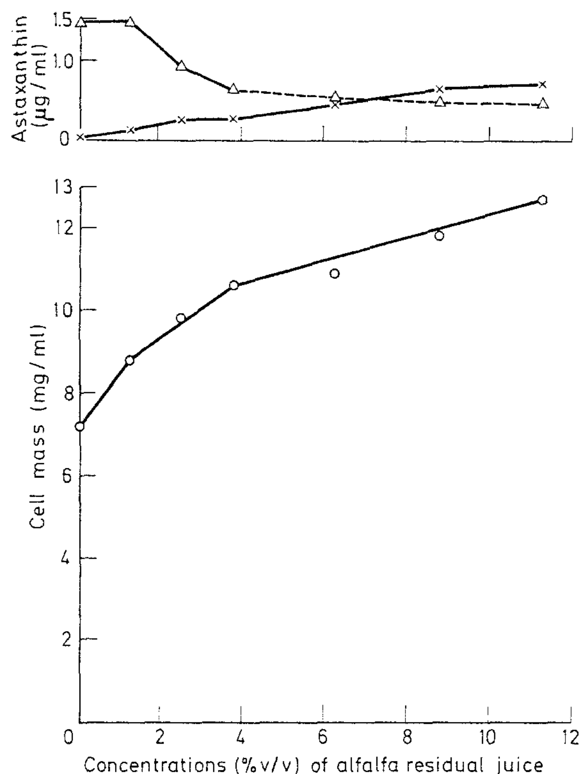


**Fig. 3.** Effect of levels of ARJ on growth and astaxanthin production by *P. rhodozyma.* Various levels of the juice were added to the semisynthetic medium similar to that of Fig. 1. △ Astaxanthin: *dotted lines* represent pigment possibly adsorbed on yeast cells and assayed as astaxanthin; X Pigment adsorbed on yeast cells (assayed as astaxanthin); O Cell mass (mg/ml)

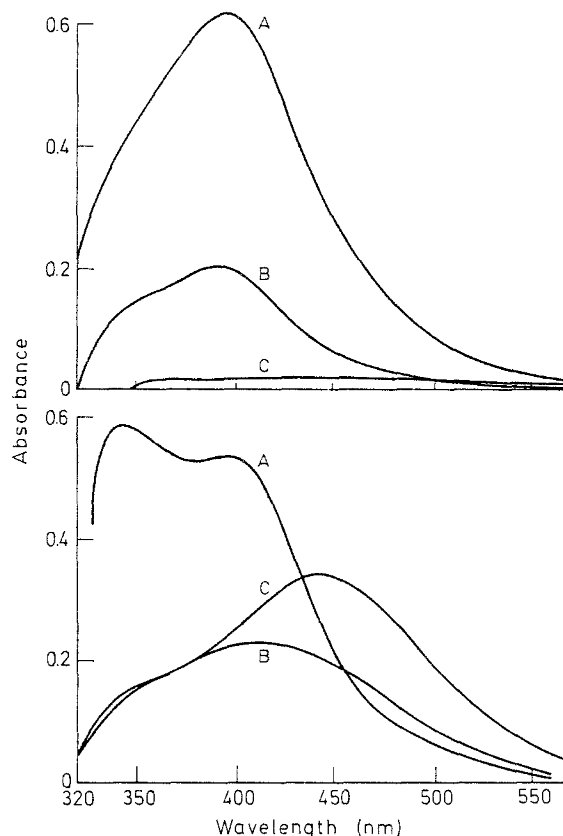


**Fig. 4.** Absorption spectra (in acetone) of pigments extracted from washed cells of *P. rhodozyma* grown in basal medium with or without ARJ. The basal medium was equivalent to the semisynthetic medium used in Figs. 1 and 3. *Top:* washed cells not treated with HCl. *Bottom:* washed cells treated with HCl. *A* medium contained 11.25% (v/v) ARJ; *B* medium contained 2.5% (v/v) ARJ; *C* basal medium (no ARJ)

acetone treatment of cells grown without ARJ in the medium. However, the mild acid treatment facilitated extraction of pink pigments which had typical absorption spectrum of carotenoids (i.e., with absorption peak at approximately 450 nm). In contrast, pigments were extractable from cells grown in presence of ARJ (2.5% and 11.25% v/v), with and without the acid treatment. The pigments from the two levels of juice were typically greenish-yellow in appearance. Some slight differences were observed (Fig. 4) in the breadths of the absorption spectra of samples derived from each level of juice, but they were probably caused by the acid treatments mentioned previously. Pigments from the two levels of ARJ were generally identical and their absorption spectra peaked at approximately 390 nm. A higher peak was observed at the higher level of ARJ indicating that the amount of pigment adsorbed on the yeast cells was related to the actual concentration in the medium.

**Table 3.** Adsorption of pigments derived from ARJ on *S. cerevisiae* 71-141

| Level of ARJ in medium (% v/v) | Absorbance of pigment in acetone ($\lambda$ = 478 nm) | Biomass (mg/ml) |
|---|---|---|
| 0 | 0 | 9.0 |
| 2.50 | 0.004 | 9.3 |
| 5.00 | 0.016 | 10.5 |
| 7.50 | 0.026 | 10.4 |

Assays were carried out after 48 h of growth

In the second experiment, a known non-pigmented yeast, *Saccharomyces cerevisiae* 71-141 (Guinness Stout yeast) was grown in media containing different concentrations (0–7.5 v/v) of ARJ. After 48 h, the yeast cells grown in presence of ARJ were darker in color, but the pigment appeared to be completely removed after three washings with water.

Acetonetreatment of washed cells, however, was able to detect rather low levels of adsorbed pigment (Table 3). Thus, adsorption of components of ARJ onto yeast cells grown in their presence is again demonstrated, but it seems that the cell surface of *P. rhodozyma* has greater affinity for the chromogens than that of *S. cerevisiae* 71-141.

In the foregoing experiments in which various levels of ARJ were added to the SSM containing 2.0% glucose, final sugar levels varied from 2.25% to 4% (the amount of sugar in the added quantity of juice was calculated from the sugar content of ARJ as reported previously (Kohler and Fremery 1975). In order to determine whether increasing sugar levels contributed to the inhibitory effect of ARJ on astaxanthin production by *P. rhodozyma,* media (100-ml amounts) were formulated to contain a constant level of sugar (2%) derived partly from varying levels of ARJ and partly from the SSM (components of the SSM other than glucose were as usual $MgSO_4$ 7 $H_2O$, 0.02%, $KH_2PO_4$, yeast extract, and peptone each 0.5%). After a 48 h growth of *P. rhodozyma,* results of the experiment (not shown) were identical to those of Fig. 3. Biomass increased with increasing levels of juice. In contrast, astaxanthin formation was virtually absent above approximately 2.5% v/v of the juice. Thus higher sugar levels in SSM supplemented with ARJ were not responsible for the observed inhibition of astaxanthin formation. ARJ apparently, has components inhibitory to pigmentation in *P. rhodozyma.*

## Discussion

In this study, alfalfa residual juice (ARJ) has been shown to support high yields of cell mass of *P. rhodozyma.* Similar effects of the juice on other yeasts have been reported by other workers (Parades-Lopez and Camargo 1973; Mudgett et al. 1980). The greater susceptibility to autolysis of *Phaffia* cell mass grown in the ARJ than that grown as a control in peptone-yeast extract-salts medium (semi-synthetic medium, SSM) could be attributed to the possible presence of larger amounts of proteinaceous material in the juice than in the SSM. It has been reported that presence of protein in growth media predisposes yeast to autolysis (Hough and Maddox 1970). The effect of glucose supplementation in enhancing yields of *Phaffia* cell mass in ARJ (Fig. 2) is to be expected; there is published evidence that yields of biomass (mg/ml) increase with rising glucose concentrations in growth medium (Johnson and Lewis 1979). The present study also showed that glucose supplementation minimised autolysis of the yeast in ARJ. Since release of proteolytic enzymes is thought to be the first step in yeast autolysis (Hough and Maddox 1970), the adverse effect of glucose on the phenomenon may be due to its repressive effect on the enzymes. The two effects of supplementary glucose (namely, enhancement of cell yield and repression of autolysis in ARJ) could be useful if *P. rhodozyma* is intended for use as a source of single cell protein.

The inability of *P. rhodozyma* to synthesize in ARJ its potentially useful pink carotenoid, astaxanthin, was also demonstrated in this study. The failure of supplementary nutrients to promote pigmentation of the yeast showed that the inhibition of astaxanthin formation was not due to deficiency in the juice of any essential nutrients or to their limited availability for absorption by the yeast. Some inhibitors of astaxanthin formation were possibly present in ARJ and clearly deserve to be elucidated. Several strains of *P. rhodozyma* were found to be susceptible to the inhibition; all of them probably synthesize astaxanthin through the same pathway which was adversely affected by the juice. It was also shown that, above 3.7% (v/v) level of ARJ, there was total inhibition of astaxanthin production, though chromogens in the juice could adsorb on the surfaces of the yeast cells and appear as artefacts in astaxanthin analyses. That the chromogens showed greater affinity for *P. rhodozyma* than for *S. cerevisiae* 71-141 was indicative of possible differences in the chemical components of the surfaces of the cells of the two yeasts. It is well known that *P. rhodozyma* contains capsular material (Miller et al. 1976).

In contrast to the observations made with ARJ, brewer's malt wort and molasses were suitable substrates for growth and pigmentation of *P. rhodozyma.* The former (i.e., malt wort) has been used to boost astaxanthin production (Johnson and Lewis 1979). Satisfactory results were also achieved in this work when the two substrates were combined in equal proportions. However, their possible utilisation for largescale production of *P. rhodozyma* for application in animal feeding would depend on economic considerations.

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