Effect of *Bacillus ciroulans* WL-12 in Mixed Culture on the Extractability of Astaxanthin from *Phaffia rhodozyma*

BY

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DAVIS

"The Widest Room On The Earth Is The Room For Improvement"

D E D I C A T I O N

This study is dedicated to my wife Rose,

and to Chinazom and Uchenna, our children.

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I. INTRODUCTION

In recent years, large-scale cultivation of yeasts and other micro-organisms for nutritional and other purposes, is widely practiced throughout the world. Post-harvest treatment of a yeast mass to disrupt the cell walls is essential to enhance extractability and intestinal absorption of nutrients and other metabolites. Acid and alkaline treatments are of limited use because some desired yeast metabolites such as carotenoids are sensitive to the chemicals (Davies, 1976). Mechanical disruption of cell walls is laborious and hardly applicable on a commercial scale. Phaff (1977) proposed the use of enzymatic cell wall digestion to supplement mechanical disruption methods. Extracellular yeast cell wall hydrolytic enzymes seem appropriate because they are produced by a wide variety of micro-organisms (Phaff, 1977). Unfortunately, the enzymes are usually inducible, and require presence of appropriate yeast cell walls for optimal production in culture media; preparation of the cell walls is tedious and laborious and not suitable for large-scale production or continuous culture.

A mixed culture, containing the desired yeast and a lytic enzyme-producing organism, is proposed. Simultaneous growth of the two organisms could lead to induction of the lytic system and digestion of the yeast cell walls. A commercially attractive feature of the proposed mixed culture is that the lytic enzyme complex would require no expensive purification or concentration

Bacillus circulans WL-12, has been extensively studied as a lytic enzyme producing bacterium. It was originally isolated on a medium containing cell walls of baker's yeast as the carbon source (Tanaka and

Phaff, 1965). Its lytic activity on the cell walls of other ascomycetous yeasts (Tanaka et al., 1966), Piricularia oryzae and other filamentous fungi (Tanaka et al., 1970), and the basidiomycetous yeast Phaffia rhodozyma (Johnson et al., 1979) have been demonstrated and provide considerable evidence of the versatile ability of the bacillus to adapt to different inducers.

Phaffia rhodozyma is a salmon-pink yeast. Its current economic potential derives from its high content of astaxanthin (approaching 800-900 µg/g yeast, depending on conditions in submerged culture) (Johnson and Lewis, 1979). The pigment is in demand as a dietary supplement for pen-reared salmonids which require adequate flesh coloration for consumer acceptance. The pigment produced by Phaffia is readily absorbed from the animal gut when the broken yeast without cell wall is added to the diets of animals; it effectively colors the flesh of pen-reared salmonids (Johnson et al., 1977) and also helps impart desirable golden color to the egg-yolk and flesh of poultry (Johnson et al., 1980). The yeast also contains a high level of unsaturated fat, protein and vitamins that contribute to good growth of animals. These attributes enhance the potential industrial utility of Phaffia rhodozyma as a source of astaxanthin in animal diets. Unfortunately, its cell walls must be modified by suitable methods to enhance pigment availability for absorption (Johnson et al., 1979).

Enzymatic digestion of the cell walls of Phaffia rhodozyma appears very attractive because it is gentle and nondestructive to carotenoids. In consequence, Johnson et al. (1978) successfully utilized the extracellular cell wall lytic enzymes of Bacillus circulans WL-12 to treat the yeast; the enzymes were induced during growth of the bacillus on

heat-killed Phaffia rhodozyma cells. Though modification of the yeast cell walls (shown by extractability of astaxanthin by acetone-treatment of cell mass) was achieved, some reduction in pigment concentration due to the heat treatment, was observed. In the present study, a mixed culture of the two organisms has been developed and utilized to achieve lytic enzyme formation and yeast cell wall modification. The mixed culture has been grown under different conditions and their effects on extractability of astaxanthin determined.

In these studies, defined or semi-defined media have been employed for growth of the organisms. In commercial fermentation processes, cheap relatively undefined substrates are used for economic reasons. Carbohydrate-rich waste products of food industries such as whey, corn steep liquor, molasses, starch wastes, etc, are commonly used. In recent years, alfalfa residual juice (alfalfa solubles), a by-product of leaf-protein extraction from the alfalfa plant (Kohler and de Fremery, 1975) has attracted some attention as a potential fermentation medium. It has been successfully used without modification or pretreatment, for production of single-cell protein from a yeast, Candida sp. (Parades-Lopez and Camargo, 1973) and from Candida utilis (Mudgett et al., 1980). The present work also tested its potential as a substrate for growth and pigmentation of Phaffia rhodozyma. Limited success was achieved. The substrate permitted adequate growth of the yeast, but the formation of carotenoid was inhibited.

II. LITERATURE REVIEW

Yeasts, as a group of micro-organisms, are economically important. Some species are pathogenic to man and livestock; yeast pathogens of lower animals such as shrimps and plants, have also been described. Other yeasts contribute to spoilage of foods and beverages. These aspects have been reviewed in many publications (Ahearn, 1978; Beuchat, 1978; Gentles and La Touche, 1969; and Phaff et al., 1978). On the credit side, yeasts may serve as sources of biological chemicals, and as indispensable agents in many food and beverage fermentation processes. Brewing, wine-making and bread-baking are examples of fermentations that have attained international and commercial significance. Yeasts have also become recognized sources of nutrients and other metabolites used in the food industry and as single cell protein.

This review is primarily concerned with useful attributes of yeasts, especially those amenable to commercial exploitation. In the first section, an overview of yeasts and yeast products that are currently promising or in commercial use in food and feed manufacture, will be presented. Advantages of yeasts and their products over other sources will be emphasized as much as possible. Some problems associated with nutritional applications of yeasts will be discussed especially those caused primarily by the rigid nature of the cell wall. The possible role of the red yeast *Phaffia rhodozyma* in commerce will be explored.

A. APPLICATIONS OF YEASTS AND YEAST PRODUCTS IN FOOD AND FEED MANUFACTURE

Harrison and Rose (1970) classified industrial uses of yeasts into three categories based on the relationship between the yeast product and the biochemistry of the organism. One category, which contains a poorly-defined group of products resulting from interaction of yeast enzymes with substrate, will not be treated further because it is not associated with food manufacture. The second class of products are those involved in baking and in alcoholic beverage production. The first category includes a wide variety of yeasts and products, most of which are intrinsic components of the cell, or are metabolites accumulated in the cell under some cultural conditions.

Yeasts in baking and in alcoholic beverage fermentations.

In this application, fermentative yeasts especially strains of Saccharomyces are used. Their products include CO_2, alcohols, such as ethanol and glycerol, and various fusel oils produced during fermentative metabolism. CO_2 is recoverable in some beverage fermentations as a saleable pure product in gaseous, solid or liquid form (Harrison and Rose, 1970). However, generally, yeast products are not recovered in pure forms in baking and alcoholic beverage production. They contribute to the desired complex flavor and texture of the finished beverage or leavened product. It is noteworthy that in these ancient industries no other group of organisms could replace the yeasts. In San Francisco sour dough bread process, the bacterium Lactobacillus sanfrancisco is responsible for souring the dough (Kline and Sugihara, 1971); however, it coexists rather than competes with the yeast Saccharomyces exiguus

responsible for the leavening action (Sugihara et al., 1971).

Yeasts and yeast products as food and feed supplements.

As mentioned previously, the organisms and products involved in this section are diverse. Fermentative and non-fermentative yeasts are involved. Products include dry whole yeast cells, lipids, macromolecular constituents such as proteins, enzymes and nucleic acids, breakdown products such as amino acids, purines and pyrimidines, and extraction compounds such as co-enzymes and vitamins. Harrison and Rose (1970) did not include organic acids and carotenoids, but they probably belong to this category, as would complex products like yeast extracts (autolysates). In general, the yeast products are used either for nutrient enrichment, or to enhance flavor, color and some functional attributes of foods.

Beuchat (1978) described some organic acids used in food manufacture. One of them, citric acid, is employed in many foods and soft drinks because of its flavor and its buffering properties. The mold, Aspergillus niger, has been traditionally employed in surface culture for the fermentative production of citric acid with molasses as the substrate. In recent years, n-alkane-utilizing yeasts, especially Candida lipolytica (perfect form: Saccharomycopsis lipolytica), have shown considerable promise in citric acid production (Marchal et al., 1977). This development was a result of intensive research in the 1960's on hydrocarbon utilization by yeasts (Shennan and Levi, 1974). Ikeno et al. (1975) have shown that mutant strains of Candida lipolytica could produce high levels of citric acid on substrates other than n-paraffins; examples of the substrates are natural oils such as palm oil, fatty acids, glycerol and ethanol. A problem caused by contamination of citric acid by

isocitric acid could be minimized by suitable choice of carbon substrate (Treton et al., 1978) and by mutagenesis (Akiyama et al., 1972). These findings improve prospects for commercial scale use of yeasts in citric acid manufacture. Beuchat (1978) has described the advantages of yeasts over Aspergillus niger, which include the ability of the former to utilize many substrates and quicker fermentation.

Yeasts, for a long time, have been known to be rich in the amino acid lysine, and were thought to be potentially useful for supplementing cereals and cereal products, such as corn, rye and malt (Spanyer and Thomas, 1956; Mojonnier et al., 1955). Strains of Saccharomyces cerevisiae and Candida utilis were even shown to increase their synthesis of lysine in a corn steep liquor medium containing suitable precursors, α-amino adipic acid and α-ketoadipic acid (KAA) (Broquist et al., 1961). Though the accumulated lysine was not released into the culture medium, it appeared not to be bound to protein and was extractable with hot water. When the yeast cells were added to a lysine-deficient diet, they promoted growth of chicks. Jensen and Shu (1961) successfully replaced corn-steep liquor with molasses in production of lysine-rich baker's yeast, using KAA as the precursor. Since KAA was expensive, Tanner et al. (1977) achieved production of lysine enriched baker's yeast by varying cultural conditions such as inoculum size, temperature, pH and others. It was suggested that the variations in cultural conditions probably affected levels of lysine precursors in the homocitrate-aminoadipic pathway involved in lysine biosynthesis. In general, the production of lysine-enriched yeast was thought very promising for enrichment of cereal foods such as bread, which are deficient in the amino acid. It should be noted that chemically-synthesized lysine is available for food

fortification, but the use of such synthetic products has been questioned (Samoqyi, 1969).

Yeasts were also known to be rich in vitamins, especially those of the B group (von Loesecke, 1946). However, riboflavin seems to be the only one produced industrially by microbiological means; strains of the yeasts Eremothecium ashbyii and Ashbya gossypii are employed (Beuchat, 1978). According to Stanier et al. (1976), the microbiologically-based vitamin production has survived competition with chemical industries. Large amounts synthesized by the yeasts are readily harvested by concentration of culture broth and are used as animal feed supplements (Phaff et al., 1978).

Enzymes derived from yeasts also find applications in the food industry. Two of them, lactase and invertase, appear to be particularly important. Lactase is traditionally produced by Kluyveromyces fragilis and Kluyveromyces lactis (Aunstrup, 1977) by fermentation of lactose in whey derived from the dairy industry. Beuchat (1978) has presented extensive information on various aspects of the fermentation of whey for biomass, ethanol and lactase production. A commercially-available lactase produced by Kluyveromyces lactis is preferred to many other sources for hydrolysis of lactose in milk and milk products because its optimum pH (6.8-7.0) is near that of milk (≅ 6.6). The lactase hydrolyzed products are acceptable organoleptically; in addition, digestibility by lactose-intolerant individuals is improved (Guy et al., 1974).

Invertase is another enzyme currently produced in commercial quantities from yeast (Cochrane, 1961), and strains of Saccharomyces cerevisiae and S. carlsbergensis are traditionally important (Boing, 1982). Candida utilis has also been shown to produce high yields of invertase when grown

on sauerkraut waste (Hang et al., 1973). This waste product promoted higher yields (up to 672,000 Units/g of dried yeast) of the enzyme than a peptone-sucrose-yeast extract broth (approximately 207,500 Units/g dry yeast). The process has been put into commercial use (Cousin, 1980). Much invertase is used in the confectionary industry to convert sucrose to invert sugar in order to prevent undesirable crystallization of the disaccharide in confectionary products.

Yeasts have been considered as potential sources of fats and oils usable in foods. Species of Rhodotorula and Lipomyces were rich in fats; their lipid contents approached 60% of cell dry weight (Whitworth and Ratledge, 1974). Though some filamentous fungi had comparable concentrations of fat, yeasts had higher fat coefficients (gram fat produced per 100g glucose or sucrose). In addition, since yeasts had faster growth rates and their fermentations could be easily manipulated, they were preferred to the filamentous fungi as potential substitutes for conventional plant and animal oils and fats. Unfortunately, cost analyses of yeast fat production on alkanes and molasses showed that they had no easily foreseeable advantages over the conventional sources. Nevertheless, Rose (1961) stated that microbial fat (presumably yeast fat) was useful in Germany during the World Wars.

Closely related to lipids are sterols and carotenoids. It has been shown that some strains of Saccharomyces cerevisiae can accumulate up to 10% ergosterol (dry-weight basis) (Dulaney et al., 1954). Irradiation with ultra-violet light can transform some of the sterols into vitamin D_2 (calciferol), and Phaff et al. (1978) stated that the method is used for production of yeast rich in vitamin D for human nutrition. Carotenoids, especially β-carotene, are recognized food colorants. The carotene

also serves as a precursor of vitamin A in foods and feeds. Yeasts, especially species of Rhodotorula (Bujak, 1961) and Cryptococcus (Phaff et al., 1978) were known to produce β-carotene, but yields were considered commercially unattractive. Filamentous fungi belonging to Zygomycetes, especially mated cultures of Choanephora spp. and Blakeslea spp. were thought to be more promising; cultures of Blakeslea trispora are employed commercially (Ciegler, 1965; and Whitworth and Ratledge, 1974). There seems to be a continuing interest in fungal production of carotenoids; this is obvious from recent production of β-carotene super-producing mutants of Phycomyces spp. by Murillo et al. (1978). In recent years, a red yeast Phaffia rhodozyma has shown considerable commercial promise as a dietary source of astaxanthin for pen-reared salmonids. The fish require adequate flesh coloration for consumer acceptance, and a diet containing 15% red yeast was shown to induce pigmentation in rainbow trout in 43 days (Johnson et al., 1977). The responsible carotenoid, astaxanthin, comprises over 85% of the yeast's total carotenoids (Andrewes et al., 1976), and could reach concentrations approaching 800-900 µg/g, depending on submerged culture conditions (Johnson and Lewis, 1979). Crustacean wastes which are currently used as sources of astaxanthin in fish diets (Saito and Regiers, 1971; Spinelli et al., 1974), have comparatively lower levels of carotenoid than Phaffia rhodozyma. In addition, they (the wastes) may cause manganese deficiency in the fish due to their abnormally high calcium contents (Meyers and Rutledge, 1971). These deficiencies of crustacean wastes, and obvious presence of nutritive components in Phaffia rhodozyma, enhance its potential value as a supplement in salmonid diets. The yeast was also shown to help impart desirable golden color to egg-yolk and flesh of poultry when fed to the birds

(Johnson et al., 1980). Color enhancement of egg-yolk caused by the yeast was clearly evident when it was added to diets in combination with marigold flower pigments and yellow corn.

There is current global interest in yeasts as sources of protein and other nutrients for man and animals. Their high content of vitamins, especially those of the B group, has been referred to earlier in this review. As a protein source, whole yeast cells (yeast biomass) in wet or dried form, or protein concentrates prepared therefrom, may be used. Since ancient times, man has consumed yeast biomass through baked and fermented foods, but the nutritional contribution of such biomass is difficult to assess. Recently, Reed (1982) stated that in modern white bread, yeast protein contributes about 5% to the total protein content. Use of yeast biomass in animals feeds is also an old practice; it began with addition of spent brewer's yeast to livestock feeds. Braude (1942) has comprehensively reviewed the development of the practice and the nutritional benefits of using yeast in various animal rations. The Germans were the first to propagate yeast for use in human food; they grew the yeast Candida utilis (also called torula yeast) in wood hydrolysates and in sulfite waste liquor from pulp and paper industry. Since then other yeasts have been grown commercially on carbohydrates for food and feed applications. They include Saccharomyces cerevisiae grown on molasses, Kluyveromyces fragilis grown on whey (Peppler, 1968), and a mixed culture of Candida utilis and Endomycopsis fibuligera grown on potato processing wastes (Jarl, 1969; Skogman, 1976). The mixed culture has been called "the Symba Process", and the role of E. fibuligera is to furnish, by its amylolytic activity, reducing sugars for growth of C. utilis. Though both organisms grow simultaneously, more than 90%

by weight of Symba yeast is C. utilis, a recognized food yeast. One further development in commercial propagation of yeasts is the use of hydrocarbons as substrates; these include gas-oil, n-paraffins, kerosine, and derived products such as ethanol and methanol. A detailed world-wide compilation of various hydrocarbon based fermentation processes has been made by Laskin (1977). Yeasts especially Candida spp. constitute the dominant group of organisms.

In order to clarify the nutritive value of yeasts, their vitamin and amino acid patterns have been determined by various workers. Bressani (1968) reviewed some data on the amino acid and vitamin composition of torula and brewer's yeasts. Wasserman (1961) presented similar information on Kluyveromyces fragilis and compared it with data on other yeasts. All the data generally indicated that yeasts were rich in vitamins and lysine, and were balanced in others except the sulfur-containing amino acids such as methionine and cystine. Many feeding experiments on laboratory animals and man confirmed the deficiency of yeast in the sulfur-containing amino acids (Bressani, 1968). Other studies, however, showed that yeast protein (supplied as biomass) was a good supplement for cereals and other foods. Additions of torula yeast to rice, corn, wheat, cottonseed and sesame improved protein quality of the foods as measured by gains in body weight and protein efficiency ratios (PER) in experimental animals. Since the tested foods were deficient in lysine, it was concluded that the role of added yeast was to make up the deficiency. Addition of yeast to soybeans did not achieve similar results because both were deficient in methionine. As a result of these and other experiments it was concluded that yeasts would become an excellent protein feedstuff for use in diets if supplemented with methionine (Bressani, 1968; Smith

and Palmer, 1976; Wang and Fields, 1979). Recently, methionine over-producing mutants of Saccharomyces cerevisiae (Cheowtirakul, 1980) and Saccharomycopsis lipolytica (Morzycka et al., 1976) have been produced. This development would enhance the nutritive quality of the strains and would obviate the need for use of synthetic methionine in diets.

In addition to its nutritive value, yeast biomass may serve other purposes in foods. Reed (1982) states that inactive dried food yeasts may contribute to flavor when added in small concentrations to fabricated foods such as baked goods, baby foods, soups and others. Yeasts fortified with water-soluble vitamins are also available in the form of tablets or dried powders for therapeutic use. A torula yeast product has been described as a flavor enhancer or extender in many food products; in some meat products, its use permits a 20 to 50% reduction in pepper and other spices (Anon., 1974).

As mentioned previously, yeast protein may be applied to foods in the form of concentrates derived from whole cells. Such concentrates may have functional attributes in addition to their nutritive value. Schnell et al. (1976) has described the nutritive and functional characteristics of Torutein, a protein concentrate derived from Candida utilis grown on ethanol. The protein is rich in lysine and is a complement to cereal-based products. Addition of 5% Torutein not only increases PER of corn cereal by 40%, it also markedly improves extensibility of dough as well as the texture and flavor of such products as doughnuts and wheat flour tortillas. Reed (1982) believes that the term single cell protein (SCP) is more suitable for protein concentrates than for whole cells (biomass), although it is popularly used for both. After concentrating protein from yeast cells, glycan which contains primarily

cell wall debris may be used as thickener for salad dressings, soups and other food items. Of greater interest is the finding that isolated comminuted cell walls of baker's yeast (yeast glycan) had a strong effect in reducing cholesterol levels in rats fed a hypercholestrerolemic diet (Robbins and Seeley, 1977). It was thought that the glycan might be partly serving as dietary fiber. Yeast RNA may also be concentrated after separation of protein and may be processed further to yield flavor-enhancing nucleotides. Yeast autolysates (extracts) which are also useful as flavoring agents and as condiments may also be prepared from yeast biomass. Peppler (1978) and Reed (1982) have written an extensive chapter and a review article covering global yeast propagation and production of various yeast-derived products which find applications in food and feed manufacture.

Microbial protein has been under intensive study in the last decade as a means of alleviating global shortage of protein. Microorganisms are thought to have advantages over conventional sources of protein, namely, higher plants and animals. Kihlberg (1972) has discussed the advantages. Briefly, microorganisms, in general, have a higher growth rate, higher protein content and can be easily modified genetically to improve their amino acid composition. Microbial propagation requires only a small land area and can be carried out in continuous culture and independent of climatic changes. In addition, single cell protein production can be based on various raw materials which may be available in large quantities and for long periods depending on locality. Among the microorganisms, protozoa have received little consideration; they have not been cultivated as a direct source of food (Bunker, 1968) and would not compete with yeasts. Various experts (Anon., 1979; Bunker,

1968; Beuchat, 1978; Kihlberg, 1972; and Reed, 1982), have reviewed the relative merits and problems associated with various microbial groups as sources of protein. All the groups tend to be deficient in sulfur-amino acids when compared with conventional proteins such as whole egg, and may cause digestive or allergic problems depending on the individual consumer (animal or man), and the level consumed. However, the yeasts are the most preferred source of SCP. Indeed, Moo-Young (1976) found in a world-wide survey of commercial SCP facilities that 42 out of 54 plants use yeasts. Many workers (especially those cited earlier), have provided possible reasons for the preferential use of yeasts. They include, age-long acceptance of yeasts as food and fodder material and their relative lack of toxicity or pathogenicity in comparison with other microbial groups. In addition, the yeasts have a higher growth rate and higher content of vitamin B than other fungi, they are non-dependent on photosynthesis unlike the algae and they have less nucleic acid and are easier to recover from the culture medium than the bacteria. It would appear that there is ample justification for the preferential use of yeasts and their products in human and animal nutrition.

Some problems associated with use of yeast in foods.

Despite their widespread acceptance, some problems limit use of yeasts in foods. One major problem is the high nucleic acid content of the yeast cell (indeed of microorganisms generally!). Inclusion of large amounts of yeast or yeast protein in human food is undesirable. This is because uric acid resulting from nucleic acid degradation cannot be metabolized by man, and could be deposited in the joints with consequent risk of gout. Man lacks the enzyme uricase which oxidizes uric

acid to the more soluble allantoin. This problem would not arise in animal feeding because they can metabolize uric acid. Edozien et al. (1970) fed different levels of Candida utilis to humans and found that daily feeding of 45 g yeast containing 2.9 g nucleic acid was sufficient to raise serum uric acid to unusually high levels. They deduced that maximum intake should be about 2 g of nucleic acid per day to avoid risk of health problems. This means that for a yeast crop to be used in human feeding, its nucleic acid content must be reduced to a safe level. Considerable research has gone into this problem and promising methods have now been developed for reducing the nucleic acid content of yeast biomass. The methods have been discussed and the merits and demerits of each one analyzed by Peppler (1978), Kharatyan (1978), and Kihlberg (1972). In general, they include regulation of growth rate (and consequently nucleic acid synthesis) by environmental manipulation, mechanical and chemical disruption of cells to leach out nucleic acids, and use of exogenous and endogenous ribonuclease with or without heat shock. Trevelyan (1976) has developed an autolytic method. In general, some of the methods can reduce yeast nucleic acid to about 1%. This progress, together with the reasonably safe applicability of yeast in animal feeding, would probably continue to maintain the high place of yeast in the food industry.

Another problem connected with nutritional use of yeast is the limited digestibility or extractability of intracellular components of intact yeast. Mitsuda et al. (1969) found digestibility to rats of proteins extracted from torula cells to be 95.8%, whereas that of intact cells was only 64.6%. Protein in intact cells of Candida sp. was also shown to be appreciably less susceptible to an in vitro pepsin digestion than the protein isolate removed from the cells by alkaline treatment (Mitsuda

et al., 1970). The protein efficiency ratios (PER; grams of gain in body weight per gram of protein consumed) for Saccharomyces cerevisiae fed to mice (Hedenskog and Mogren, 1973; Mogren et al., 1973) and to rats (Omstedt et al., 1975), were found to be 18-37% greater with mechanically disintegrated lyophilized cells than with intact lyophilized cells. Johnson et al. (1980) found that astaxanthin produced by the yeast Phaffia rhodozyma could not be deposited in the skin of rainbow-trout when intact yeast was included in the feed, in contrast, efficient deposition occurred when yeast cells were mechanically ruptured or enzymatically digested before inclusion into the feed. When the red yeast was fed to birds (Johnson et al., 1980), astaxanthin from broken yeast and astaxanthin contained in oil extracted with solvent (acetone) after mechanical disruption of yeast, were deposited in egg-yolks; the pigment was not biologically-available from intact yeast cells. Mahoney et al. (1974) were unable to extract lactase from fresh cells of Kluyveromyces fragilis unless the cell walls were ruptured; solvent-induced autolysis and homogenization with glass beads were effective in permitting extraction of the enzyme. These studies indicate that the integrity of the yeast cell must be disrupted to improve extractability or biological availability of its metabolites. It is obvious that the major limiting factor is the cell wall because its rigidity maintains the shape and strength of the yeast cell (Phaff et al., 1978). In order to examine further the practical importance of the yeast cell and methods available for its disruption, its chemical components will be reviewed.

B. THE STRUCTURE AND SIGNIFICANCE OF THE YEAST CELL ENVELOPE

The term 'cell envelope' may be used to include all structures comprising the surface on the yeast cell. In every yeast, the cell wall normally forms an integument surrounding the whole cell. However, in some, for example Phaffia rhodozyma (Miller et al., 1976), the wall may carry an externally-secreted capsular material; yeast capsules can be considerably heterogeneous in chemical composition (Phaff, 1971). Hair-like structures termed fimbriae have also been found on the cell walls of many yeasts (Poon and Day, 1975). That the fimbriae may be unrelated to capsules could be inferred from their presence in relatively uncapsulated yeasts such as Saccharomyces and Schizosaccharomyces.

Chemical composition of the yeast cell envelope.

There is a great deal of information on the chemical composition of the yeast cell envelope which has been revealed by physical, chemical and enzymatic analyses. The literature has been reviewed frequently (Barlnicki-Garcia, 1968; MacWilliam, 1970; Phaff, 1971, 1977; Rose, 1978). Only the basic features will be reviewed in this presentation.

According to MacWilliam (1970), the cell envelope of various strains of brewing yeasts comprise 6-27% dry weight of the cell and contains glucan (30-45%), mannan (30-45%), protein (10-25%), chitin (0.5-1.0%), phosphate (0.1-2.0%) and lipid (6%). Many factors can affect the relative proportions of the cell wall components. They include (in addition to strain differences) culture age (Masschelein et al., 1963), substrate availability (carbon(glucose) and nitrogen (NH_4) (McMurrough and Rose, 1967), phosphate availability (Ramsay and Douglas, 1979) and growth factor (such as inositol) deficiency (Power and Challinor, 1969).

Occurrence in some yeasts, of different morphological types, for example mycelial and blastospore forms in Candida albicans (Chattaway and Holmes, 1968) and Paracoccidioides brasiliensis (Kanetsuna et al., 1969), and ellipsoidal and triangular forms in Trigonopsis variabilis (Sentheshanmuganathan and Nickerson, 1962), may also introduce variations in amounts of various cell wall components. These possibilities are essential to this study because Phaffia rhodozyma contains typical vegetative and chlamydospore forms (Miller et al., 1976).

1. Glucans

The glucan component of the yeast cell wall is a highly branched polysaccharide. In Saccharomyces cerevisiae there are at least three separable molecular species, namely, an alkali-insoluble, acetic acid insoluble β-(1-3) glucan (Manners et al., 1973a), an alkali-insoluble, acetic acid soluble β-(1-6) glucan (Manners et al., 1973b) and an alkali-soluble β-(1-3) glucan (Fleet and Manners, 1976). There is electron microscopic evidence that the alkali-insoluble glucans (β-(1-3)) occur in the cell wall as a tightly-structured microfibrillar network (Kopecka et al., 1974); they are thought to be primarily responsible for the shape and structural integrity of the wall.

In addition to the β-linked glucans, α-(1-3) glucans are present in the walls of some yeasts and many higher fungi. These alkali-soluble polysaccharides appear to be absent in the walls of Saccharomyces cerevisiae, but they have been detected in the cell walls of species of Schizosaccharomyces and Cryptococcus (Bacon, 1968) and in the red yeast Phaffia rhodozyma (Miller et al., 1976). Jones et al. (1969) showed that the level of α-(1-3) glucan synthesized by a yeast may depend on the medium of growth. In a rich medium (glucose-peptone), Cryptococcus

albidus and C. terreus synthesized a normal component, but in glucose-salts medium, the level of α-(1-3) glucan was reduced. It was also observed that the cell walls of C. albidus grown in the less favorable medium had greater susceptibility to lysis by a Streptomyces spp. and Cytophaga johnsonii than those of the cells grown in the richer medium. This probably indicates that α-(1-3) glucan plays a role in maintenance of cell wall integrity.

2. Mannan

Studies on the mannan component of yeast cell walls have been reviewed by Ballou (1976). Mannan appears, on the one hand, to be complexed with protein, and on the other, with glucan. Indeed, it is often present in alkaline extracts of yeasts as mannan-protein complex mixed with some alkali-soluble glucan (Phaff, 1977). Previous workers such as Kessler and Nickerson (1959) had isolated glucan-mannan-protein complexes from yeast cell walls. In general, there are three parts to the mannan molecule. The backbone consists of an α-(1-6)-linked chain of mannose residues; to this short α-(1-2) and α-(1-3)-linked mannose side chains are linked. Structural analyses have shown that the side chains vary in length with different strains of yeasts and may contain other side groups such as phosphate (in some strains of Saccharomyces), mannosyl phosphate (in Kloekera species), and N-acetyl glucosamine (in species of Kluyveromyces) (Phaff, 1977). Then there is an inner backbone near the point of attachment of protein. This core is also known to have an α-(1-6)-linked mannose backbone and short α-(1-2) and α-(1-3)-linked mannose side chains; the backbone is linked through N-acetylglucosamine to an aspartate residue in protein. The protein also carries manno-oligosaccharide attached to it via serine and threonine residues; the short

oligosaccharides appear to have the same structure as those of the side chains of the high molecular weight mannan, and are removable by mild alkaline treatment (Sentandreu and Northcote, 1968).

The mannan-protein complex has structural (just described), and enzymic portions. The latter fraction consists of the glycoprotein enzymes, such as invertase, acid phosphatase, glucamylase (Lampen, 1968). They appear to be partly localized in the periplasmic space and some workers such as Lampen (1968) and Kidby and Davies (1970) have proposed models to illustrate the relationship of these enzymes with other components of the cell wall. According to the later model (see Appendix 1), invertase is not complexed with other wall components and is retained in the periplasmic space by an external region of the cell wall which contains disulfide bridges, serves as permeability barrier and maintains cell wall integrity.

3. Chitin

As mentioned previously in this review, some N-acetylglucosamine links the inner core of mannan with protein; this accounts for the presence of glucosamine in acid hydrolysates of walls of Saccharomyces cerevisiae. The bulk of the N-acetylglucosamine, however, is present as a β-(1-4)-linked polymer called chitin. Exhaustive extraction of mannan, glucan, protein and lipid leaves the chitin as an insoluble residue (Phaff, 1971). It has been shown that in Saccharomyces cerevisiae, the chitin is localized in bud-scars (Cabib and Bowers, 1971). Some yeasts, for example species of the fission yeast Schizosaccharomyces (Bush et al., 1974), and triangular forms of Trigonopsis variabilis (Sentheshanmuganathan and Nickerson, 1962), seem to be entirely devoid of chitin. In Phaffia rhodozyma, the polymer also appears to be lacking (Johnson

et al., 1979), while in some members of Cryptococcus (Bacon et al., 1968) and in general Rhodotorula, Sporobolomyces and Nadsonia fulvescens, the chitin content can be quite high (Kreger, 1954).

4. Lipid, Protein and Phosphate

Lipids have been isolated from yeast cell walls, but whether they are true wall components has often been questioned (Rose, 1978). They are often thought to be contaminants from the lipid-rich plasma membrane. Dyke (1964) appeared to provide some reliable evidence that lipids are intrinsic components of the cell wall. It was shown that cell wall preparation from Nadsonia elongata, which was not significantly contaminated with cytoplasmic material, contained lipid. Of particular interest was that palmitoleic acid, the major fatty acid of whole cells, was completely absent from the cell wall.

Proteins and phosphates exist in the yeast cell wall mainly as complexes with mannan. Amino acid analyses have shown that the proteins may be of great variety and may serve to cross-link mannan sub-units, thus increasing cell wall integrity (Thieme and Ballou, 1972).

Significance of the yeast cell envelope.

The envelope serves obvious protective functions for the cell. The enzymic components such as invertase, may be important in cellular nutrition. One evidence of this is the finding that sucrose-fermenting ability of a strain of Saccharomyces cerevisiae was lost when it was converted to protoplasts by enzymatic digestion of its walls (Islam and Lampen, 1962).

Besides its physiologic functions, the yeast cell envelope may have practical consequences depending on the type of yeast. In some pathogenic

yeasts such as Cryptococcus neoformans, the capsule is important in virulence (Gentles and Latouche, 1969), possibly due to its ability to inhibit phagocytosis by macrophages (Gadebusch, 1960). There is evidence that resistance of Candida albicans to polyene antibiotics is related to the integrity of the cell wall which limits diffusion of the antibiotics; stationary phase cells with thicker walls are more resistant than exponential phase organisms (Hammond and Kliger, 1974; Gale, 1975).

The mannan molecule confers serologic specificity to strains of yeast (Campbell and Gilmour, 1969). Phaff (1977) attributed this to the side chains containing α-(1-2) and α-(1-3) mannose-linked residues and other attached groups which have been described in this review; the side chains and groups vary in their length and nature with different strains of yeast. In the genus Saccharomyces, serological differences have been exploited in devising methods for identifying wild strains in the brewery (Campbell, 1968; Richards and Cowland, 1967).

Flocculence, a desirable property of brewing strains of yeast, is determined by the cell wall. MacWilliam (1970) reviewed the structure of yeast cell wall in relation to the phenomenon of flocculation. According to the review, the mannan-protein fraction of the wall is involved in the phenomenon. Specific components include phosphodiester linkages in the cell wall phosphomannan (Lyons and Hough, 1971) or carboxylic groups in wall proteins responsible for bridge formation with Ca^{2+} between the cells (Taylor and Orton, 1973; Stewart et al., 1975). There is also some evidence that the fimbriae are involved in yeast flocculation (Day et al., 1975). Cell surfaces of flocculent strains of Saccharomyces cerevisiae and S. carlsbergensis (uvarum) were found to carry more fimbriae than those of non-flocculent strains. What seems

to be interesting here is the observed ability of α-amylase and pronase to destroy flocculating ability; it was suggested that the fimbriae might be surface mannan-protein complexes.

The role of the cell wall in flocculation of yeast appears to be a desirable one. However, the structure presents problems where large crops of yeast cells are grown specifically for use as sources of nutrients and other metabolites applicable in foods. Limitations imposed by cell walls on solvent extractability and intestinal absorption of nutrients and commercially desirable metabolites of yeasts have been reviewed earlier. They necessitate appropriate post-harvest treatments designed to disrupt or modify yeast cell walls.

Methods of yeast cell wall modification.

The effects of mechanical breakage and autolysis in enhancing extraction and absorption of protein, astaxanthin and lactase from various yeasts have been pointed out previously. Other methods of yeast modification are plasmolysis, hydrolysis, enzymatic, chemical and genetic (mutagenesis). Many of the procedures have been reviewed by Tannenbaum (1968), with emphasis on their application in single cell protein processing.

Mechanical disintegration of yeasts for various purposes is an efficient procedure in laboratory practice. Some industrial scale homogenizers have been described and shown to have potential utility in extraction of protein, enzymes and possibly other products of yeasts (Follows et al., 1971; Hedenskog and Mogren, 1973; Mogren et al., 1974). It is doubtful whether the instruments are commercially available in large quantities and at moderate prices. Mechanical disruption of yeast cell

walls, in general, would be expensive in view of large energy required for the process (Phaff, 1977).

Thermal processing of yeast in aqueous or oil suspensions and drum-drying, were effective in improving nutritive value of yeast protein as shown by high protein efficient ratios (PER) in mice (Mogren et al., 1973). Spray-drying, apparently milder than drum-drying, was not effective. Since drum-drying did not rupture the cell wall (as shown in electron micrographs), its effect in increasing nutrient availability (as shown by PER) was thought to be due to adverse effects on the selective permeability of the plasma membrane. It is important to note that heat treatment would be inapplicable to yeasts meant for use as sources of carotenoids and possibly enzymes, because the components would be considerably denatured. For example, Johnson et al. (1978) observed an appreciable reduction in astaxanthin content of Phaffia rhodozyma by heating the yeast at 80°C for only five minutes.

Autolysis and plasmolysis utilize the cell's indigenous enzymes for dissolution of the cell walls. The former is achieved by incubating the cells at pH 6.5 and at 45-50°C for up to 14 hr (Hough and Maddox, 1970). It has been used in extraction of commercial enzymes such as lactase (Mahoney et al., 1974) and invertase (Cochrane, 1961). In plasmolysis a high concentration of sodium chloride (up to 25%) is used to accelerate autolysis of yeast. The two procedures, together with hydrolysis are used in preparation of commercial yeast extracts used as flavoring agents (Acraman, 1966; Johnson, 1977). The latter procedure (hydrolysis) involves disruption of yeast walls with strong acid (HCl); it may be destructive to some nutrients such as tryptophan (Kihlberg, 1972) and would be undesirable for carotenoid extraction. Johnson et al.

(1980) suggested possible use of autolysis for treating Phaffia rhodozyma to enhance bio-availability of the carotenoid astaxanthin. Since many chemicals (commonly organic solvents) have been used to accelerate autolysis of many yeasts, some discrimination might be essential if the product would be used as food or feed.

Some chemical methods which have been applied to extraction of yeast metabolites include use of concentrated urea, sodium hydroxide and thiol reagents. Soaking with a strong solution of urea (8M) was effective in protein extraction (Mitsuda et al., 1969), but Kihlberg (1972) has mentioned possible formation of questionable substances such as homocitrullin and cyanate during urea soaking. Concentrated sodium hydroxide has been used for protein extraction from yeast (Lindblom, 1974; Vananuvat and Kinsella, 1976; Hedenskog et al., 1970; Hedenskog et al., 1973). Some of these workers achieved satisfactory extraction of protein with the alkali only after mechanical disintegration of cells; this would complicate the procedure and increase cost in a commercial situation. In addition, alkali can denature some amino acids such as lysine, cystine and arginine (Kihlberg, 1972). Carotenoids are also labile to alkali (Davies, 1976) and the procedure would not be useful for disrupting cell walls of carotenoid producing yeasts. Thiol reagents were applied to enhance protein extraction from yeast by Shetty and Kinsella (1978). Since the reagents activated proteolytic enzymes which hydrolyzed extracted protein, initial control of the procedure was considered necessary to avert the hydrolysis. However, the pungent odor of some of the thiols such as dithiothreitol would probably preclude their use in commercial treatment of food or feed yeast.

Genetic procedures (i.e. mutagenesis) have been used to modify yeast

cell walls for possible commercial application. Harada et al. (1972) isolated a wall-defective mutant of a hydrocarbon assimilating Candida sp. Protein from the mutant showed greater susceptibility (than the parent) to extraction by alkali and to in vitro pepsin digestion. The mutant was also more susceptible than the parent to homogenization in a Manton-Gaulin homogenizer; 100% versus 37.9% disruption were achieved in 7.5 minutes disruption time. Mehta and Gregory (1981) isolated mutants of Saccharomyces cerevisiae and Candida utilis with increased susceptibility to digestive enzumes. Unfortunately, the observed cell wall deficiency in some of the mutants did not appreciably enhance efficiency in utilization of their protein by rats. Johnson et al. (1980) has suggested development of capsule-deficient mutants of Phaffia rhodozyma as a possible method of improving utilization of its carotenoid astaxanthin. It is pertinent to mention that long-term stability of wall-defective mutants would be difficult to predict and that this would probably retard their possible commercial applications.

A method of yeast cell wall modification that has considerable commercial potential is enzymatic digestion by means of wall-lytic enzymes. Though it was proposed by Phaff (1977) for possible application in protein extraction, it would be useful for other yeast products such as carotenoids which are labile to many physical and chemical cell wall disruption methods. Sources and basic characteristics of cell wall lytic enzymes will now be reviewed in relation to their potential utility in yeast processing.

C. SOURCES AND CHARACTERISTICS OF YEAST CELL WALL LYTIC ENZYMES

Enzymes with lytic activity on components of the yeast cell wall belong to two broad categories, namely, endogenous lytic enzymes produced by the yeast itself, and lytic enzymes produced by other living organisms.

Endogenous lytic enzymes of yeast.

Knowledge in this category of enzymes has accumulated as a result of attempts to understand some morphogenetic phenomena which probably necessitate some localized wall softening in the yeast cell cycle. These phenomena include budding, mating, ascus lysis, cell elongation, autolysis and others. Much work in the field has emphasized glucanases of yeast, probably because of the well established role of glucans in maintenance of cell wall integrity. However, yeast associated chitinases and xylanases have also been found. Phaff (1977, 1979, Villanueva et al., 1979), have reviewed the literature on endogenous lytic enzymes of yeasts. The available information indicates that levels and patterns of activity of the enzymes can vary considerably among yeasts. Instability of some of the enzymes make studies difficult. It is also clear that relatively little work has been done on wall lytic enzymes of basidiomycetous yeasts. With regard to their potential practical utility, Phaff (1977) emphasized that limited knowledge of the regulation (including activators and inhibitors) of the yeast-associated enzymes, is a major stumbling-block. In this respect, it is noteworthy that Shetty and Kinsella (1978) claimed that thiol reagents which enhanced extractability of protein from brewer's yeast, activated the yeast's wall-associated glycosidases. Their claim was based on release of soluble

oligosaccharides from yeast cells during incubation with the thiol reagents. It would appear that further research might unravel methods of utilizing yeast endogenous enzymes to modify the cell and improve its commercial utility.

Yeast cell wall lytic enzymes from other sources.

The main sources are the digestive juice of the garden snail and suitable culture fluids of certain microorganisms. In general, the lytic enzymes occur as complex mixtures of hydrolytic enzymes capable of degrading some or all the major components of the yeast cell wall. The hydrolytic components of lytic mixtures are usually detected by application of suitable assay procedures and may vary in relative proportions and combinations depending on the source. The snail digestive juice for example, contains glucanases, lipases, phosphatases and possibly chitinases (Anderson and Millbank, 1966), the lytic complex of Oerskovia sp. CK contains glucanases (β-(1-3) and β-(1-6)), mannanase, protease and amylase (Obata et al., 1975), the lytic complex of Bacillus circulans WL-12 contains glucanases (α-(1-3), β-(1-3), and β-(1-6)), xylanases and chitinase (Johnson et al., 1978, 1979). Some of these and other lytic enzymes, in crude or purified forms, have become potent tools in structural analysis of fungal (including yeast) cell wall polysaccharides, and in protoplast formation (Fleet and Manners, 1977; Kopecka, 1975; Kopecka et al., 1974; Villanueva and Acha, 1971). At least one lytic enzyme has been used for isolation of DNA from yeast (Shurygin et al., 1978).

The lytic enzymes of the snail digestive juice are available commercially, but their limited quantities restrict their use to laboratory

studies. In contrast, microbial sources of lytic enzymes are thought to be more promising for industrial use, especially in treatment of yeast to enhance recovery of useful intracellular metabolites. Microorganisms that produce lytic enzymes are of great variety (Kopecka, 1975; Phaff, 1977; Sugimori et al., 1972; Villanueva and Acha, 1971). They include unicellular bacteria especially Bacillus spp. (Tanaka and Phaff, 1965), myxobacteria and actinomycetes (Jones et al., 1969) and filamentous fungi (Sugimori et al., 1973). Only the bacteria will be discussed further in relation to their potential use in yeast processing.

Enzymes that lyse yeast cell walls are produced by Arthrobacter, Bacillus, Cytophaga, Oerskovia and Streptomyces (Phaff, 1977). In addition to obvious morphological differences among the genera, the composition of the lytic enzymes complexes also vary, as illustrated by the composition of the lytic complexes of Bacillus circulans and Oerskovia sp. previously described (Obata et al., 1975; Johnson et al., 1979). However, β-glucanases, especially β-1-3-glucanases appear to be common components of these complex mixtures of enzymes; this is consistent with the role of glucans in maintenance of yeast cell wall integrity. In some of the lytic mixtures, the isolated β-glucanases (chiefly β-1-3, but also β-1-6), acting alone are sufficient to achieve lysis of yeast cell wall preparations (Tanaka and Phaff, 1965) or whole viable cells (Kitamura et al., 1975); in the latter case, an endo-β-1-3-glucanase which lysed viable yeast cells was termed zymolyase. In other cases, alkaline proteases from Oerskovia xanthineolytica and Arthrobacter sp. were shown to act synergistically with their respective endo-β-1-3-glucanases in lysis of others strains of Saccharomyces cerevisiae (Scott and Schekman, 1980; Vrsanska et al., 1977a). Another glucanase,

α-1-3-glucanase produced by Bacillus circulans WL-12 appears to play an important role in lysis of cell walls of yeasts containing α-1-3-glucan in their cell walls, for example Phaffia rhodozyma (Johnson et al., 1979).

Most lytic systems known are inducible; a suitable preparation of yeast cell walls must be present as carbon source in the culture media for optimal induction of the enzyme systems. This means that efficiency of a lytic system is not only determined by the overall capability of the producer, but also by the nature of the cell wall inducer. For example, in an experiment called the cross-induction test, Tanaka and Phaff (1966) showed that Bacillus circulans WL-12 which was inherently capable of lysing cell walls of Schizosaccharomyces pombe, could not do so when other yeasts including Saccharomyces cerevisiae served as inducers of its lytic enzyme complex. A culture filtrate, derived from growth of Oerskovia sp. on viable cells of S. cerevisiae, differed in its effects on viable cells of other yeasts and was totally unable to lyse cells of Rhodotorula rubra and Sporobolomyces salmonicolor (Mann et al., 1972). The resistance of the yeasts was thought to be due to their relationship to basidiomycetes; cell walls of that group of yeasts differ structurally from those of ascomycetous yeasts (Kreger-van Rij, 1971). Many basidiomycetous yeasts were also resistant to lysis by an enzyme of Arthrobacter GJM-1 induced by baker's yeast cell walls (Vrsanska et al., 1977b).

These limitations of lytic enzymes, caused primarily by differences among the producers and among yeast cell walls, must be taken into account in any scheme for processing yeasts enzymatically. Some preliminary screening is essential to demonstrate unequivocally that a given organism has lytic ability on the walls of a desired yeast. One suitable method

of testing for lytic activity is the cup-plate assay. Yeast cell walls are incorporated in agar and a crude culture fluid of the lytic organism dropped in wells in the agar plate. If lytic enzymes are present, a clearing zone is observed visually (Tanaka and Phaff, 1965). Similarly, a bacterium may be streaked on a nutrient agar plate containing the cell walls of the yeast; development of lysed zones around the streak will then serve to indicate lytic ability on the cell walls. An alternative is to suspend the yeast cell walls in buffer, add the enzyme source, and to follow spectrophotometrically the decrease in turbidity caused by the enzyme solution. In all the tests, heated or viable yeast cells may be used to replace cell walls (Yokotsuka et al., 1974). Because the glucans of the yeast wall contain principally β-1-3 and β-1-6 bonds, some workers have tested the ability of lytic enzymes to hydrolyze β-1-3 and β-1-6 polymers, laminarin and pustulan respectively. Such tests would not be useful approaches in evaluating the practical utility of lytic enzymes. It has been shown that fractions of lytic enzymes showing appreciable laminarinase and pustulanase activities may be deficient in yeast cell wall lytic activity (Fleet and Phaff, 1974). Besides, Mann et al. (1978) observed considerable variations (7.5-fold) in ratios of lytic to laminarinase activity of various strains of Oerskovia and have argued that laminarinase activity of crude enzymes is not a satisfactory indicator of cell wall lytic activity. In general, reliable evaluation of the lytic potential of an organism or its enzyme system must be based on assays using appropriate yeast cell walls as substrates.

Other characteristics of a lytic organism may be important in assessment of its commercial potential. For example, many strains of Oerskovia xanthineolytica had predatory activity on Saccharomyces cerevisiae in

cross-streaks on agar plates; the morphology of the cells varied with stages of the culture and included a motile phase (Mann et al., 1978). In addition, their activity on viable cells quickly lysed these cells in mixed cultures in absence of osmotic support (Mann et al., 1972). The potential significance of these features will be apparent from a review of some methods in which lytic systems could be utilized in large-scale processing of yeast.

A simple enzymatic treatment of yeast would involve an addition of a culture fluid containing a lytic system to a harvested washed yeast mass. Adoption of this approach is limited by the inducible nature of lytic systems; yeast cell walls cannot be prepared on a large-scale for inclusion in culture media. Since many lytic organisms are now known to attack viable cells, the need for cell wall preparation could be avoided. Indeed, an Arthrobacter sp. has been grown on Saccharomyces fragilis for production of lytic enzyme complex in batch and continuous flow fermentors; lytic activity was induced by whole yeast cells (Rowley and Bull, 1977). Enzymes produced in such fermentation might be useful in treatment of yeast mass harvested from cultures. A second possible approach is to inoculate the lytic organism directly onto fresh or heat-killed yeast mass. Only an organism that does not totally lyse yeast cells could be used in this method because release of some intracellular products into the medium might complicate their recovery. The heat treatment might be necessary because some lytic organisms have no effect on viable cells (Webley et al., 1967). Johnson et al. (1978) successfully used Bacillus circulans WL-12 to modify the cell walls of Phaffia rhodozyma after killing the yeast by treatment at 80°C for 5 minutes. Unfortunately, the heat treatment caused some decrease in the content

of astaxanthin in the yeast. Clearly, heat treatment of Phaffia rhodozyma would be undesirable. Otherwise, a temperature lower than 80°C or a shorter time period should be used to minimize pigment losses. It is likely that intracellular yeast proteins that may have nutritive value could also be lost through heat treatment.

A third possibility in large scale enzymatic treatment of yeast would be to grow the lytic organism in mixed culture with the desired yeast. Simultaneous growth of the two could lead to induction of the lytic system and modification of the yeast cell walls. The preferred lytic organism should have attributes that would guarantee possible coexistence with the desired yeast; it should have no predatory characteristics like the Oerskovia spp. mentioned previously and should be able to digest yeast walls without effecting total lysis of the cells. Before a consideration of Bacillus circulans WL-12 as a potential candidate in this respect, some industrial applications of mixed cultures will be reviewed.

D. INDUSTRIAL APPLICATIONS OF MIXED CULTURES

It is well known that mixed populations are characteristic of natural environments such as the soil, water, air, bodies of plants and animals. As a result, fermentations of wastes (such as those involved in composting and sewage treatment), and natural products, are mediated by mixed cultures. Among the fermentations of natural products are those involved in food production, for example sauerkraut, cheese and other dairy products, and some alcoholic beverages. Knowledge of the types of organisms involved in a given fermentation has led to the use of

defined strains often termed "starter cultures"; some of these may be mixed cultures of different species or strains but will not be considered here. The primary interest here is on mixed cultures of organisms which were not naturally associated with known fermentations, but which were brought together to achieve specific objectives in industry.

Many advantages of mixed cultures over pure cultures have been claimed in fermentation systems (Larsen et al., 1978). They include, among others, increased yield and higher food value of product, more efficient utilization of mixed substrates, higher growth rates, improved process stability, and lower incidence of contamination. Many combinations of microorganisms can be employed to achieve desired ends. However, this discussion will be limited to those in which at least one member is a yeast.

It appears that most of the mixed cultures which have employed yeasts were designed primarily to produce single cell protein (in form of biomass). Carbohydrate-rich substrates including waste products from the food industry, have been employed. Unfortunately, the yeast intended for cultivation often lacked the enzymatic machinery for utilizing the polymeric substrates, starch and cellulose. Another organism (probably a yeast), was therefore added to the culture to hydrolyze the substrate and furnish the reducing sugars needed for growth of the desired yeast. This principle was the basis of a mixed culture on starch wastes, of two yeasts, Endomycopsis fibuligera and Candida utilis (a recognized food and feed yeast). The process has current industrial application; it is called the "Symba Process", and has been extensively described by Jarl (1969), Skogman (1976) and Wiken (1972). Amylolytic enzymes produced by the former yeast hydrolyze the starch to reducing sugars

which are utilized by the faster growing Candida utilis. It is estimated that more than 90% of the final product (Symba yeast) is Candida utilis. Mixed cultures based essentially on the same principles, but utilizing alkali-treated barley straw as the substrate, have been described for Trichoderma viride (a cellulolytic fungus) and Candida utilis or Saccharomyces cerevisiae (Peitersen, 1975), and Cellulomonas sp. (a cellulolytic bacterium) and C. utilis (Kristensen, 1978). In general, mixed cultures produce useful biomass from otherwise waste materials and reduce their pollution effects on the environment.

In some mixed cultures, one of the components appears to stimulate growth of the other, possibly by production of a growth factor required for its growth. For example, a strain of Candida lipolytica which could not grow on unsupplemented mineral salt-n-alkane medium, achieved good growth in presence of Candida intermedia (Miller and Johnson, 1966). The mixed culture grew more rapidly on n-alkanes than did C. intermedia. Though workers did not examine the substance which stimulated C. lipolytica in the mixed culture, some growth factors secreted by the other component may be involved. It has been shown that Mycotorula japonica which could scarcely grow in a pure culture, grew well in a mixed culture containing Bacillus subtilis (Miura et al., 1973). The bacterium was shown to excrete desthiobiotin which served as a source of biotin required for growth by the yeast.

Two yeasts, Candida lipolytica and C. tropicalis achieved higher growth rates in mixed culture than in pure culture on an n-paraffin fraction of crude oil and on glucose (Achremowitz et al., 1977). About 18% higher yields of biomass was observed in the mixed culture than in the individual cultures. Apparently, a mutual stimulatory effect of

both yeasts occurred in mixed culture. Kamel (1979) reported simultaneous growth of two approved food grade yeasts, Candida utilis and Saccharomyces cerevisiae on waste carbohydrates from date processing (containing primarily glucose and fructose). The yeasts grown together yielded a higher protein content (52.4%) at a shorter fermentation time (8 hours) than did either yeast grown separately (49.4% for C. utilis, and 50.2% for S. cerevisiae) even with longer time of growth (12 hours). Again it can be assumed that the two yeasts had some mutually beneficial effects in the mixed culture.

Sulfonated lignin (in beech wood pulping wastes), a substance highly resistant to microbial attack, was reduced by use of a mixed culture (Ban et al., 1979). An adapted culture containing two Trichosporon spp. (yeasts), two Arthrobacter spp., Pseudomonas putida and Chromobacterium violaceum (all bacteria), was used. Ratio of the bacteria to the yeast was 80:20, as established by counting. The fermentation was carried out in a fermentor at 35°C and an initial pH of 8.5. In 24 hours, about 50% of the calcium lignosulfonate in the wood pulping waste was metabolized by the organisms and single cell protein produced as by-products.

The foregoing examples illustrate how suitable mixed cultures can produce or enhance yield of useful biomass on otherwise waste products. There is also evidence that some organic acid conversions can be mediated by mixed cultures. Bull (1980) described some organic acid fermentations developed in Japan. One of them involves a yeast, Pichia membranaefaciens. In the mixed culture, species of Rhizopus, especially Rhizopus arrhizus (Takao and Hetter, 1977) first converts carbohydrate (glucose) to fumaric acid. The yeast (P. membranaefaciens), with high fumarase activity, then converts the fumarate to malic acid.

Many other applications of mixed cultures have been described. They include other processes for production of single cell protein (including lysine-enriched yeast) from waste products, and authentic chemical compounds such as ethanol from cellulosic wastes. Most of the processes are described in the patent literature and a comprehensive review has been presented by Haas et al. (1980).

The usefulness of mixed culture in production of yeast biomass from a variety of substrates does not solve the problems caused by the structural integrity of the cell walls. Many of the problems have been discussed in earlier sections of this literature review. Modification of yeast cell walls by means of lytic enzymes of microbial origin was suggested (Phaff, 1977) as an attractive approach because of the nondestructive effect of the enzymes on yeast metabolites. As an alternative to the use of lytic enzymes per se, a mixed culture containing a desired yeast and a suitable lytic enzyme-producing organism was proposed; this approach has not been done before and would represent a novel mixed culture fermentation. Bacillus circulans WL-12 which produced a lytic enzyme, has considerable potential for mixed culture application. It is unicellular throughout its life cycle and has no predatory activity on yeast. The lytic enzyme complex of the bacillus is versatile and is inducible by ascomycetous and basidiomycetous yeasts (Tanaka and Phaff, 1966; Johnson et al., 1979). The composition of the enzyme complex shows that it is deficient in proteolytic and mannanase activity (Tanaka and Phaff, 1965); thus its hydrolytic activity is limited only to the glucan portion of the yeast cell wall. These features of Bacillus circulans WL-12 are considered important because they would guarantee its co-existence with a yeast in mixed culture. Many lytic organisms, especially strains

of Oerskovia xanthineolytica, have predatory activity on yeast at some stage of their life cycle (Mann et al., 1978). In addition, many species of Oerskovia (Obata et al., 1975; Mann et al., 1978) and Arthrobacter spp. (Rowley and Bull, 1977; Vrsanska, 1977b) have proteases and mannanase in their lytic systems and can effect total lysis of yeast cells; this would be undesirable in mixed culture.

III. MATERIALS AND METHODS

MICROORGANISMS

All microorganisms used in this study were obtained from the culture collection of the Department of Food Science and Technology, University of California, Davis (U.C.D.). They included six strains of *Phaffia rhodozyma* (U.C.D. F.S.&T. Culture Collection Nos. 67-202, 67-203, 67-210, 67-385, 67-484, 68-653C), *Saccharomyces cerevisiae* 71-141 (Guinness Stout yeast), and *Bacillus circulans* WL-12.

The strains of *Phaffia* were obtained as stock cultures on slants of malt extract agar. After a transfer to YM (Difco) agar slants, followed by incubation for 48 hr at 20°C, they were stored at 4°C and subcultured every two months. *S. cerevisiae* was obtained and maintained on wort (brewer's wort) agar. *B. circulans* was received on slant containing baker's yeast cell walls as the sole carbon source (Tanaka and Phaff, 1965). It was then transferred to another slant containing 0.67% yeast nitrogen base (YNB) (Difco), agar (Difco) 1.8% cell walls of *Phaffia rhodozyma* 0.2%, and .05M potassium phosphate buffer, pH 6.5. Freshly inoculated slants were incubated for 48 hr at 30°C, then kept at 4°C and maintained in the same medium by bi-monthly transfers.

GROWTH CONDITIONS

All fermentations were carried out in batch cultures. Routine mixed cultures were grown in 500 ml baffled side-arm flasks (Bellco Glass Inc., Vineland, N.J.) containing 120 ml of medium and shaken at 200 rpm (Orbital shaker, New Brunswick Scientific, Edison, N.J.) at 20°C for 72-100

hr. The medium was defined and contained 0.7% YNB, 1.5% glucose and phosphate or phthalate buffers at various concentrations and pH; glucose and the buffered basal salts solutions were always sterilized separately at 121°C for 15 mins. The same defined medium was used when cell crops of strains of Phaffia rhodozyma were grown in pure cultures for use in various experiments. When the yeast was grown in alfalfa residual juice (ARJ), the concentrated juice was diluted ten-fold (to contain approximately 2% sugar) in .02M phthalate buffer, pH 5.6. Brewer's malt wort was diluted (with distilled water) to 2% sugar (measured as soluble solids with a refractometer). When molasses was used as the growth substrate, it was diluted twenty-fold. The dilution was determined to contain approximately 5.5% soluble solids, and preliminary tests showed that lower yields of cell mass and astaxanthin were obtained at higher and lower dilutions. Nutrients added to the juice or to the molasses were autoclaved separately.

Fermentor mixed culture experiments were conducted in 5-liter vessels with 3-liter working volumes of medium. Sterile air was supplied at the rate of 19L/min and agitation was at 600 rpm. Temperature was maintained at 20°C. The medium was essentially a modification of that of Johnson and Lewis (1979), and contained in 3 liter, 1.5% cerelose (CPC International, Englewood Cliffs, N.J., U.S.A.) (autoclaved separately), and 0.IM potassium phosphate buffered solution of $(NH_4)_2SO_4$, 2.0%; KH_2PO_4, 1.0%; $MgSO_4 \cdot 7H_2O$, 0.5%; $CaCl_2 \cdot 2H_2O$, 0.1%, yeast extract (Difco) 0.2%, at pH 7.0. In both shake-flask and fermentor experiments, a 10% (v/v) solution of sterile antifoam (FG-10, Dow Corning) was added intermittently to control foaming.

Inocula for the shake-flask mixed cultures or for pure cultures

of Phaffia rhodozyma used in allied experiments, were routinely prepared by aseptically transferring stock cultures of the yeast and of Bacillus circulans WL-12 into individual 250 ml Erlenmeyer flasks containing 50 ml of sterile medium and incubating at 20°C (the yeast) and at 30°C (the bacillus) for 20 hr. The media contained 0.7% YNB, 1.5% glucose and 0.5% phytone (BBL, Maryland, U.S.A.), in .05M potassium phosphate buffer, pH 6.5. A semi-defined medium consisting of 0.5% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 0.5% peptone and 0.5% glucose in .02M phthalate buffer, pH 5.6, was used for growing the yeast inoculum for the experiments with the ARJ, and incubation was for 28 hr.

Cells were collected aseptically by centrifugation at 800 rpm for 10 min (Servall automatic refrigerated centrifuge) and washed twice with sterile distilled water. Those grown for the mixed culture and allied experiments were resuspended to turbidities of 170 klett units (yeast) and 150 (Bacillus), using a Klett-Summerson photoelectric colorimeter (model 800-3, Klett-manufacturing Co., N.Y., U.S.A.), equipped with a red filter; the suspensions contain approximately 1.5 mg/ml of both organisms on a dry weight basis. Yeast cells grown for the ARJ experiments were resuspended to a turbidity of 300 klett units. 2 ml of each suspension was used as inoculum for appropriate experiments in shake flasks.

In fermentor experiments, inocula were prepared by aseptically transferring 10 ml portions of the uncentrifuged inoculum cultures described above, to secondary inoculum flasks where the organisms were grown again as pure cultures for 20 hr. The secondary flasks were identical to the mixed culture shake-flasks, and contained 120 ml volumes .05M phosphate buffered YNB (0.7%), 1.5% glucose, and 0.5% phytone, at pH 6.5; i.e.

the secondary inoculum medium had the same composition as that of the primary inoculum. Cells from the secondary flasks were centrifuged, washed with sterile distilled water, and the organisms resuspended separately to a turbidity of 600 klett units. 25 ml of the yeast suspension and variable volumes of that of the bacillus, were used as the final inocula for the fermentor mixed culture fermentations.

PREPARATION OF CELL WALLS OF PHAFFIA RHODOZYMA

Yeast mass used as a source of cell walls, was grown for 40 hr at 20°C in the 5 liter fermentor (working volume 3 liter). The medium contained in gram per liter: glucose, 10 g; peptone (Difco) 5 g; yeast extract, 3 g; and malt extract (Difco) 3 g. pH was adjusted to 5.8 with 2NHCl prior to autoclaving. Inoculum was developed in the same medium through the three step procedure described for the mixed culture except that entire contents of the secondary inoculum flask was emptied into the fermentor as the final inoculum. Temperature of the culture was maintained at 20°C, aeration (with sterile air) was at the rate of 191/min, and agitation was at 600 rpm, i.e. the conditions employed were similar to those described previously for the fermentor mixed culture.

Yeast cell mass harvested from the fermentor was washed twice with chilled distilled water. The method employed in preparation of cell walls was a modification of that of Melvin (1975). The washed cells were resuspended in chilled .1M Tris buffer, pH 8.5 to create a thick slurry and inactivate endogenous autolytic enzymes possibly present. The slurries were mixed with equal volumes of glass beads (0.45-0.50 mm) in special bottles designed for the chamber of Braun mechanical

cell homogenizer (Bronwill Scientific Inc., Rochester, N.Y.); a head-space of approximately one-quarter of total volume was left in each bottle. Cell disruption was accomplished by vibrating the bottles in the homogenizer. In the process, temperature was maintained just above freezing by delivering suitable amounts of liquid carbon dioxide into the instrument chamber. Usually 70-85% cell breakage (determined by light microscopy) was achieved in 3.5 min disruption period. The homogenates (from all the bottles used) were pooled, decanted from the beads which readily settled in a glass beaker, and centrifuged at 800 rpm for 20 min at 4°C. After washing in the same buffer (0.1 Tris, pH 8.5) twice to remove pigments, the washed cells and walls were subjected to a second disruption treatment in the homogenizer for another 1.5 min. This process produced a final 90-95% cell breakage. The walls were then washed four times with the buffer and four times with chilled distilled water. Cell wall suspensions were finally poured into plastic petri dishes (85 mm diameter), lyophilized and stored at 0°C.

DETERMINATION OF YEAST CELL WALL LYTIC ACTIVITY OF SAMPLES OF MIXED CULTURE FILTRATES

Qualitative and quantitative assays were employed. The qualitative assay was a modification of the agar cup plate method of Tanaka and Phaff (1965). Lyophilized cell walls of Phaffia rhodozyma (0.1%) and purified agar (Difco) 0.85%, were evenly suspended in 0.1M potassium phosphate buffer, pH 6.5 and dispensed into large test tubes in 20 ml amounts. After autoclaving for 6 min to melt the agar and inactivate endogenous glucanases, the contents of each tube was poured into a plastic

petri dish (85 mm diameter) and allowed to set. The dishes were kept at 4°C and used within three weeks. In the assay, wells (6 mm diameter) were made in the agar with a no. 2 cork borer. 0.05 ml amounts of enzyme solutions or culture filtrates were added to the wells and the plates were incubated at 30°C. After 24 hr, zones of clearing around the wells were taken to indicate presence of lytic enzyme activity in the culture filtrates.

Quantitative determination of lytic activity was based on reduction in turbidity of suspensions of autoclaved Phaffia rhodozyma cell walls. The reaction mixture contained 2 ml of culture filtrate (or suitable dilution in 0.05M phosphate buffer, pH 6.5), and 0.5 ml of a suspension containing the yeast cell walls (3 mg/ml) and sodium azide (0.04%) in 0.05M phosphate buffer, pH 6.5. The mixture was incubated at 30°C with mild rotation on a roller drum. Absorbance of the reaction mixture was followed at intervals of up to 100 hr, at 600 nm in a Spectronic 20 (Bausch and Lomb) colorimeter. For each assay, a control tube also contained the same mixture with 0.5 ml distilled water substituted for the cell wall suspension. Percent decrease in turbidity was calculated by using the following equation which was adapted from Tabata and Terui (1962):

$$\% \text{ decrease} = \frac{Abs_0 - Abs_t}{Abs_0} \times 100$$

where Abs_0 denotes the initial absorbance of the reaction mixture, and Abs_t the value at time t.

Fig. 1 shows the reduction in absorbance of the reaction mixture as a function of enzyme concentration (dilution of culture filtrate) and incubation time. In general, the reduction rate is linear for 20-60 min depending on the enzyme concentration. Lytic activity (expressed as lytic units per ml of culture filtrate) was determined at 60 min. Amount of enzyme which caused a 50% reduction in turbidity of the mixture was assigned 20 lytic units. As percent decrease in turbidity was directly proportional to enzyme concentration within an approximate range of 0-22% (Fig. 2) enzyme solutions showing high lytic activities were suitably diluted with 0.05M potassium phosphate buffer, pH 6.5 prior to quantitation of lytic activities.

Fig. 1. Reduction in turbidity of Phaffia rhodozyma cell wall suspensions caused by various dilutions of lytic enzyme (culture fluid) produced in mixed culture buffered by 0.1M potassium phosphate pH 7.0.

0.05M phosphate buffer pH 6.5 was used as diluent.

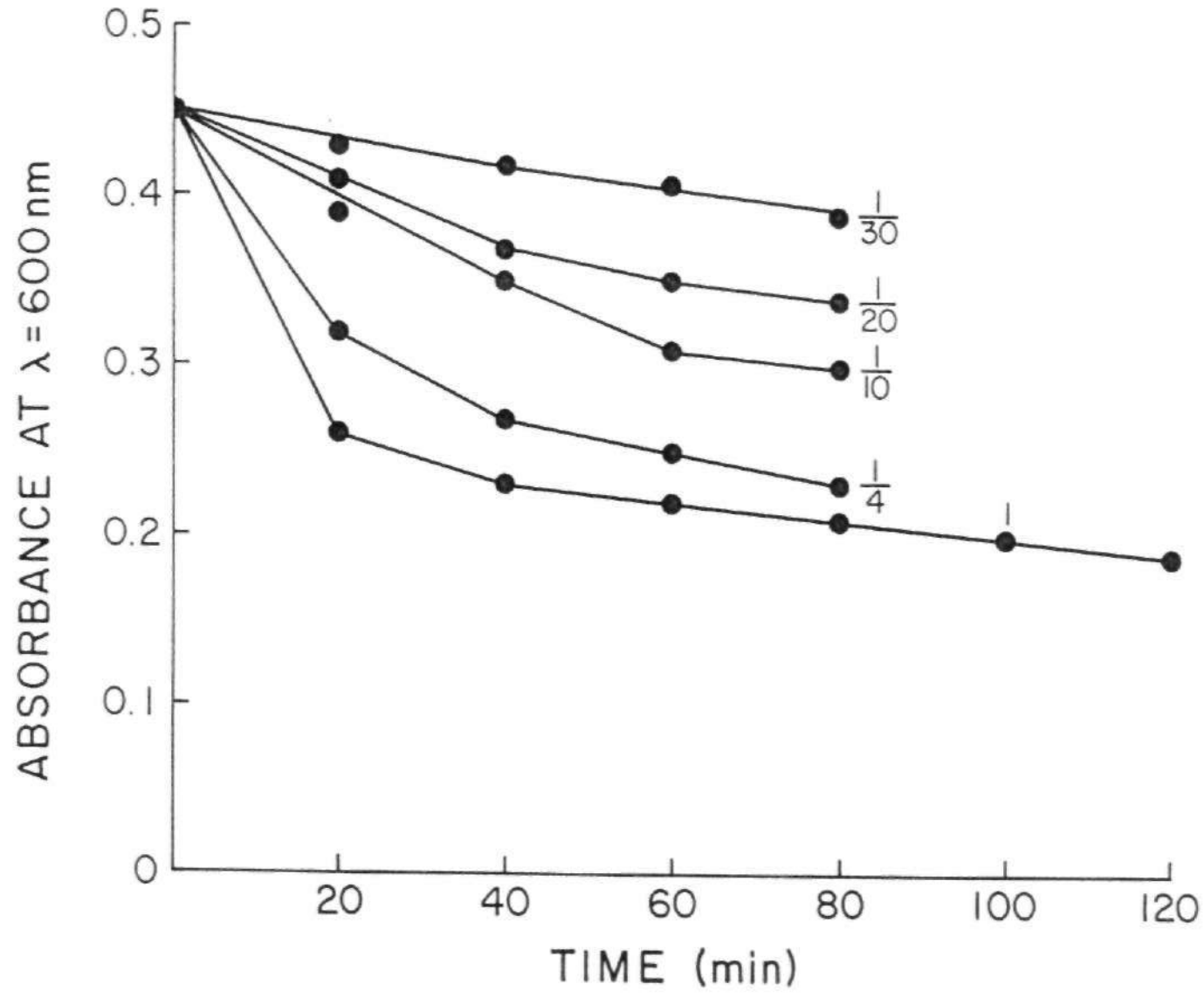
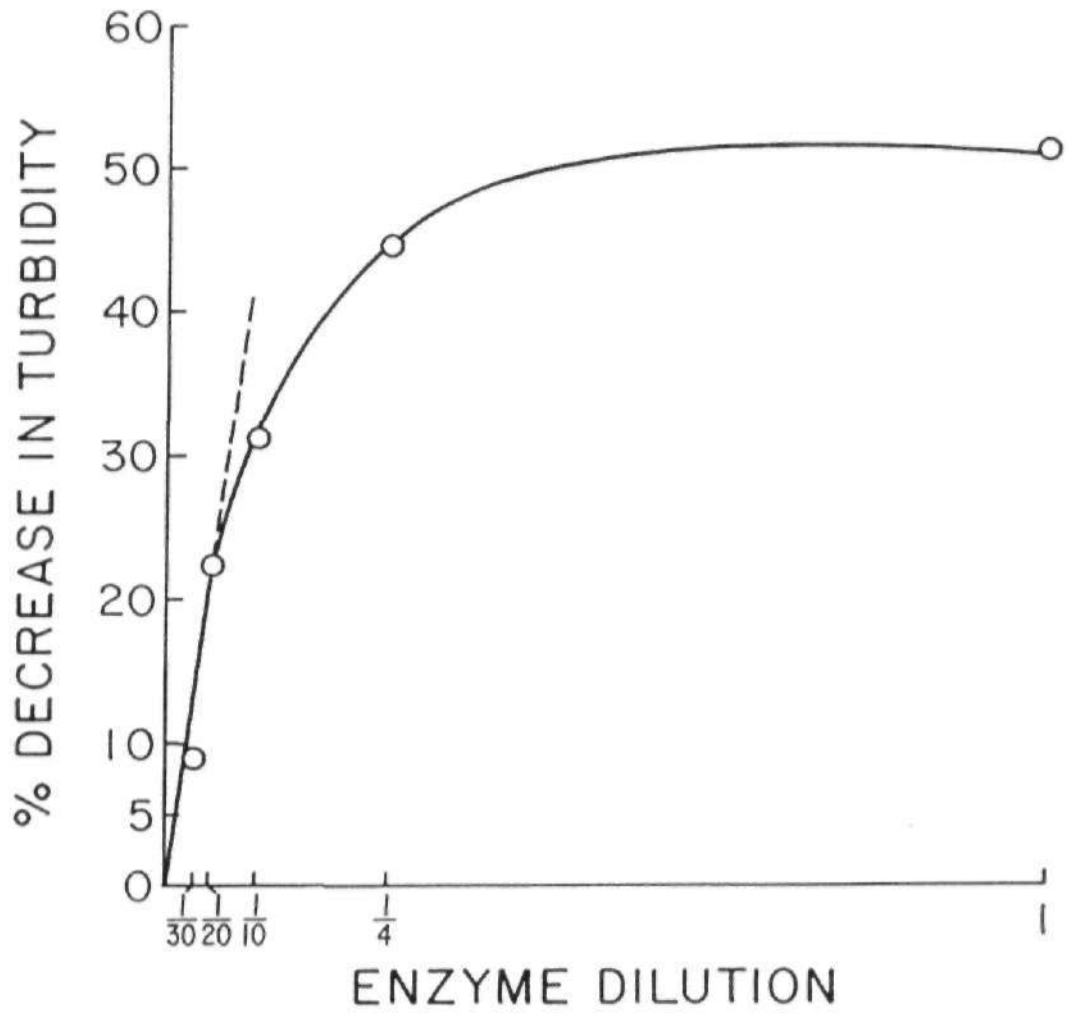

0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT λ = 600 nm
20
40
60
80
100
120
TIME (min)
1/30
1/20
1/10
1/4
1

Fig. 2. Relation between dilution of lytic enzyme and per cent decrease in turbidity of suspensions of Phaffia rhodozyma cell walls.


% DECREASE IN TURBIDITY
60
50
40
30
20
10
5
0
1/30
1/20
1/10
1/4
1
ENZYME DILUTION

QUANTITATIVE ANALYSES

1. pH

All measurements were performed using a pH meter (Orion Research, model 601A digital ionalyzer).

2. Cell growth

This was measured in terms of biomass production, and as viable counts of the microorganisms. In determination of biomass, culture samples, taken at suitable times, were centrifuged and washed twice with cold distilled water. Then they were suspended in sufficient water to transfer to pre-dried, cooled and tared aluminum pans, and dried in a vacuum oven at 70°C for 18 hr. Biomass was expressed as cell dry weight (mg/ml). In the determinations, any cells of Bacillus circulans which did not sediment when mixed culture samples were centrifuged, were ignored in calculations of cell dry weight.

In viable count determinations, serial dilutions of samples were made in sterile distilled water, and suitable aliquots spread in duplicate nutrient agar (Difco) (for Bacillus circulans) and potato dextrose agar (Difco) (for Phaffia rhodozyma) plates. The plates were incubated at 30°C and 20°C respectively. Colonies were counted after 30 hr and 60 hr incubations respectively, and viable counts computed as colony-forming units per ml (cfu/ml) of sample.

3. Residual glucose

This was determined as reducing sugar by the Nelson-Somogyi method described by Whistler and Wolfrom (1962).

4. Extraction and estimation of astaxanthin.

Astaxanthin content of cell pellets was determined as 'free' and as 'total' astaxanthin. If both assays were necessary for any sample, the 'free' astaxanthin was determined first by direct treatment of centrifuged cell mass with a suitable volume of acetone. The amount of freely extractable pigment was taken as a relative measure of the integrity of yeast cell wall at the time of assay, and was used to regulate both heating time and temperature in the 'total' astaxanthin assay. Method employed for the 'total' assay was a modification of that of Nakayama et al. (1953). Cell mass was treated with 2N HCl and subjected to a mild heat treatment which ranged from 80°C to boiling in a water bath for 1-2 min, followed by rapid cooling, then acetone extraction. Heating time and temperature were regulated to a minimum to avoid heat destruction of carotenoids (Mrak et al., 1949). Concentrations of astaxanthin in acetone extracts were estimated by measuring the absorbances at λmax = 478 nm with a Beckman DB Spectrophotometer. The specific absorption coefficient $A^{1\%}_{1cm}$ = 1600 (Andrewes et al., 1976) and the formula provided by Davies (1976) were used in calculations of astaxanthin concentrations. When desired, percent extractability of astaxanthin was calculated for any sample by employing the formula:

$$\% \text{ extractability} = \frac{\text{freely extractable astaxanthin } (\mu g/ml)}{\text{total astaxanthin } (\mu g/ml)} \times 100\%$$

ELECTRON MICROSCOPY

Samples to be examined by transmission electron microscopy (TEM) were processed as follows:

Pelleted washed cells were fixed in cold 2% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 for 10 hr (Hector, 1980). After a rinse with the buffer, a portion of the sample was withdrawn, and treated for 1-2 hr at room temperature with a solution containing equal amounts of fresh 6% glutaraldehyde, 0.2M cacodylate buffer pH 7.3, and a stock solution of ruthenium red prepared according to the procedure of Luft (1971). This was followed by three rinses in cacodylate buffer. Samples treated with and without ruthenium red were post-fixed in cold 1% O_sO_4 according to Kellenberger's method described by Audrey (1961). After a rinse with buffer, and then water, the fixed materials were dehydrated in a graded series of ethanol solutions and embedded in Epon-Araldite resin. Thin sections were cut on an LKB ultratome IV microtome, mounted on 300 mesh grids, and post-stained with uranyl acetate and lead citrate. Microscopy was carried out with a Zeiss 109 transmission electron microscope.

Chemicals.

All chemicals were, where possible, of analytical grade. D-glucose, solvents (acetone and toluene), calcium carbonate, magnesium sulfate, sodium chloride and citrate, potassium, phosphate salts and potassium hydrogen phthalate, urea, ammonium sulfate and ammonium chloride were obtained from Mallinkrodt, St. Louis, MO., U.S.A.; sucrose, D-mannitol, cellobiose, D-xylose, xylan (from larchwood), oleic and linoleic acids, ergosterol and cholesterol, inositol, dithiothreitol, methyl green and trisma base were from Sigma; maltose, L-arabinose and β-ionone were from ICN Pharmaceuticals; soluble starch and ammonium phosphate from J.T. Baker Chemical Co., calcium pantothenate from Nutritional Biochemicals, and fructose from Fisher Scientific Co., N.J., U.S.A. Other miscellaneous

chemicals, stains or dyes were obtained from the Dept. of Food Science and Technology or the Electron Microscope Facility at U.C. Davis.

IV. RESULTS

INHIBITION OF BACILLUS CIRCULANS WL-12 BY PHAFFIA RHODOZYMA IN MIXED CULTURE

In preliminary studies, the primary objective was to assess the ability of Phaffia rhodozyma and Bacillus circulans WL-12 to grow compatibly and synthesize their respective products, namely, astaxanthin and yeast cell wall lytic enzyme complex. An initial pH of 6.1 was chosen as a compromise between the requirement for optimum growth and pigmentation of the yeast (≅ 5.0; Johnson and Lewis, 1969), and that for the bacterial growth and lytic activity (≅ 6.5; Tanaka and Phaff, 1965). Growth medium was buffered by potassium phosphate (0.05M). For comparison, the organisms were also grown in pure cultures under identical conditions. Results presented in Fig. 3a show that Phaffia rhodozyma exhibited identical patterns of growth in the pure and mixed culture systems. Its logarithmic growth began immediately, and maximal population was achieved in approximately 36 hr and remained fairly stable up to 50 hr. Satisfactory pigmentation was also achieved; average total yields of astaxanthin at 48 hr were 1.30 µg/ml in mixed culture, and 1.40 µg/ml in pure culture. Bacillus circulans also grew luxuriantly in pure culture. After an initial lag of approximately 10-12 hr, its growth rate was comparable to that of the yeast. Its maximum population which was attained at about the same time as that of the yeast (i.e. at 36 hr) was greater, and declined rather rapidly. In contrast, the bacillus was grossly inhibited in mixed culture, even though its initial population (6.8×10^5 plotted as log cfu/ml = 5.83) was about four times that of Phaffia rhodozyma (1.6×10^5 plotted as log cfu/ml = 5.20).

Fig. 3a. Inhibition of growth of Bacillus circulans WL-12 by Phaffia rhodozyma in mixed culture.

Symbols: o B. circulans WL-12: pure culture

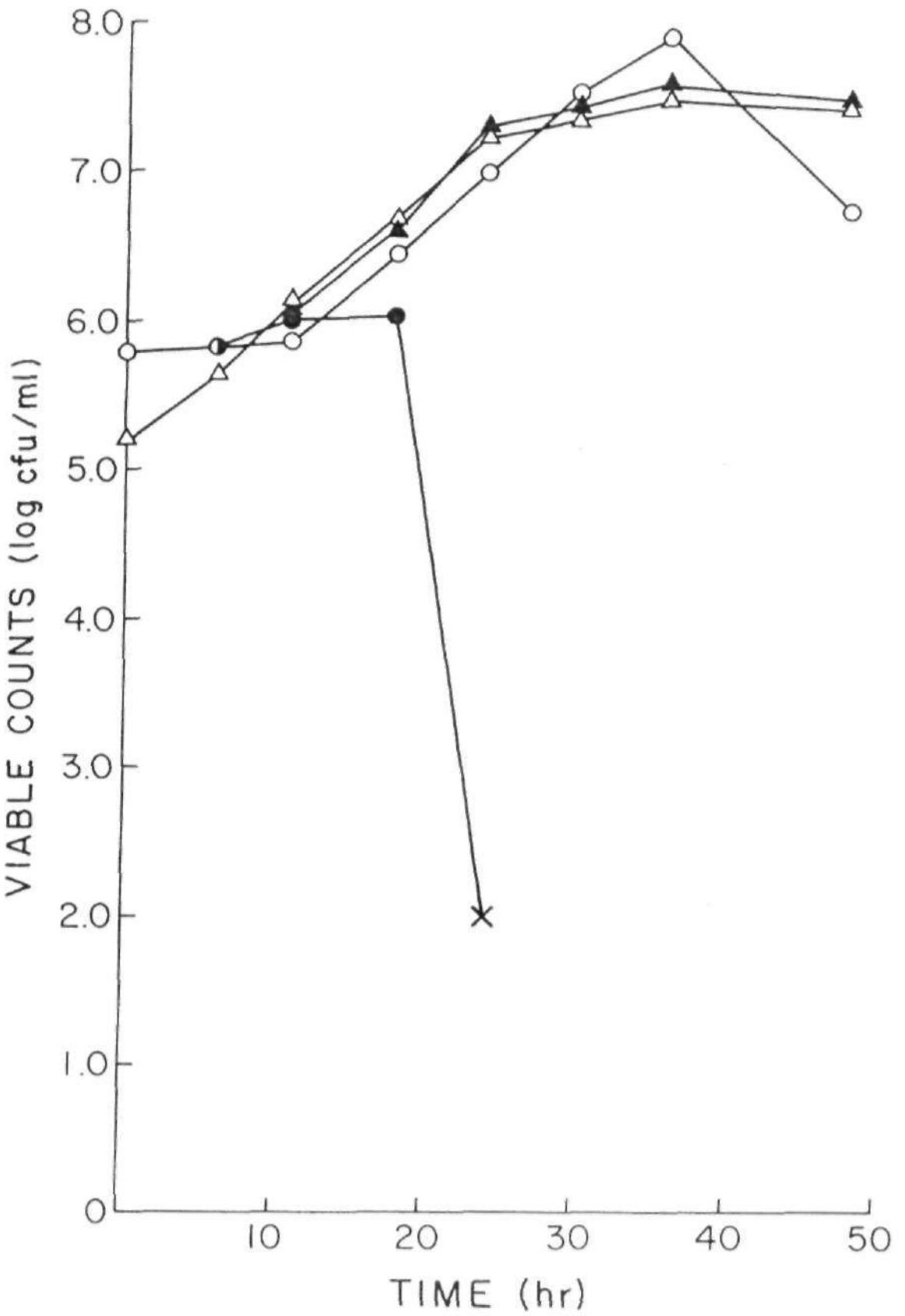
• B. circulans WL-12: mixed culture

Δ P. rhodozyma: pure culture

▲ P. rhodozyma: mixed culture

Medium was buffered by 0.05M potassium phosphate at pH ∿ 6.1.

X indicates that actual viable count at that point was below log 2.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
VIABLE COUNTS (log cfu/ml)
10
20
30
40
50
TIME (hr)

Little growth of the bacillus occurred from 6-8 hr, but by 24 hr, the initial population of approximately 6.8 x 10^5 had declined to below 1.0 x 10^2. At 50 hr, neither lytic activity nor freely-extractable astaxanthin was detected in the mixed culture.

Changes in pH of culture media, were also monitored during growth of the organisms. The data presented in Fig. 3b show that growth of Bacillus circulans WL-12 in pure culture caused a relatively gradual decline in pH. In contrast, there was a more rapid drop as well as a lower final pH in the mixed culture; its resemblance with that of the pure culture of Phaffia rhodozyma suggested that increased acidity of the medium caused by the metabolic activities of the yeast was probably the major factor that was detrimental to bacterial viability in the mixed culture.

EFFECTS OF pH CONTROL ON VIABILITY OF BACILLUS CIRCULANS WL-12 IN THE MIXED CULTURE

Initial studies in this work showed that populations of Bacillus circulans WL-12 could retain appreciable viability at pH 5.5. The work of Johnson et al. (1978), also showed that this level of pH was satisfactory for action of the lytic enzyme complex of the bacillus on Phaffia rhodozyma cell walls. Therefore, it was considered desirable to maintain minimum pH at approximately 5.5 in subsequent mixed culture fermentations, by application of suitable methods of pH control.

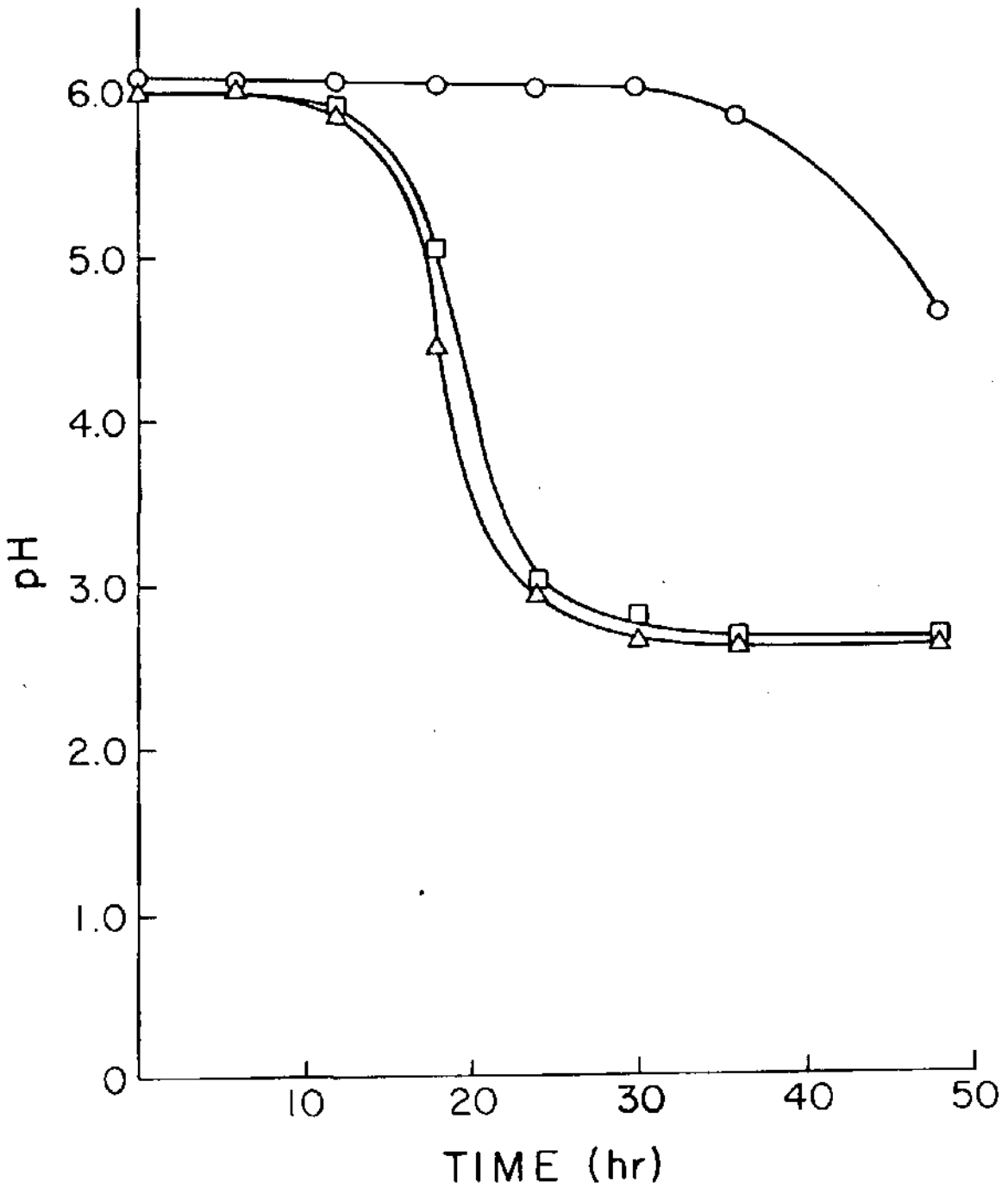
Substitution of phthalate buffer (0.05M) for phosphate, retarded the rate of pH decline; however, bacterial viability and lytic activity were again impaired. pH was approximately 5.5 from 17-19 hr and dropped

Fig. 3b. Changes in pH of the medium during growth of pure and mixed cultures of Bacillus circulans WL-12 and Phaffia rhodozyma.

Symbols: o Pure culture of B. circulans WL-12

Δ Pure culture of Phaffia rhodozyma

▫ Mixed culture


6.0
5.0
4.0
3.0
2.0
1.0
0
pH
10
20
30
40
50
TIME (hr)

to around 4.70-4.80 from 35-50 hr (Fig. 4B). Attempts were made to control medium pH by periodic additions (manually) of suitable volumes of sterile 2.5N NaOH. This procedure appeared to improve viability of the bacillus; average colony-forming units (cfu) of up to 1.4×10^5 per ml were detected in the cultures after 50 hr (Fig. 4A & B) (as opposed to below 1.0×10^1 cfu/ml in uncontrolled flasks). In addition, supernatants of culture samples showed detectable lytic activity (qualitative assay). Unfortunately, there was no detectable evidence of modification of Phaffia rhodozyma cell walls; astaxanthin was not extractable by direct treatment of cell pellet with acetone. This was attributed to low efficiency and variability of the manual method of pH control; in eight separate 50 hr experiements, minimum pH which could be maintained in the mixed cultures ranged from 5.01-5.62.

In general, the results indicate that control of the pH of the mixed culture is critical for bacterial survival and for its hydrolytic activity on the cell walls of Phaffia rhodozyma. The importance of using a reliable method of pH control was emphasized.

EXTRACTABILITY OF ASTAXANTHIN FROM pH-CONTROLLED MIXED CULTURE AFTER A HOLDING PERIOD

Prospects for successful application of the mixed culture to enzymatic modification of the cell walls of Phaffia rhodozyma was enhanced when it was observed that pigment was freely extractable from contents of some flasks which had been abandoned on laboratory bench (approximately room temperature, ≅ 23-25°C) for some days after previous fermentation failures. Since the flasks had been subjected to periodic

Fig. 4. Growth of Bacillus circulans WL-12 and changes in pH in mixed culture with Phaffia rhodozyma, with and without extrinsic pH control.

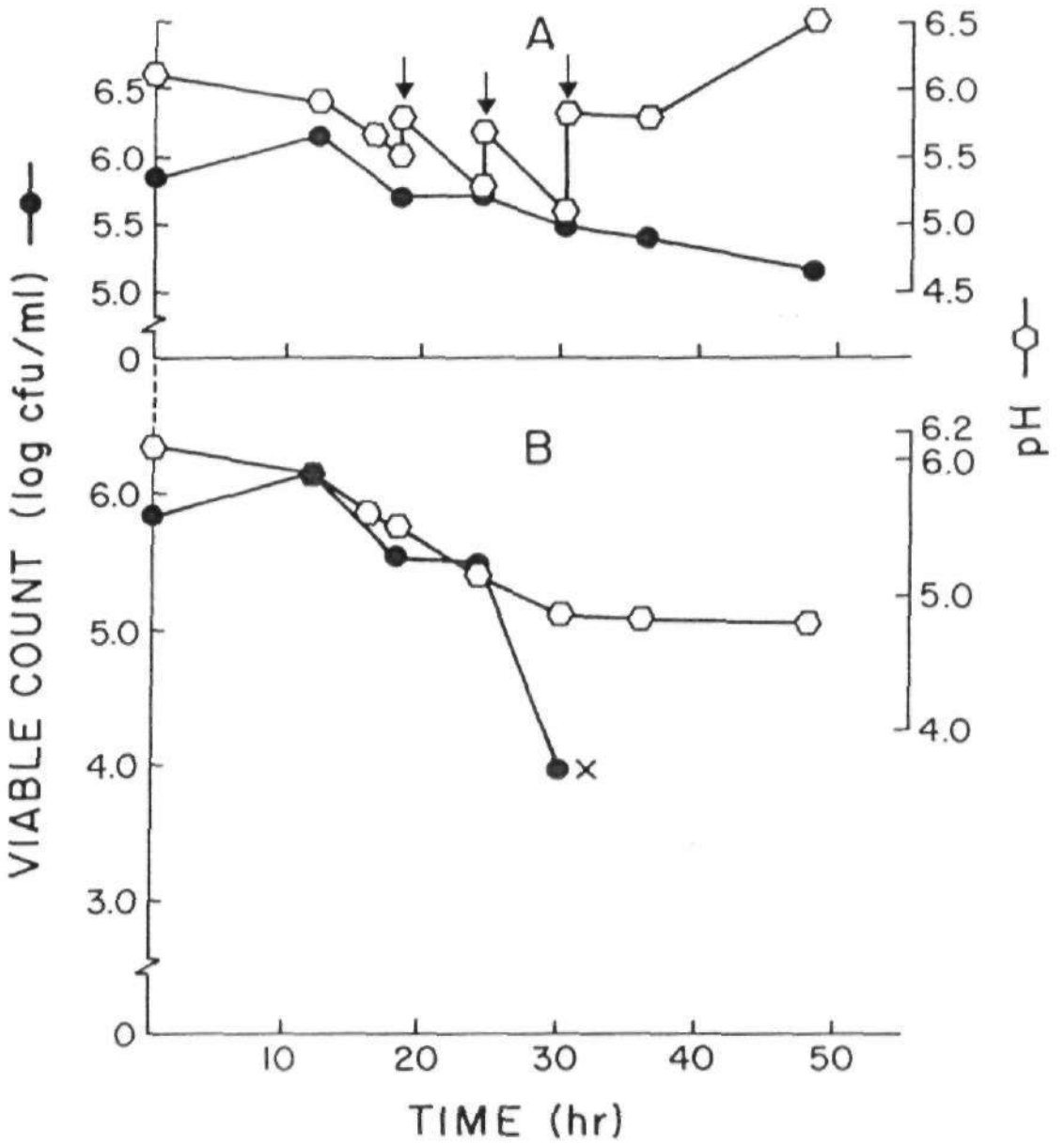
A. Culture with pH control: inverted arrows indicate points of addition of 2.5N NaOH.

B. Culture without pH control: X indicates that viable counts after 30 hr were lower than log 4.

Media for A and B were buffered with 0.05M phthalate buffer pH 6.1.

Symbols: o pH

• viable counts of B. circulans WL-12


A
B
6.5
6.0
5.5
5.0
0
6.5
6.0
5.5
5.0
4.5
6.2
6.0
5.0
4.0
6.0
5.0
4.0
3.0
0
x
10
20
30
40
50
TIME (hr)
VIABLE COUNT (log cfu/ml)
pH

pH adjustments, it appeared that a 'holding' period was required after culture growth. It was further observed that the holding period before astaxanthin became extractable varied (5-9 days) among the flasks; this was probably due to the different degrees of efficiency achieved in manual pH adjustments. In a separate experiment in which equal volume of mixed culture contents were held at 30°C and 20°C, extractability of pigment was detected in the flasks after 5 and 9 days respectively. This showed that the 'holding' temperature was probably important and provided further evidence of an enzymatic basis for the yeast cell wall modification process.

In order to clarify these observations, a comprehensive exploratory experiment was carried out to determine the overall effects of various treatments on extractability of astaxanthin from mixed culture of Bacillus circulans and Phaffia rhodozyma. The results presented in table 1 clearly show that the yeast cell wall modification depended on lytic activity of the medium; the activity was apparently enhanced by the final pH adjustment (to ≅ 6.9) and the higher (30°C) holding temperature. In a subsequent study (Fig. 5), it became clear that there was a resurgence of bacterial growth and considerable increase in lytic activity during the holding period.

In another experiment, a mixed culture which had undergone no intermittent pH adjustments, was finally brought to 6.9 at 42 hr and incubated at 30°C. Levels of Bacillus circulans WL-12 (up to 1.2×10^8 cfu/ml) and lytic activity (≅ 214 u/ml) and extractability of astaxanthin were later achieved. It appeared that very low levels of the bacillus could survive the low pH established by Phaffia rhodozyma and thence re-initiate growth when exposed to relatively good environmental conditions.

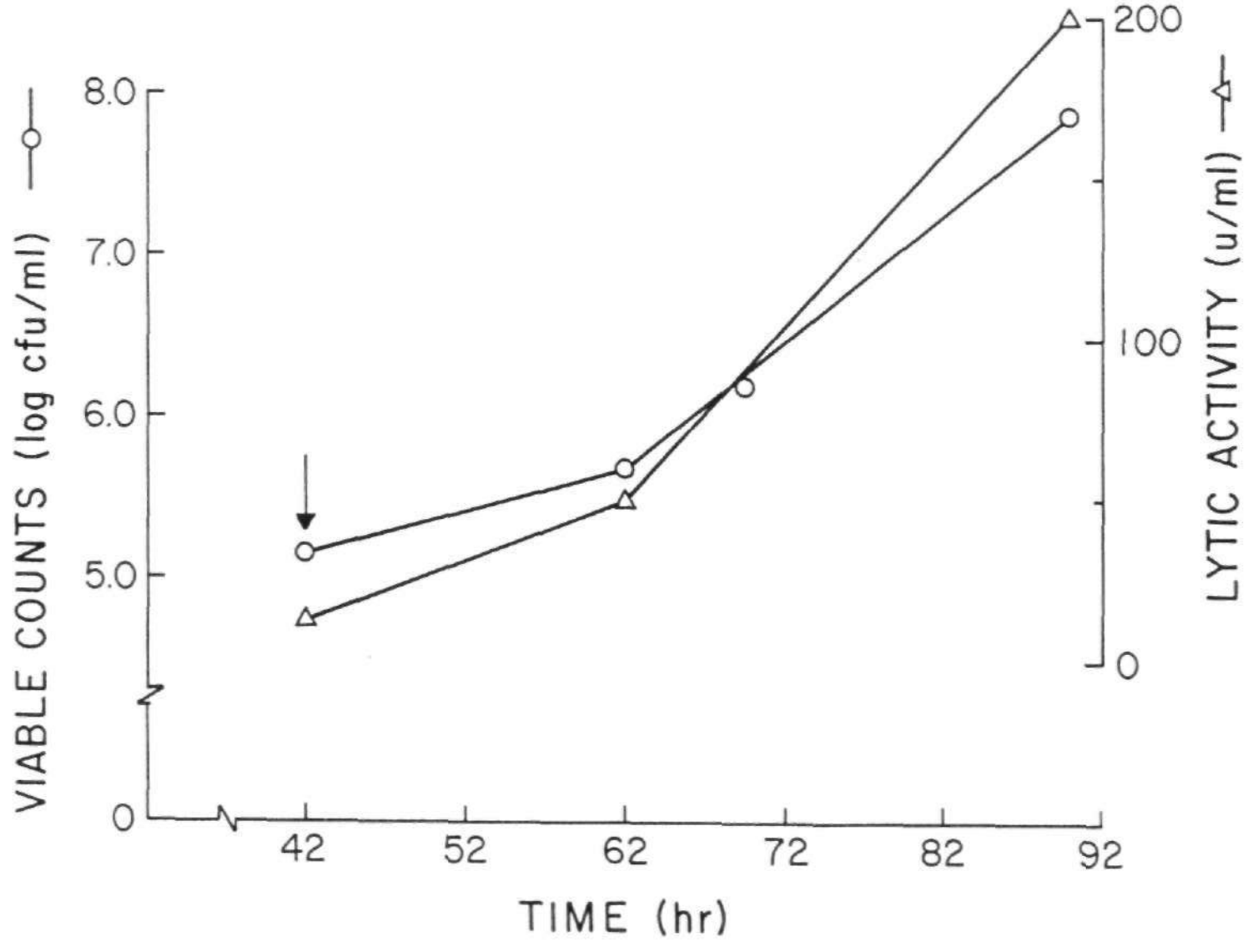
Table 1. Effects of some treatments on **extractability of astaxanthin from a mixed culture of** *B. circulans* and *P. rhodozyma*.

Method of culture	Lytic activity at 42 hr	pH adjustment (at 42 hr) to 6.9	Holding temp (°C) after 42 hr	Relative* Lytic activity	Extractability* of astaxanthin
Mixed	+	+	20 30	+ +++	++ +++
Mixed	+	-	20 30	+ ++	- +
Pure (Phaffia)	-	+	20 30	- -	- -

* after 3 days holding period.

All cultures were buffered with .05 M pthalate at pH 6.1. pH was controlled during growth of the mixed cultures by additions of alkali as shown in Fig. 4. At 42 hr, pH was increased further to 6.9 where indicated above. Equal portions of each culture were then held (with agitation at 200 rpm) at 20 and 30°C as indicated.

Fig. 5. Growth of Bacillus circulans WL-12 -o- and development of lytic activity -Δ- in mixed culture after final pH adjustment to 6.9 and incubation at 30°C.
Arrow indicates time of final pH adjustment.


VIABLE COUNTS (log cfu/ml)
LYTIC ACTIVITY (u/ml)
8.0
7.0
6.0
5.0
0
200
100
0
42
52
62
72
82
92
TIME (hr)

The procedure in which pH was adjusted only once appeared to be easy. However, since the results reported above were observed after 80-84 hr following pH adjustment, as opposed to comparable events at 40-48 hr under conditions of intermittent pH control (Fig. 5), the latter approach was adopted in subsequent experiments.

INFLUENCE OF AGITATION ON EXTRACTABILITY OF ASTAXANTHIN

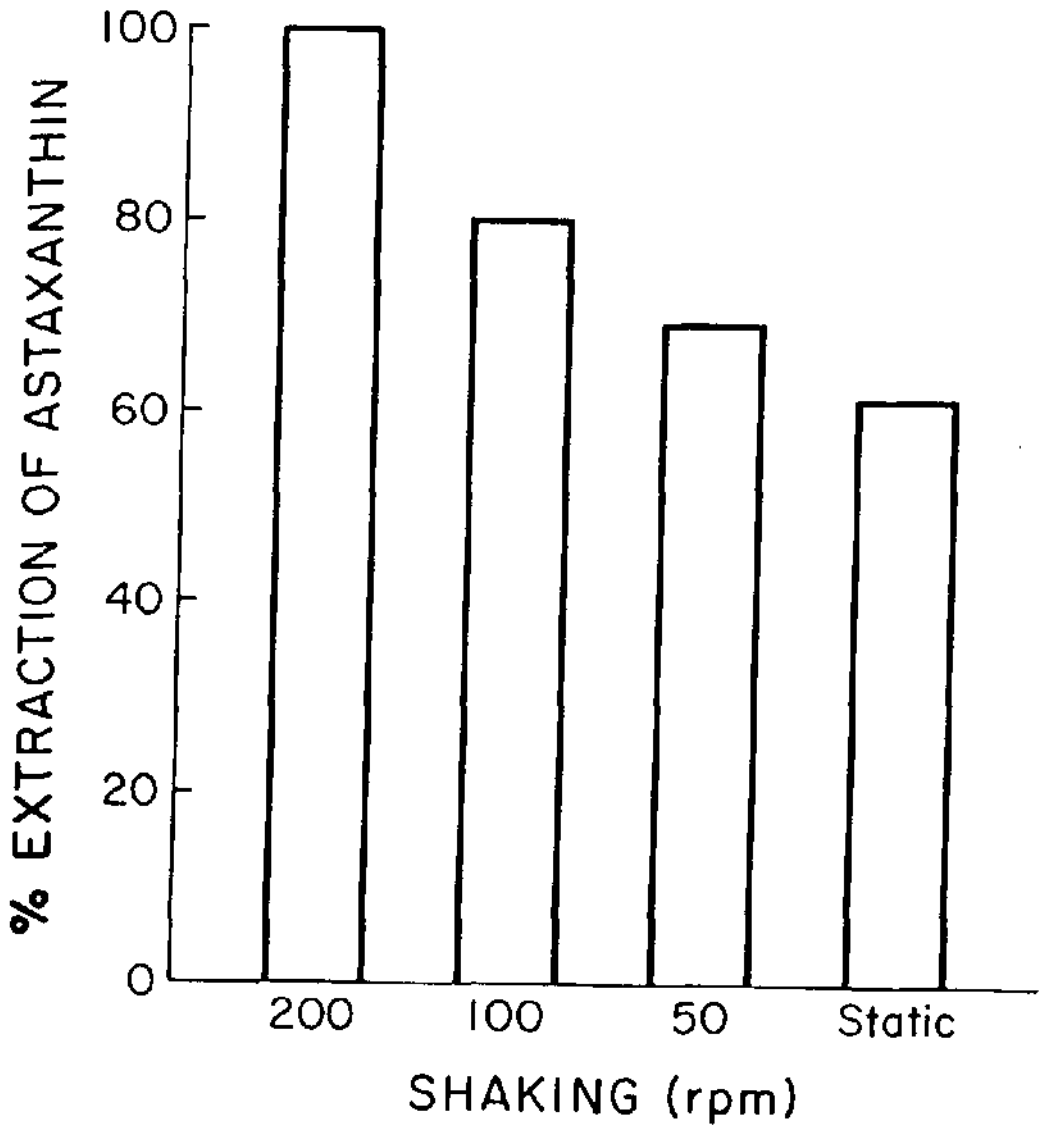
It was desirable to determine the effect of agitation on yeast cell wall modification during the holding period. Contents of two mixed culture flasks (which had received pH adjustments as in Fig. 4), were pooled, brought to pH 6.9 at 42 hr and divided among four similar flasks. All were incubated at 30°C; three parts shaken at 200, 100 and 50 rpm while the fourth was kept stationary. They were assayed for extractability of astaxanthin after two days. Fig. 6 shows that agitation accelerated the rate of yeast cell wall hydrolysis, pH was found to increase in all the flasks during the period of yeast cell wall modification, and was especially remarkable in the shaken flasks. For example, final pH in shaken flasks ranged from 7.25-8.01, while that of the unagitated sample was ≈ 7.1.

The observed rise in pH may be due to leakage of nitrogenous substances from the yeast, Phaffia rhodozyma; Lewis and Phaff (1963) have shown that agitation enhanced the leakage in brewer's yeast. It is also possible that growth and metabolic products of Bacillus circulans contributed to the rise in pH; Tanaka and Phaff (1965) reported that growth and production of β-1,3 glucanase by the bacillus, was promoted by agitation.

Fig. 6. Effect of agitation on efficiency of yeast cell wall digestion, measured by extractability of astaxanthin.

Contents of mixed culture flasks which received intermittent pH adjustments during growth were pooled at 42 hr and brought to pH 6.9. The pool was shared among four similar flasks, and shaken as indicated or left static at 30°C for two days.

$$\text{\% extraction of astaxanthin} = \frac{\text{amount of extractable astaxanthin (µg/ml) after 2 days}}{\text{amount of total astaxanthin (µg/ml) at zero time}} \times \frac{100}{1}$$


% EXTRACTION OF ASTAXANTHIN
100
80
60
40
20
0
200
100
50
Static
SHAKING (rpm)

EFFECT OF pH AND HOLDING TIME ON EXTRACTABILITY OF ASTAXANTHIN

Up till now, yeast cell wall modification has depended on a revival of bacterial growth during the holding period of the mixed culture. It was essential to determine the optimum or suitable range of pH that would yield the desired outcome. Mixed cultures were pooled and divided into aliquots which were adjusted to pH values ranging from 6.2 to 7.5. Total astaxanthin (μg/ml) was determined for each aliquot and the value was used thoughout in computing percent extraction of astaxanthin. All the aliquots were incubated at 30°C with shaking at 200 rpm, and extractability of pigment was followed as a function of time. Fig. 7 shows that digestion of the yeast cell walls proceeded most rapidly at pH 7.5. However, efficiencies achieved after two days were maximal and fairly comparable for all the pH values tested. The observed decline in efficiency of extraction after two days is probably due to autolysis of the yeast; pH 7.5 also appeared to promote the most rapid autolysis. The optimum pH range for holding the mixed culture appears to be 6.2-6.8 because it is the range at which autolysis proceeds for minimum rate for at least up to three days. In general, Fig. 7 suggests that the cell mass be harvested as soon as over 90% extraction is achieved, to avoid losses due to autolysis.

EFFECT OF HOLDING TEMPERATURE AND TIME ON EXTRACTABILITY OF ASTAXANTHIN

The course of digestion of *Phaffia rhodozyma* cell walls was also examined in mixed cultures held at various temperatures after growth in order to determine the most suitable range or optimum. Data presented

Fig. 7. Effect of pH adjustment and holding time after growth of the mixed culture on extractability of astaxanthin from Phaffia rhodozyma.

Mixed cultures, grown in different flasks with intermittent pH adjustments as in Fig. 4, were pooled at 42 hr, and divided into aliquots which were finally adjusted to pH values ranging from 6.2 to 7.5. They were incubated at 30°C with shaking at 200 rpm and extractability of astaxanthin monitored.

Symbols: ○ pH 6.2

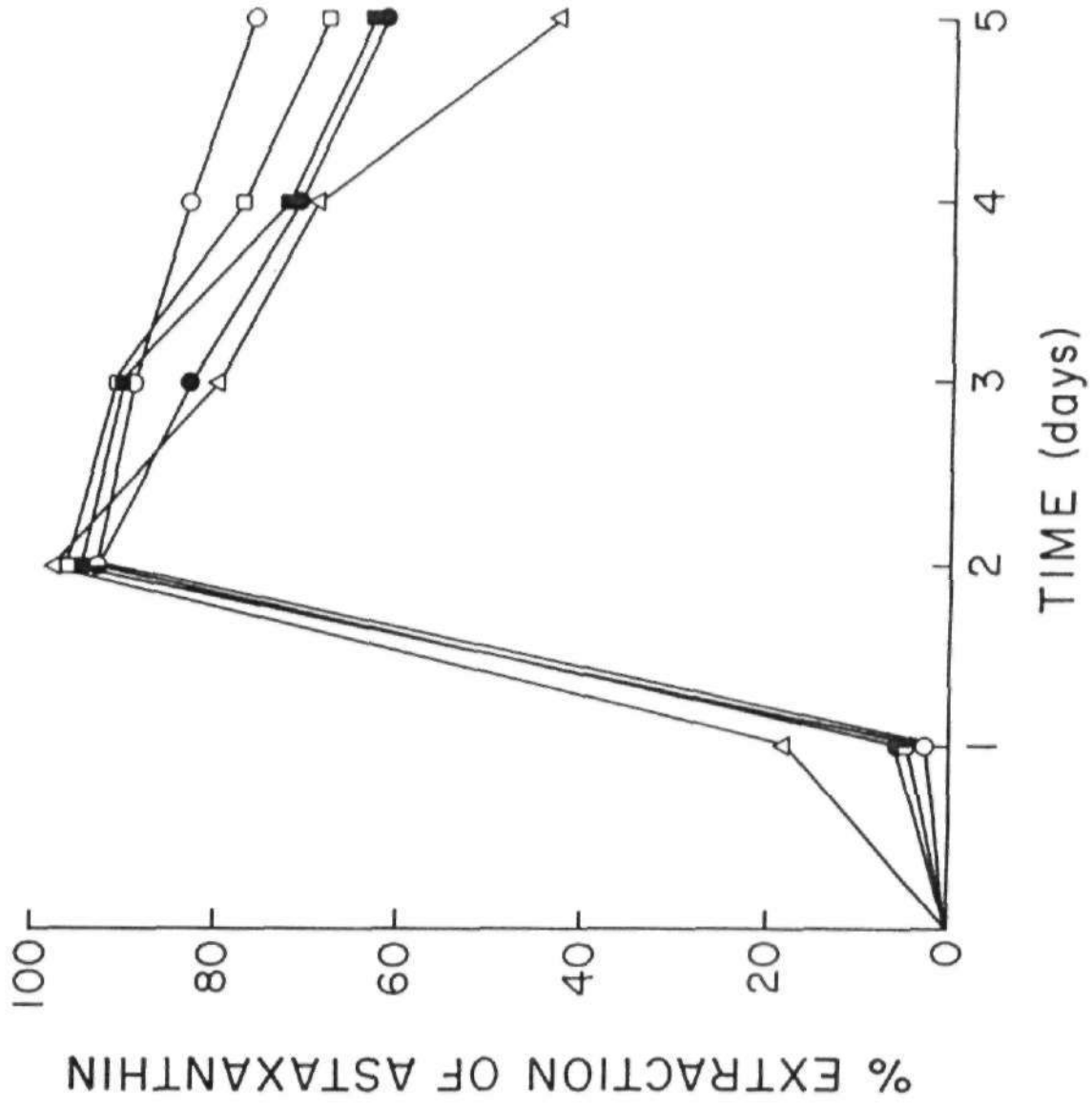
□ pH 6.5

■ pH 7.0

● pH 7.5

△ pH 7.5

% Extraction was computed as in Fig. 6.


% EXTRACTION OF ASTAXANTHIN
0
20
40
60
80
100
TIME (days)
1
2
3
4
5

in Fig. 8 show that 30°C and 37°C were very favorable to yeast cell wall digestion: maximum extractability of astaxanthin was achieved in 40-60 hr in cultures held at the two temperatures. Autolysis appeared to proceed more rapidly at 37°C. Cultures incubated at 20°C and 24°C showed some extractability of astaxanthin only after 40-46 hr lag. Maximum cell wall modification was not achieved even at 144 hr. As expected, even at the same time interval, a pure culture of Phaffia rhodozyma treated and held at 30°C, had no detectable evidence of cell wall modification.

Phase contrast microscopic observations were carried out at intervals on the pure culture as well as on the mixtures held at 30°C and 37°C. All cultures were identical at 12 hr, regardless of the holding temperature, and showed normal budding cells with extensive surface granulation. The pure culture remained the same even at 48 hr when observations were discontinued. In contrast, the mixed cultures held at 30°C and 37°C, showed abundant bacterial growth (at 40 hr) with considerable degranulation and transparency at the surfaces of yeast cells; all the cells however retained normal shapes. By 48 hr, refractile bacterial spores were abundant while observable numbers of yeast cells appeared to be reduced, apparently due to autolysis.

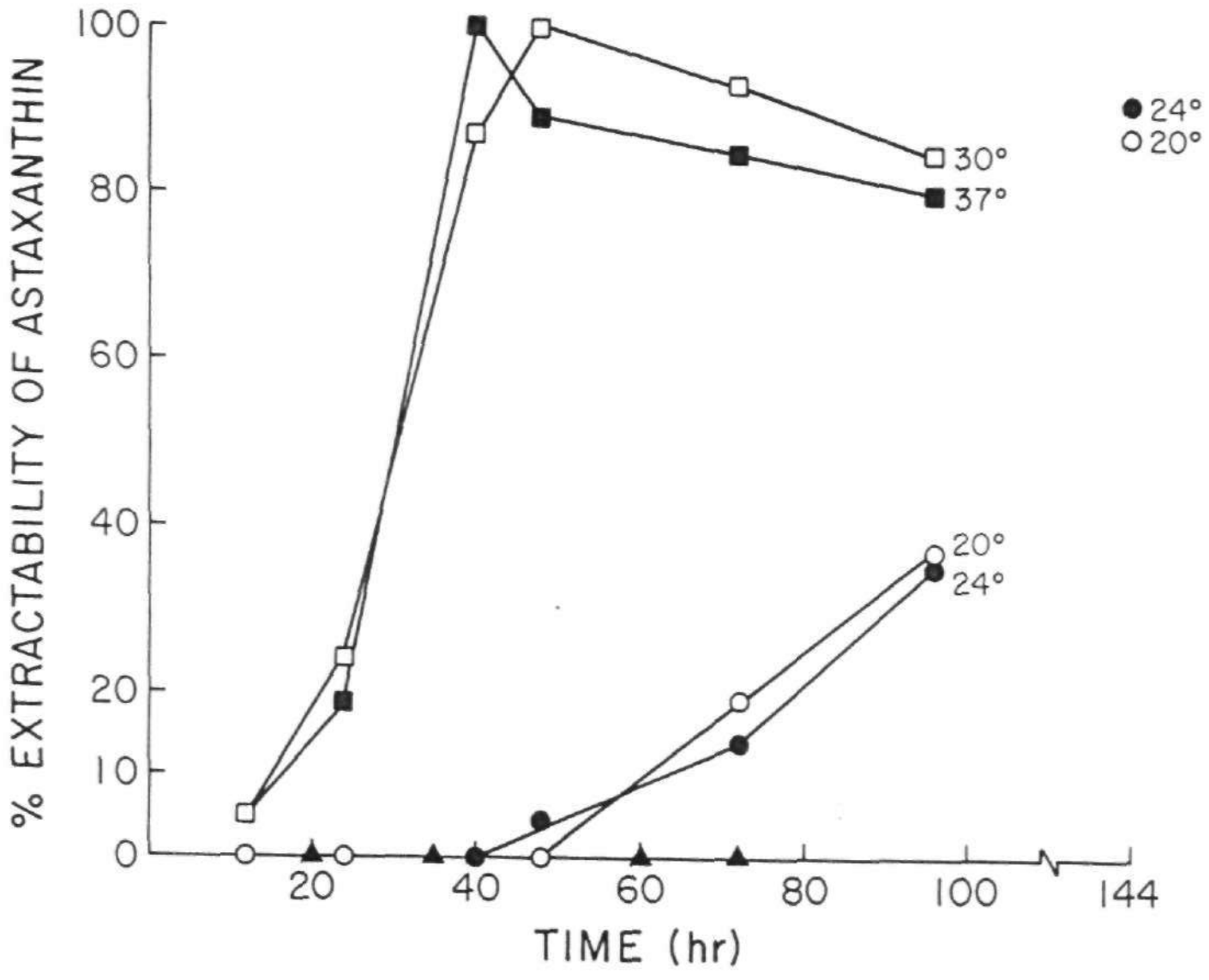
SUMMARY AND SOME CONCLUSIONS

In the foregoing section of this thesis, it has been shown that the red yeast Phaffia rhodozyma inhibited Bacillus circulans WL-12 in mixed culture, in a medium in which their pure cultures grew satisfactorily. The inhibition has been attributed primarily to low pH caused

Fig. 8. Effect of holding temperature and time on extractability of astaxanthin from Phaffia rhodozyma after final pH adjustment to 6.5 of the mixed culture.

Shake-flask mixed cultures and a pure culture of P. rhodozyma, were grown as usual, at 20°C with intermittent pH adjustments. At 42 hr, the mixed cultures were pooled and the pool was brought to pH 6.5, as was the pure culture. The pool was divided into aliquots which were then held at different temperatures with shaking at 200 rpm. The pure culture was similarly held at 30°C. Extractability of astaxanthin was assayed in all flasks as a function of time.

Symbols:
- o Mixed culture 20°C
- • Mixed culture 24°C
- ▫ Mixed culture 30°C
- ■ Mixed culture 37°C
- ▲ P. rhodozyma pure culture 30°C (control)


% EXTRACTABILITY OF ASTAXANTHIN
100
80
60
40
20
10
0
20
40
60
80
100
144
TIME (hr)
30°
37°
20°
24°
24°
20°

by metabolism of the yeast under the relatively low initial pH and low buffer concentration employed. It suppresses lytic activity of the bacillus and consequently digestion of the yeast cell walls, and prevents extractability of astaxanthin. Control of medium pH during growth of the mixed culture minimizes the inhibition. Up to this point, manual pH control has been utilized to achieve extractability of astaxanthin, but it is tedious and laborious and renders the culture susceptible to contamination. Moreover, its relative inefficiency necessitates a holding period after yeast growth, and upward temperature and pH shifts are essential to optimize further growth of and lytic enzyme production by the bacillus. In so far as extractability of astaxanthin was achieved, the mixed culture holds considerable commercial promise because modern fermentation equipment utilizes automatic pH control systems.

Since this study is primarily interested in developing a system that would guarantee optimum growth of Bacillus circulans WL-12 and Phaffia rhodozyma, some attempts would be made to achieve pH control intrinsically by using a suitable buffer concentration and initial pH.

DEVELOPMENT OF A MIXED CULTURE OF BACILLUS CIRCULANS WL-12 AND PHAFFIA RHODOZYMA

Selection of a suitable concentration and pH of buffer.

Mixed cultures were grown for 100 hr, in media buffered with phthalate or phosphate at different molar concentrations and pH. In some cases, the buffers were supplemented with varying amounts of $CaCO_3$ (as a pH regulator). Suitability of each buffer system to sustain

viability of Bacillus circulans WL-12 in presence of Phaffia rhodozyma was determined from two criteria: presence of detectable cell wall lytic activity in culture supernatants, and a final medium pH above 6.0. Results of this screening test are presented in Table 2. Four of the buffer systems tested appeared suitable for the mixed culture. The best two are considered to be those which maintained final pH 6.64 and 6.66; these values are closest to 6.5 which has been found to be optimal for growth and lytic activity of Bacillus circulans WL-12 (Tanaka and Phaff, 1965; Johnson et al., 1978). However, 0.1M phosphate buffer pH 7.0 was chosen for subsequent studies because presence of $CaCO_3$ crystals in the other system complicated measurements of cell dry weight.

Of all the buffers tested in this survey, 0.05M phosphate buffer pH 6.5 was least suitable. However, when it was compared with 0.1M phosphate pH 7.0 (Table 3), the former appeared to promote, initially, more rapid growth and development of lytic activity, than the latter. This observation is important for two reasons: (1) It suggests that if a mixed culture of Bacillus circulans and Phaffia rhodozyma were to be subjected to intermittent pH control as was done in the first section of this study, an initial pH of 6.5 could be more appropriate than pH 6.0. (2) It shows that Bacillus circulans WL-12 is probably able to synthesize low levels of the lytic enzyme constitutively; thus low pH in the mixed culture inhibits subsequent bacterial growth and inactivates pre-formed enzymes.

Growth of Bacillus circulans WL-12 and Phaffia rhodozyma in mixed culture.

In medium containing the chosen buffer (i.e. 0.1M phosphate pH 7.0),

Table 2. Selection of buffer concentration and pH.*

Buffer System	Final pH	Total yield of astaxanthin µg/ml	Lytic** activity	Other observations:*** pigmentation in supernatant
0.05 M potassium phosphate, pH 6.5	4.99	1.12	- (0)	-
0.05 M pot. phosphate, pH 7.0	6.05	1.00	~~+~~ (4.0)	~~+~~
0.05 M pot. phosphate, pH 7.0 + 0.1% $CaCO_3$	6.66	0.68	~~+++~~ (5.0)	~~+++~~
0.05 M pot. phosphate, pH 7.0 + 0.15% $CaCO_3$	6.80	0.62	~~+++~~ (5.0)	~~+++~~
0.05 M phthalate, pH 6.5	5.20	1.25	- (0)	-
0.1 M pot. phosphate, pH 7.0	6.64	0.70	~~+++~~ (5.0)	~~+++~~
0.1 M phthalate, pH 6.5	5.78	1.20	- (0)	-

* All data are averages of results from duplicate flasks.

** Figures in brackets represent diameters of zones of clearing around wells in cell wall agar plate.

*** Visual assessment after centrifugation at 12,000 rpm for 1 hr.

Table 3. A comparison of growth and lytic activity of B. circulans WL-12 in mixed culture in presence of two buffer systems.

	Buffer systems: 0.05M phosphate, pH 6.5				0.1M phosphate, pH 7.0			
Time of growth (hr)	Final pH	Viable counts cfu/ml B. circulans	P. rhodozyma	Lytic* activity	Final pH	Viable counts (cfu/ml) B. circulans	P. rhodozyma	Lytic activity
0	6.50	6.80×10^5	1.60×10^5	-(0)	7.0	6.80×10^5	1.60×10^5	-(0)
20	6.20	1.60×10^7	1.20×10^7	~~+~~(3mm)	6.97	7.60×10^6	1.10×10^6	+(1.5mm)
30	3.97	$<1.0 \times 10^4$	3.80×10^7	-(0mm)	6.89	5.10×10^7	5.20×10^6	~~++~~(3.2mm)
40	4.23	$<1.0 \times 10^4$	3.50×10^7	-(0mm)	6.61	8.90×10^7	3.40×10^7	~~+++~~(4.5mm)
72	4.43	$<1.0 \times 10^4$	4.50×10^7	-(0mm)	6.56	1.10×10^8	2.00×10^7	~~+++~~(5.0mm)

* Figures in brackets are diameters of zones of clearing in cell wall agar plates.

both organisms begin logarithmic growth immediately after inoculation (Fig. 9A). The yeast apparently grew more rapidly, and reached stationary phase at approximately 20 hr, while the bacillus required somewhat longer time. In general, termination of growth of both organisms coincided with very low residual levels of glucose and pH approximately 6.4 in the medium. Synthesis of astaxanthin occurred mainly during the late logarithmic and early stationary phases of the growth of Phaffia rhodozyma; maximum level was reached about 50-52 hr of the fermentation and remained fairly stable for up to 72 hr.

Very low level of lytic activity (less than 5 units/ml) was detectable in culture fluid during active growth of Bacillus circulans (Fig. 9B). In contrast, high levels were produced during the stationary phase and approached 110 lytic units/ml by 75 hr. Period of maximum lytic enzyme secretion appeared to coincide with exhaustion of glucose and a slight increase in pH of the culture (Fig. 9A & B). Digestion of the yeast cell walls (indicated by extractability of astaxanthin), was associated with production of the lytic enzyme complex; however, less than 30% of total yield of astaxanthin synthesized by 48 hr was extractable. Considerable cell wall modification appeared to have occurred after this time (48 hr) and was virtually complete by 60-65 hr, of the mixed culture fermentation. It was interesting that Phaffia rhodozyma maintained a high level of viability when its pigments were nearly 100% extractable (Table 3a).

Fig. 9A. Growth of Bacillus circulans WL-12 and Phaffia rhodozyma in mixed culture. Medium was buffered by 0.1M phosphate buffer pH 7.0.

Symbols: Δ P. rhodozyma

o Total astaxanthin

□ B. circulans WL-12

X Residual glucose

▲ pH

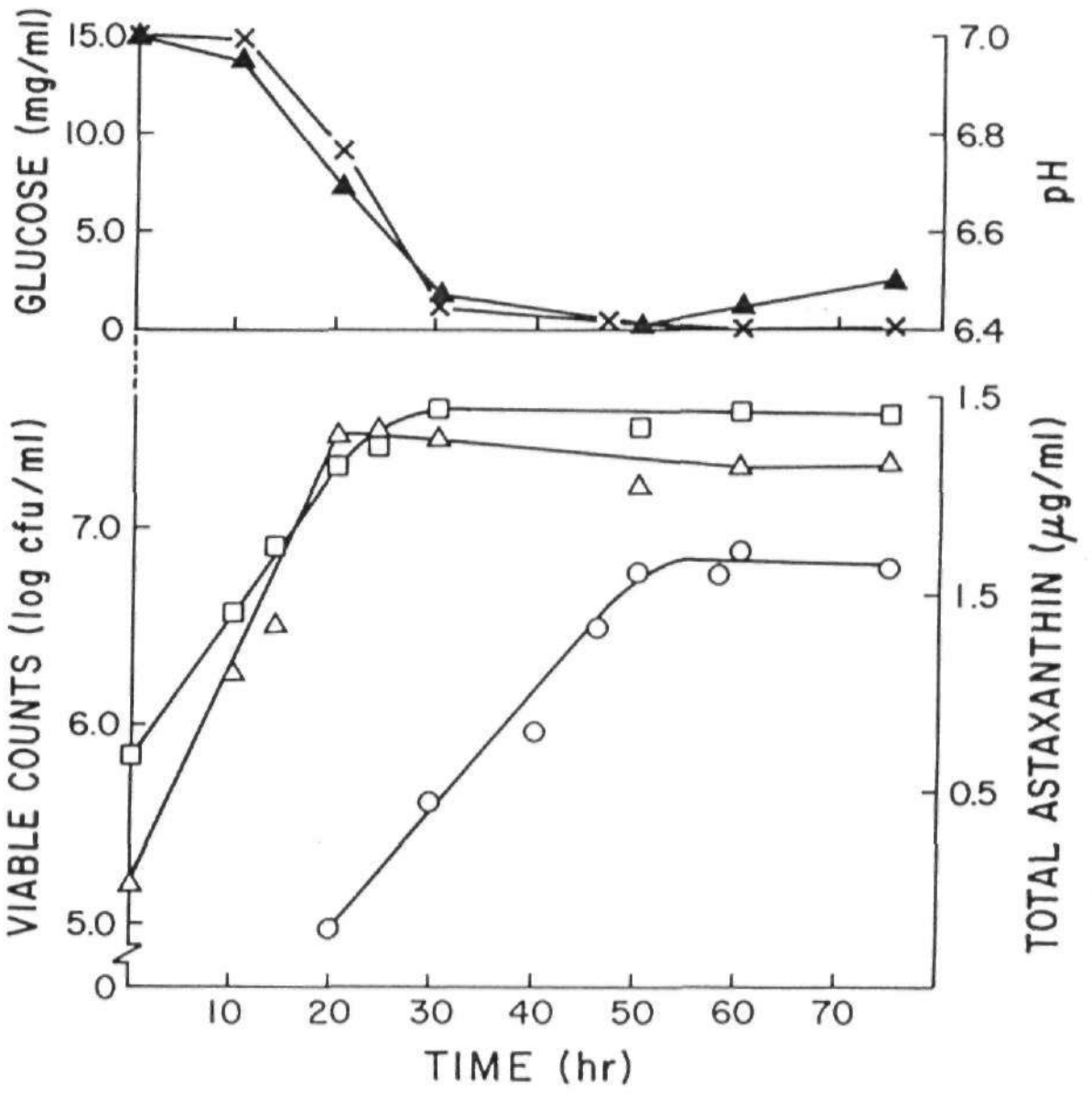

GLUCOSE (mg/ml)
15.0
10.0
5.0
0
7.0
6.8
6.6
6.4
pH
VIABLE COUNTS (log cfu/ml)
7.0
6.0
5.0
0
1.5
1.5
0.5
TOTAL ASTAXANTHIN (μg/ml)
10
20
30
40
50
60
70
TIME (hr)

Fig. 9B. Development of lytic activity and extractability of astaxanthin in a mixed culture of B. circulans WL-12 and P. rhodozyma. Data plotted were from the same culture as Fig. 9A.

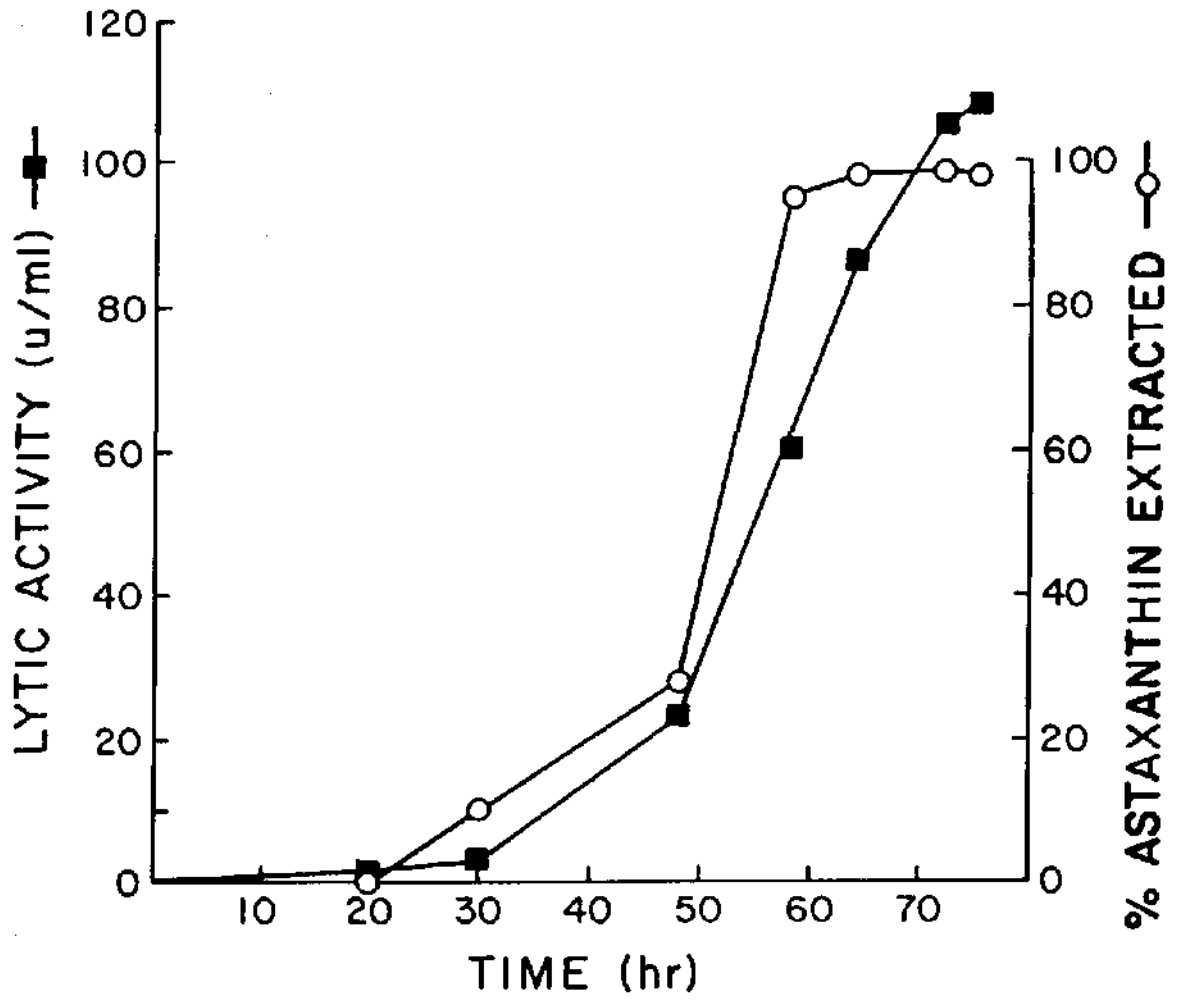

LYTIC ACTIVITY (u/ml)
% ASTAXANTHIN EXTRACTED
TIME (hr)
120
100
80
60
40
20
0
10
20
30
40
50
60
70

Table 3a. Viability of Phaffia rhodozyma and extractability of astaxanthin in mixed culture with Bacillus circulans WL-12.

Time (hr)	Viable count log cfu/ml	% Extractability of astaxanthin
60	7.3	96
75	7.2	98

INFLUENCE OF ENVIRONMENTAL CONDITIONS ON EFFICIENCY OF YEAST CELL WALL MODIFICATION IN THE MIXED CULTURE

Effect of initial pH.

Using the same molar concentration of buffer (0.1M), the initial pH of the mixed culture was varied from 6.0 to 7.8. The results are presented in Fig. 10. The range of pH examined, has negligible effect on yield of astaxanthin. However, because initial pH had great influence on the final pH (Table 4), there were some effects on viability of Bacillus circulans and activities of the lytic system. Optimum lytic activity and extractability of astaxanthin were observed in the pH range 6.8-7.4. At this range, obvious differences in final pH and viable counts of the bacillus (Table 4) had no effect on the outcome of the mixed culture fermentation. Below this range, bacterial viability and lytic activity were retarded. When the initial pH was 6.5, extractability of astaxanthin was low (45.9%), but it was considerably improved to 92.5% by incubation to 100 hr (not shown in Table 4). Above 6.8-7.4, bacterial viability appears to be favored, but there was low efficiency in cell wall modification, probably because of low lytic enzyme production.

Effect of temperature.

Table 5 shows that only a relatively narrow range of temperature (20-27°C) can support good yield and extractability of astaxanthin in mixed culture at an initial pH of 7.0. The optimal range appears to be 20-24°C. At the lowest temperature tested (15°C) there is good growth of Phaffia rhodozyma, but its cell wall digestion is hampered by

Fig. 10. Influence of initial pH of the mixed culture on lytic activity and on total and extractable astaxanthin.

Concentration of phosphate buffer in all cases was 0.1M.

Fermentation period was 75 hr.

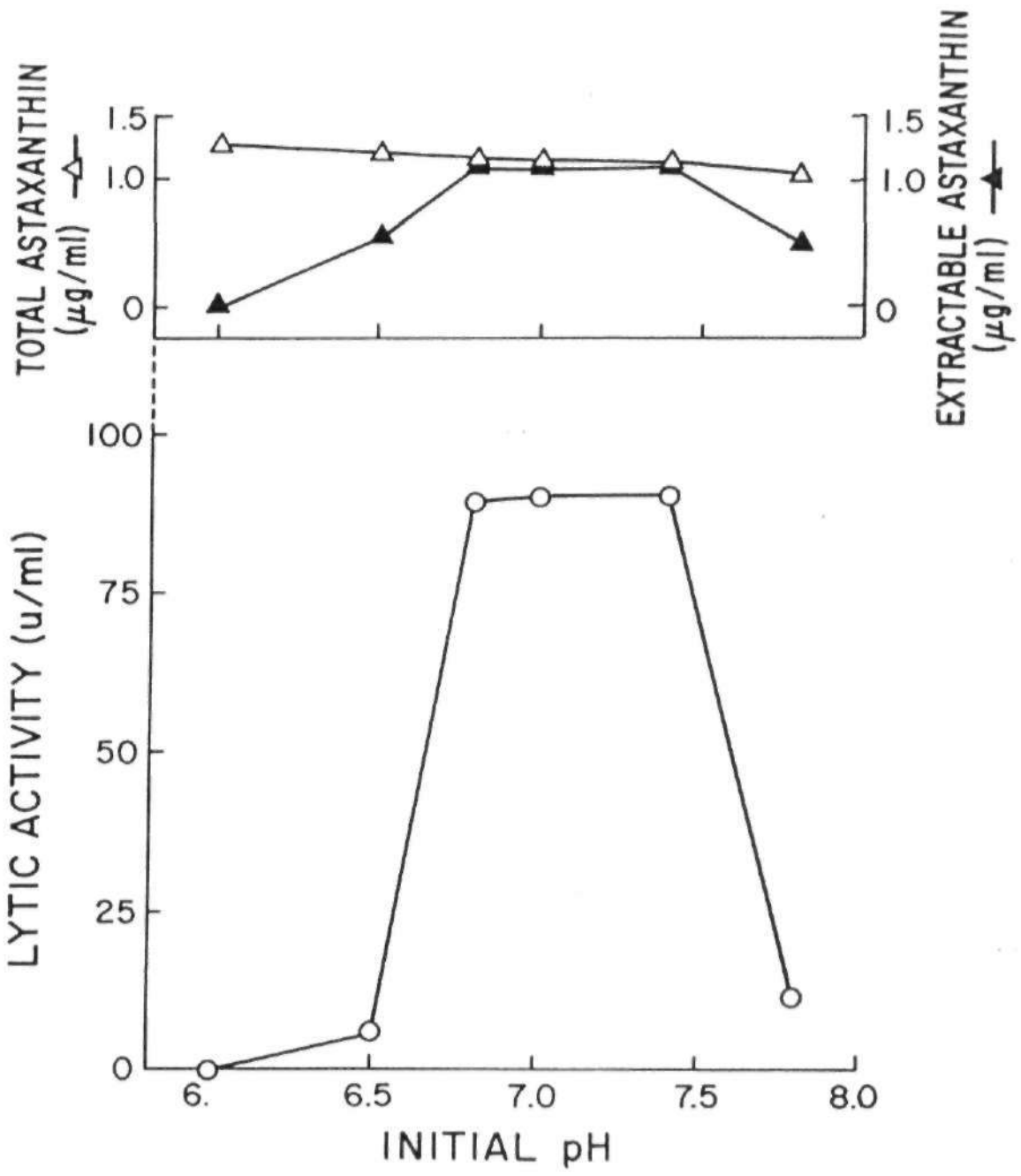

TOTAL ASTAXANTHIN (µg/ml)
1.5
1.0
0
EXTRACTABLE ASTAXANTHIN (µg/ml)
1.5
1.0
0
LYTIC ACTIVITY (u/ml)
100
75
50
25
0
6.
6.5
7.0
7.5
8.0
INITIAL pH

Table 4. Effect of final pH on cell growth and extractability of astaxanthin.

Initial pH	Final pH	Viable counts (cfu/ml) *B. circulans*	Viable counts (cfu/ml) *P. rhodozyma*	Extractability of astaxanthin %
6.0	3.60	$<1.00 \times 10^3$	2.10×10^7	0
6.5	5.78	3.20×10^6	4.30×10^7	45.9
6.8	6.25	2.20×10^7	2.60×10^7	96.6
7.0	6.47	3.10×10^7	2.50×10^7	96.6
7.4	6.83	4.00×10^7	3.80×10^7	97.4
7.8	6.98	6.60×10^7	1.00×10^8	49.5

All assays were at 72-75 hr.

Table 5. Effect of growth temp. on B. circulans WL-12 and P. rhodozyma in mixed culture.*

Temp. °C	Final pH	Lytic Activity Lytic U/ml	Viable counts (cfu/ml) B. circulans	P. rhodozyma	Yield of Astaxanthin µg/ml	Extractability of Astaxanthin (%)
15	6.59	2.6	4.90×10^6	6.20×10^7	1.13	11.50
20	6.57	95.00	3.10×10^7	2.60×10^7	1.12	96.40
24	6.51	90.00	5.00×10^7	1.00×10^6	1.00	92.00
27	6.47	88.00	6.30×10^7	$<1.00 \times 10^5$	0.84	83.3
30	5.28	1.50	4.00×10^6	$<1.00 \times 10^4$	ND	NA

ND = not detectable

NA = not applicable

* Growth period was 75 hr.

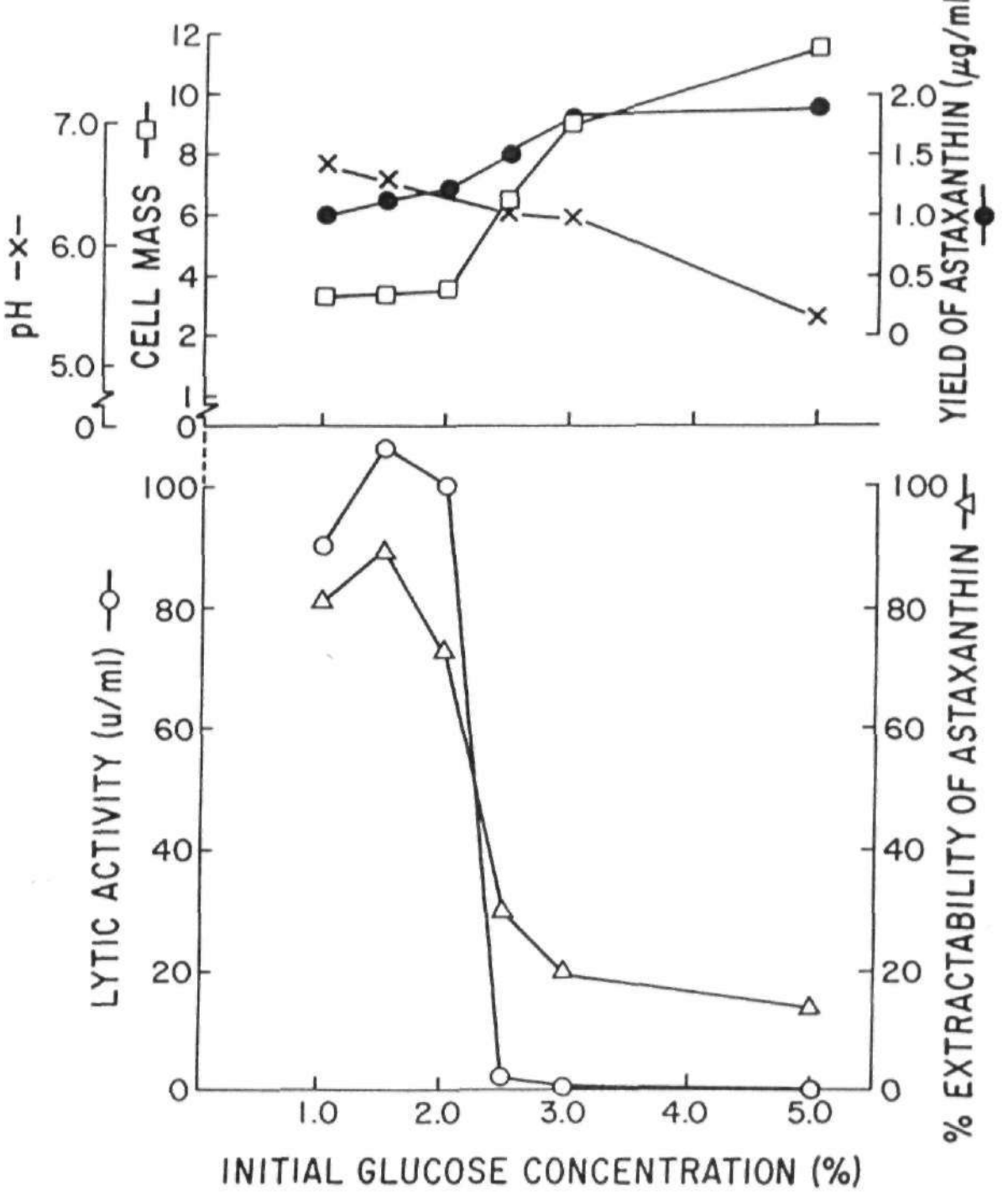
repressed growth of Bacillus circulans, and by low lytic activity. An interesting observation is that at least within the range 15-27°C, the two organisms appear to be affected by temperature in fairly opposite fashion. No growth of Phaffia was observed at 30°C. In contrast, there was abundant growth of the bacillus; the relatively low viable count probably indicates that it had entered the declining or death phase at the time of assay. Growth of the bacillus at 30°C caused a very drastic fall in pH of the medium; the low pH and relative absence of yeast probably caused the low lytic activity observed at 30°C.

Effect of initial concentrations of glucose on Bacillus circulans WL-12 and Phaffia rhodozyma in mixed culture.

Fig. 11 shows the effects on the mixed culture, of using various concentrations of glucose in growth medium. Yields of cell mass (mg/ml) (primarily Phaffia rhodozyma) as well as astaxanthin (µg/ml), rose steadily as glucose concentration increased; the most notable rise occurred between 2 and 3% glucose. There were slight increases in lytic activity and in extractability of astaxanthin at 1.5% glucose level; thereafter, they decreased, but remained relatively high at 2% glucose. Above this level of sugar, the levels of lytic activity and extractability of astaxanthin decreased fairly rapidly. The period of maximum rate of decline appears to coincide with that of optimum rise in cell mass and total astaxanthin. In general, 1-2% glucose appears to be critical for good yields of astaxanthin and yeast cell wall modification in the mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma.

Final pH decreased with increasing initial glucose concentrations and was remarkably low (about 5.42) at 5% initial glucose concentration

Fig. 11. Effect of various concentrations of glucose on growth of B. circulans WL-12 and P. rhodozyma in mixed culture.


pH –x–
CELL MASS –□–
YIELD OF ASTAXANTHIN (μg/ml)
LYTIC ACTIVITY (u/ml) –○–
% EXTRACTABILITY OF ASTAXANTHIN –△–
INITIAL GLUCOSE CONCENTRATION (%)

(Fig. 11). At that level, only approximately 3/4 of the glucose was utilized during the 75 hr fermentation period. Addition of 0.12% $CaCO_3$ partly reduced the acidity of the medium (not shown), but neither lytic activity nor extractability of astaxanthin was improved.

Effect of slow-feeding of glucose.

In earlier parts of this study, it was observed that high levels of lytic activity were produced only when glucose concentrations in the growth media declined to growth-limiting levels (see for example, Fig. 9A). This often occurred after 48 hr of growth of the mixed culture. In order to minimize or control the repressive effect of glucose on lytic enzyme formation, fed-batch mixed cultures were attempted. Each was initiated on 84 ml of medium which contained YNB and phosphate buffer in suitable concentrations to give 0.7% and 0.05M-0.10M respectively, when 120 ml of medium was reconstituted. Stock solutions of glucose were made to give final concentrations of 1.6% glucose when used to reconstitute the usual 120 ml of medium in each culture. The glucose solutions were added to the cultures according to three schedules; on the whole, each culture received a total of either 8 or 6 or 4 equally-spaced additions of glucose during the first 42 hr of the fermentation. Each addition of glucose contained 0.42 g or 0.32 g or 0.48 g in appropriate volume, depending on the schedule followed. For comparison, another culture which contained 1.6% glucose (in single addition), was included. The results of this fed-batch process are shown in Figs. 12 and 13. Generally, rate of astaxanthin formation was reduced by slow feeding of glucose in the manner adopted in this work (Fig. 12). In the first 48 hr, yield of astaxanthin appeared to be proportional to

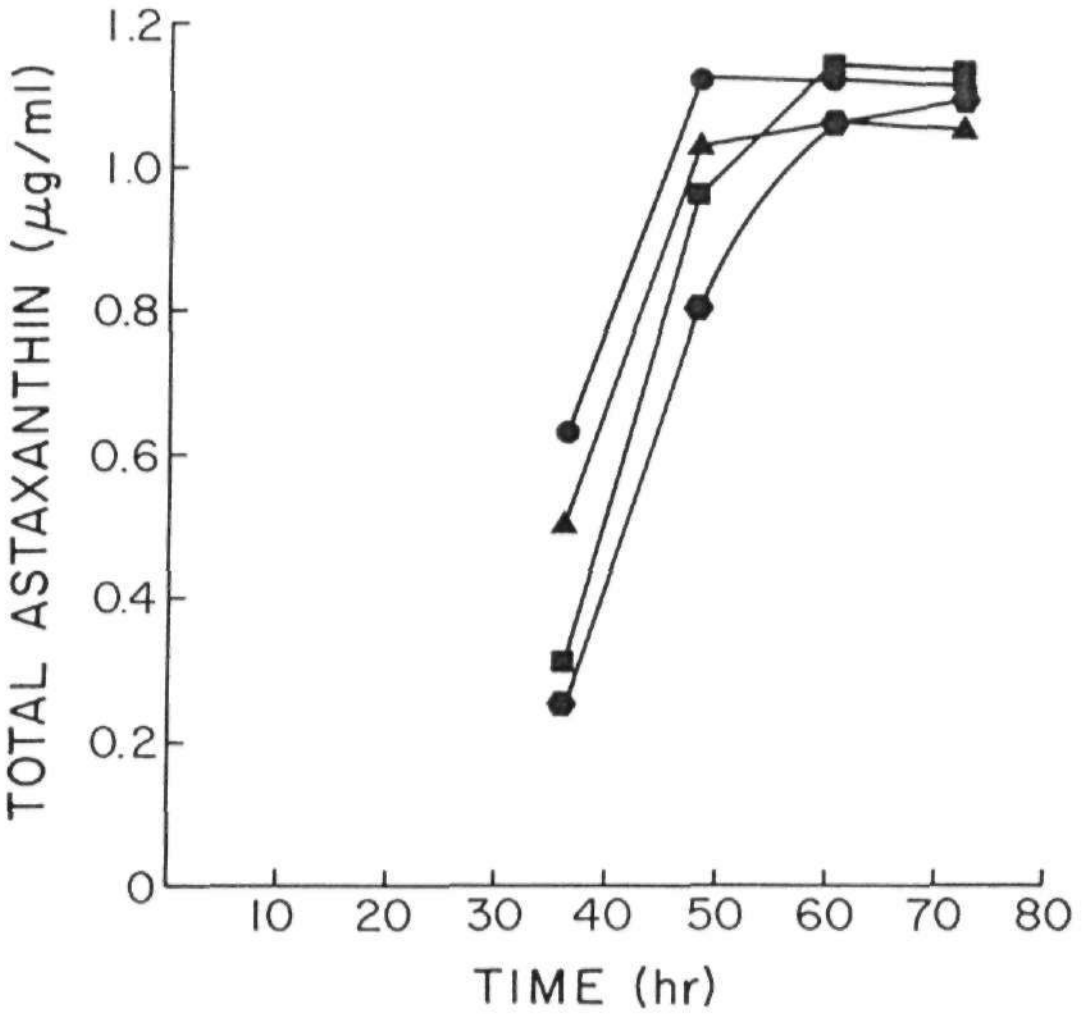
Fig. 12. Astaxanthin formation in a mixed culture fed-batch fermentation. Total amount of glucose added in each case was 1.92 g in 120 ml final volume of medium (i.e. 1.6% glucose).

Symbols: • All the glucose was added once at the beginning of the experiment.

▲ 0.48 g glucose added four times in appropriate volume of stock solution.

■ 0.32 g glucose added six times in appropriate volume of stock solution.

● 0.24 g glucose added eight times in appropriate volume of stock solution.


TOTAL ASTAXANTHIN (μg/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
80
TIME (hr)

the amount of glucose added per feed; however, overall yields were comparable for all the feeding schedules tested. Lytic activity was suppressed by the slow-feeding of glucose in the first 60 hr of the fermentations irrespective of the schedule employed (Fig. 13). Thereafter, there was a relatively rapid development of enzyme activity; it was most rapid when 0.32 g glucose was added with each feed. In cultures which received 0.24 g and 0.48 g glucose per feed, overall patterns and final lytic activities were similar. The culture in which glucose concentration was not regulated, produced the least final lytic activity within the period examined. The interval when most of the detectable lytic activity developed (60-72 hr), did not coincide with that of optimal extractability of astaxanthin (48-60 hr). In general, slow-feeding of glucose improved final lytic enzyme production, but did not accelerate lytic activity or yeast cell wall modification.

Effect of various carbon sources on growth of Bacillus circulans WL-12 and Phaffia rhodozyma in mixed culture.

The ability of carbon sources other than glucose to serve as substrates in the current mixed culture study, was investigated and the results are presented in Table 6. For each carbon source, a pure culture of Phaffia rhodozyma was set up as a control. Of the monosaccharides tested, glucose supported a higher yield of astaxanthin than fructose, even though lytic activity was more abundant in presence of the latter. Of the disaccharides tested, sucrose and maltose, in that order, promoted good yields of astaxanthin, lytic activity and extractability of astaxanthin, while cellobiose was a poor carbon source. The pentose, arabinose also appeared to be a satisfactory source of carbon for the

Fig. 13. Lytic activity and extractability of astaxanthin in the mixed culture fed-batch fermentation.

Data are from the same experiment as Fig. 12, and the symbols are similar to those of Fig. 12.

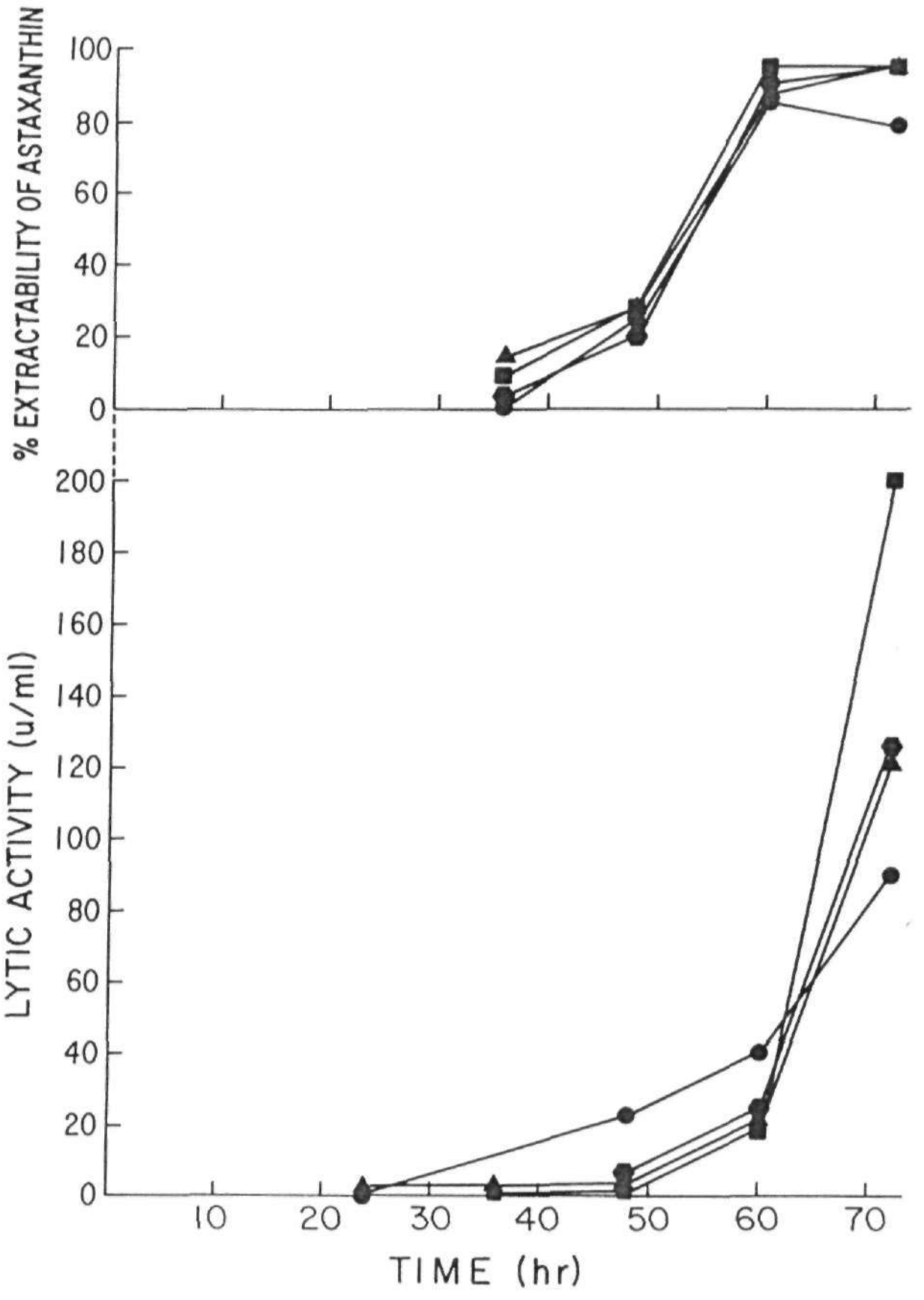

% EXTRACTABILITY OF ASTAXANTHIN
100
80
60
40
20
0
LYTIC ACTIVITY (u/ml)
200
180
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
70
TIME (hr)

Table 6. Growth of B. circulans and P. rhodozyma in mixed culture on various carbon sources.

Carbon sources		Yield of astaxanthin (µg/ml)	Yield of extractable astaxanthin (µg/ml)	Extractability of astaxanthin (%)	Lytic activity (Units/ml)
glucose	Expt.	1.30	1.19	91.5	110.00
	Control	1.32	n.t	n.t.	0
fructose	Expt.	0.70	0.60	85.70	298.00
	Control	0.85	n.t	n.t	0
sucrose	Expt.	1.43	1.38	96.50	296.00
	Control	1.40	n.t	n.t	0
maltose	Expt.	1.03	0.93	90.30	134.00
	Control	1.03	n.t	n.t	0
starch	Expt.	NYG	n.t	n.t	0
	Control	NYG	n.t	n.t	0
mannitol	Expt.	0.69	0.28	40.60	25.00
	Control	0.67	n.t	n.t	0
arabinose	Expt.	1.47	1.31	89.10	280.00
	Control	1.39	n.t	n.t	0
xylose	Expt.	trace	trace	n.t	0
	Control	trace	n.t	n.t	0
xylan	Expt.	NYG	n.t	n.t	0
	Control	NYG	n.t	n.t	0

Table 6. (cont.)

Carbon sources		Yield of astaxanthin (µg/ml)	Yield of extractable astaxanthin (µg/ml)	Extractability of astaxanthin (%)	Lytic activity (Units/ml)
cellobiose	Expt.	0.22	0.19	86.40	41.30
	Control	0.50	n.t		

n.t = not tested

NYG = no yeast growth

mixed culture fermentation, but xylose was not; indeed very little growth of Phaffia rhodozyma occurred on xylose. Mannitol supported little growth of the yeast, and very low lytic activity. The polymeric carbon sources, starch and xylan, were not suitable for yeast growth and cell wall modification under the conditions of this study. In general, sucrose, arabinose and maltose (in addition to glucose) were the best of all the carbon sources tested, because they promoted a combination of high lytic activity, and good yields of total and extractable astaxanthin.

Effect of the inoculum size of Bacillus circulans WL-12.

Another attempt to accelerate modification of the cell walls of Phaffia rhodozyma in mixed culture, was made by increasing levels of the bacterial inoculum. As described in Materials and Methods, routine mixed cultures usually employed Bacillus circulans WL-12 and Phaffia rhodozyma, at an initial ratio of 1:1, on cell dry weight basis. In order to vary the bacterial load, suitable volumes were employed to give ratios of 0.5:1, 1:1, 2:1, and 4:1 in the same total volume (120 ml) of medium. Fig. 14 shows that high bacterial inoculum size shortened or eliminated the lag phase of growth. By 72 hr, the heaviest inoculum had evidently completed the stationary phase and entered the death phase of growth. There was also a quicker development of lytic activity at the high inoculum level. By 48 hr, the lytic activity produced by the 4-fold bacterial size was fairly comparable to that of the usual 72-75 hr mixed (1:1) culture. Extractability of astaxanthin was over 80% while the values obtained at lower inoculum sizes were less than 20% (Fig. 15). Unfortunately, the lytic activity appears to accelerate

Fig. 14. Effect of bacterial inoculum size on growth and lytic activity in mixed culture.

Figures indicate ratios of Bacillus circulans WL-12 (based on dry wt of inocula). Phaffia rhodozyma was kept at 1.0 throughout.

Closed symbols = viable counts of B. circulans WL-12 at different inoculum levels.

Open symbols = lytic activity of appropriate culture filtrates.

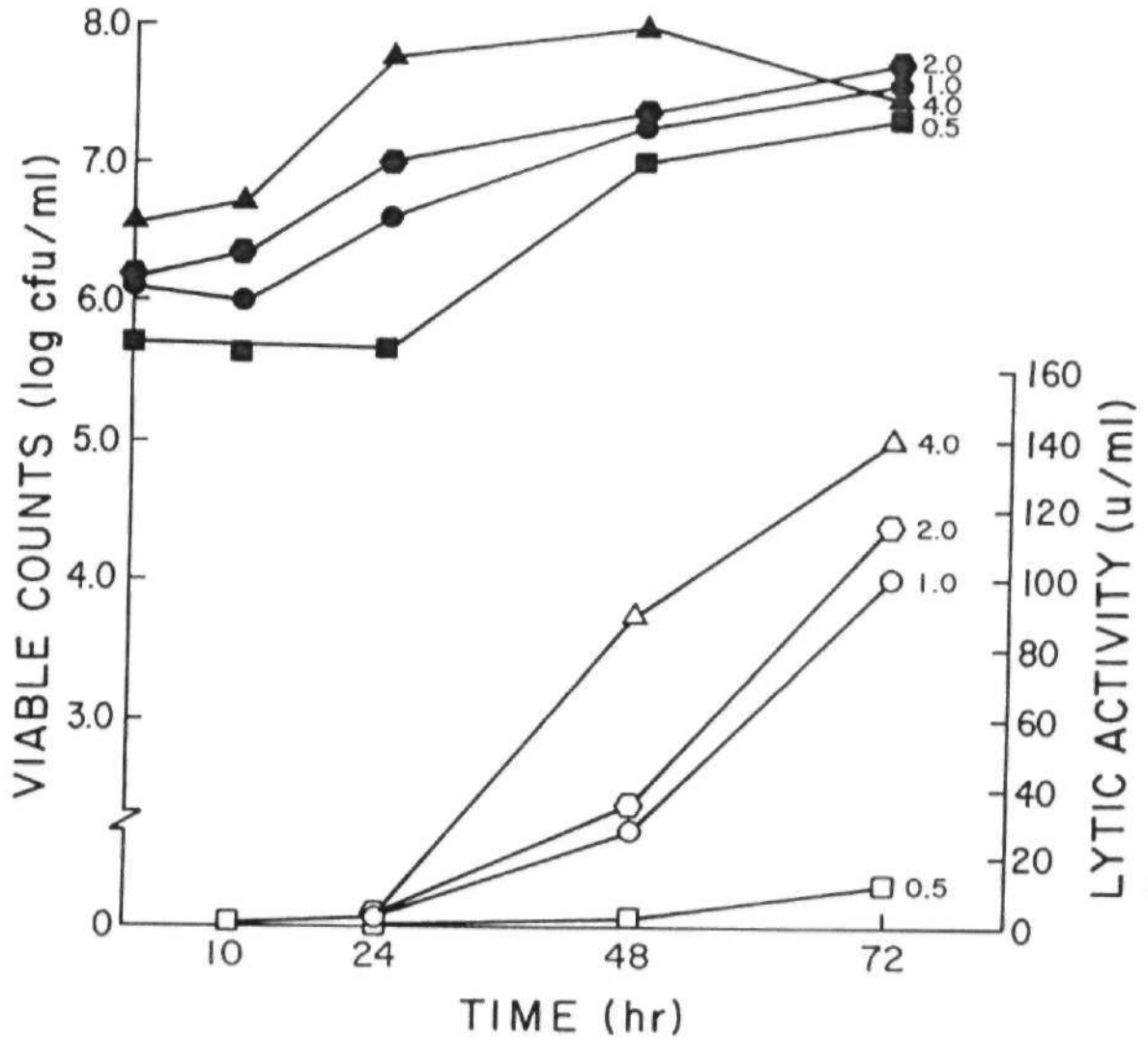
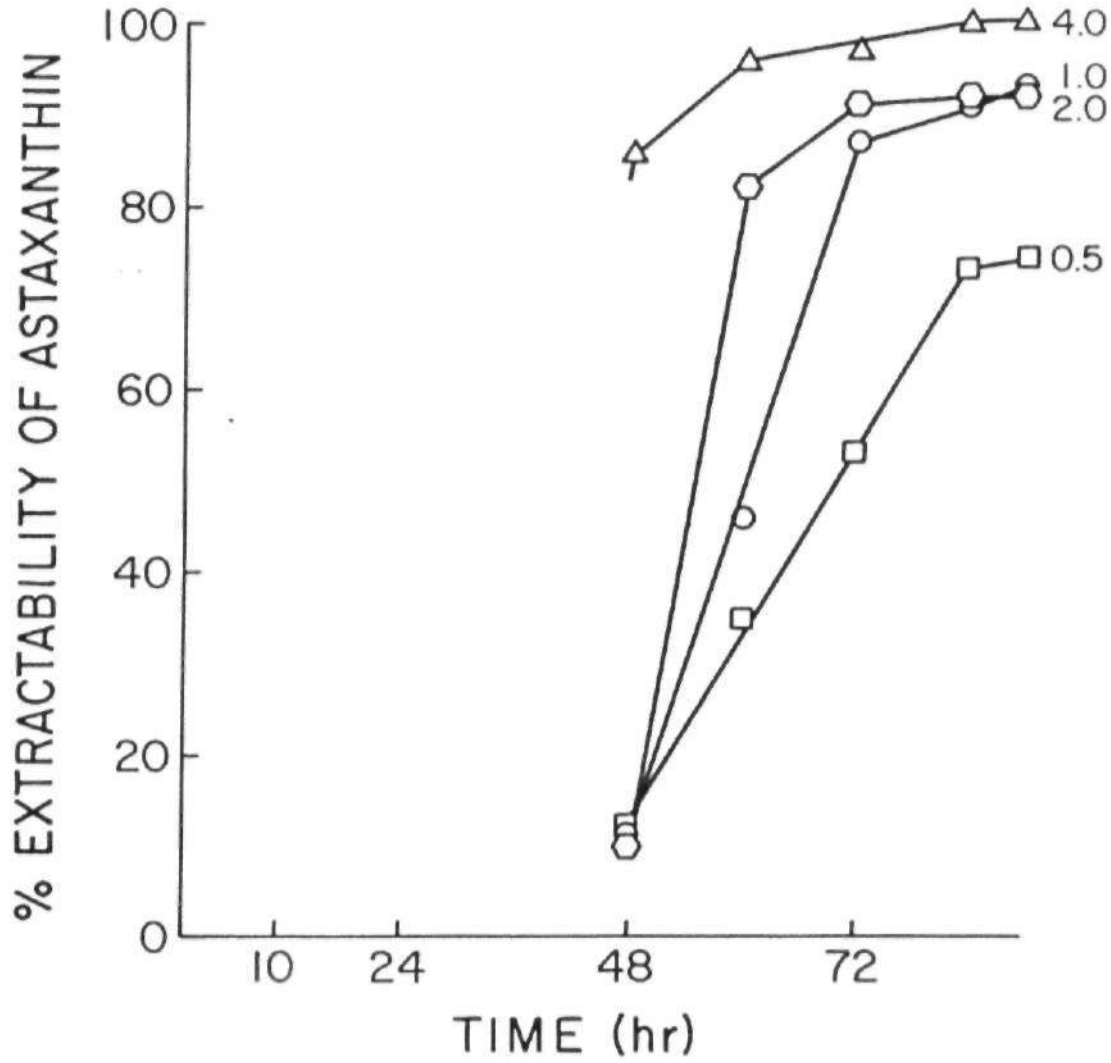

8.0
7.0
6.0
5.0
4.0
3.0
0
VIABLE COUNTS (log cfu/ml)
2.0
1.0
4.0
0.5
160
140
120
100
80
60
40
20
0
LYTIC ACTIVITY (u/ml)
4.0
2.0
1.0
0.5
10
24
48
72
TIME (hr)

Fig. 15. Effect of bacterial inoculum size on extractability of astaxanthin from the mixed culture.

Figures and corresponding symbols indicate relative inoculum sizes of Bacillus circulans WL-12 as in Fig. 14.


% EXTRACTABILITY OF ASTAXANTHIN
100
80
60
40
20
0
10
24
48
72
TIME (hr)
4.0
1.0
2.0
0.5

autolysis of Phaffia rhodozyma. This was obvious from the decline in total yield of astaxanthin (Fig. 16), as well as in the recoverable cell mass (Fig. 17). The higher the initial bacterial concentration, the earlier the initiation of the autolytic phenomenon.

As a result of the yeast autolysis, culture medium becomes slightly pigmented. This phenomenon was observed earlier in this study (see Table 2). When cultures were centrifuged at high speeds (12000 rpm for 1 hr) to remove highly modified Phaffia cells which probably remained in suspension (Johnson et al., 1978) the pigmentation in the supernatant was not completely eliminated. To determine the fate of carotenoids presumably released into the culture medium, supernatant was treated with acetone on a 1:1 (V/V) basis and kept at 4°C for 12 hr. A white precipitate (which had lytic activity after dissolution in phosphate buffer) was removed by centrifugation and electronic absorption spectrum of the yellow-brown supernatant (i.e. pigment in acetone-water) was determined. No evidence of presence of carotenoids was found; in fact the observed absorption spectrum had no peak within the range of wavelength tested (Fig. 18). Since high initial levels of Bacillus circulans WL-12 improved its effectiveness in modifying the cell walls of Phaffia rhodozyma, the effect of high inoculum levels of the yeast was examined. Results presented in Table 7, show that yields of cell mass and final pH of medium were fairly independent of the yeast inoculum size within the range tested. Total yield of astaxanthin, probably, was also unaffected; the slight differences in the data might be due to the time of assay since the lag phase of growth (and hence astaxanthin production) would be reduced at the high inoculum levels. Levels of lytic activity were high but no consistent effect

Fig. 16. Effect of bacterial inoculum size on yield of astaxanthin in the mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma.

Figures and corresponding symbols refer to relative ratios (dry weight basis) of inocula of Bacillus circulans WL-12. Phaffia rhodozyma was kept at 1.0 throughout.

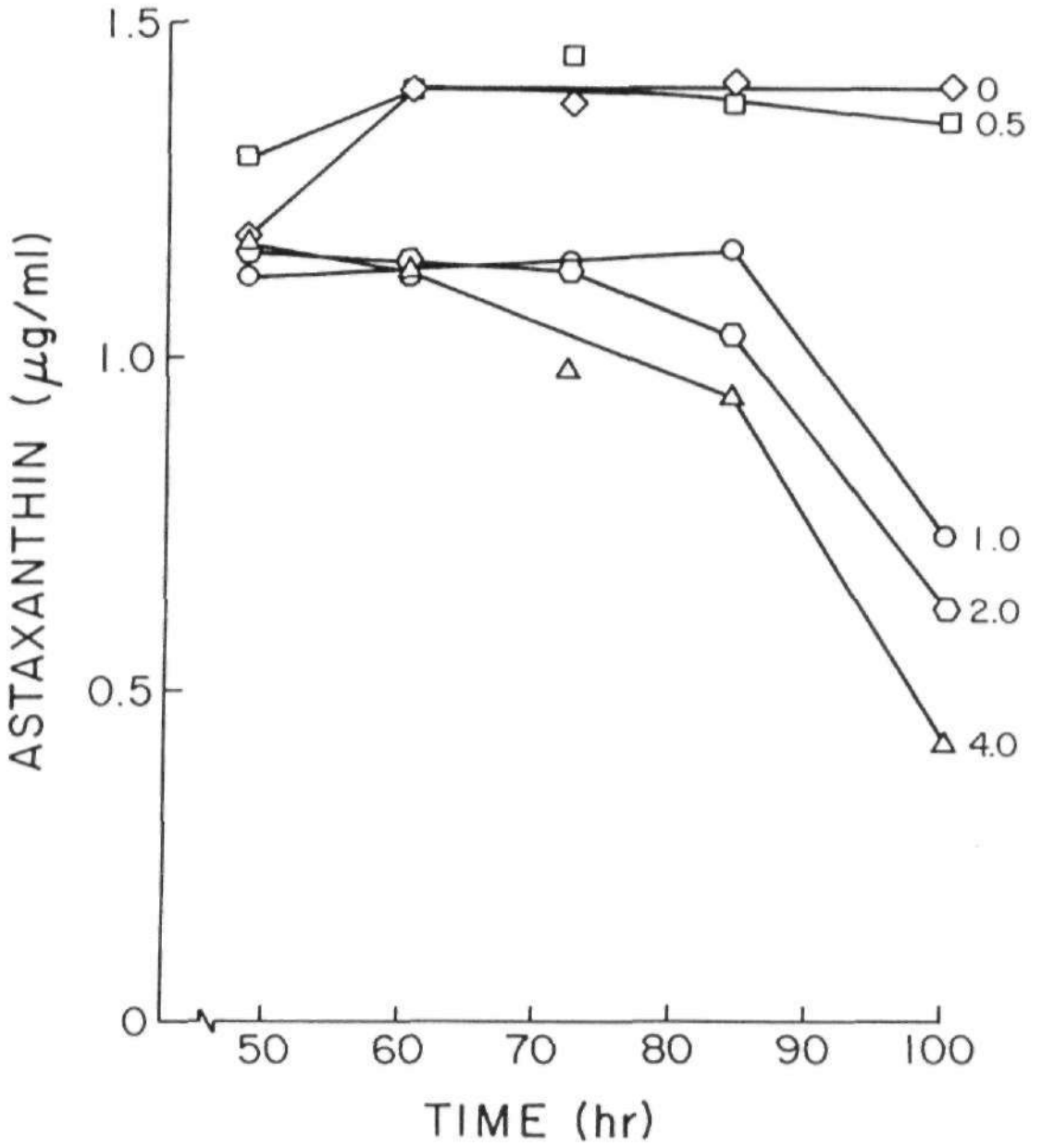

1.5
1.0
0.5
0
ASTAXANTHIN (μg/ml)
50
60
70
80
90
100
TIME (hr)
0
0.5
1.0
2.0
4.0

Fig. 17. Effect of the inoculum size of B. circulans WL-12 on cell mass recovered from the mixed culture with P. rhodozyma.

Ratio of yeast (Phaffia) was kept at 1.0 throughout.
Date were obtained from the same experiment as Figs. 14, 15 and 16.

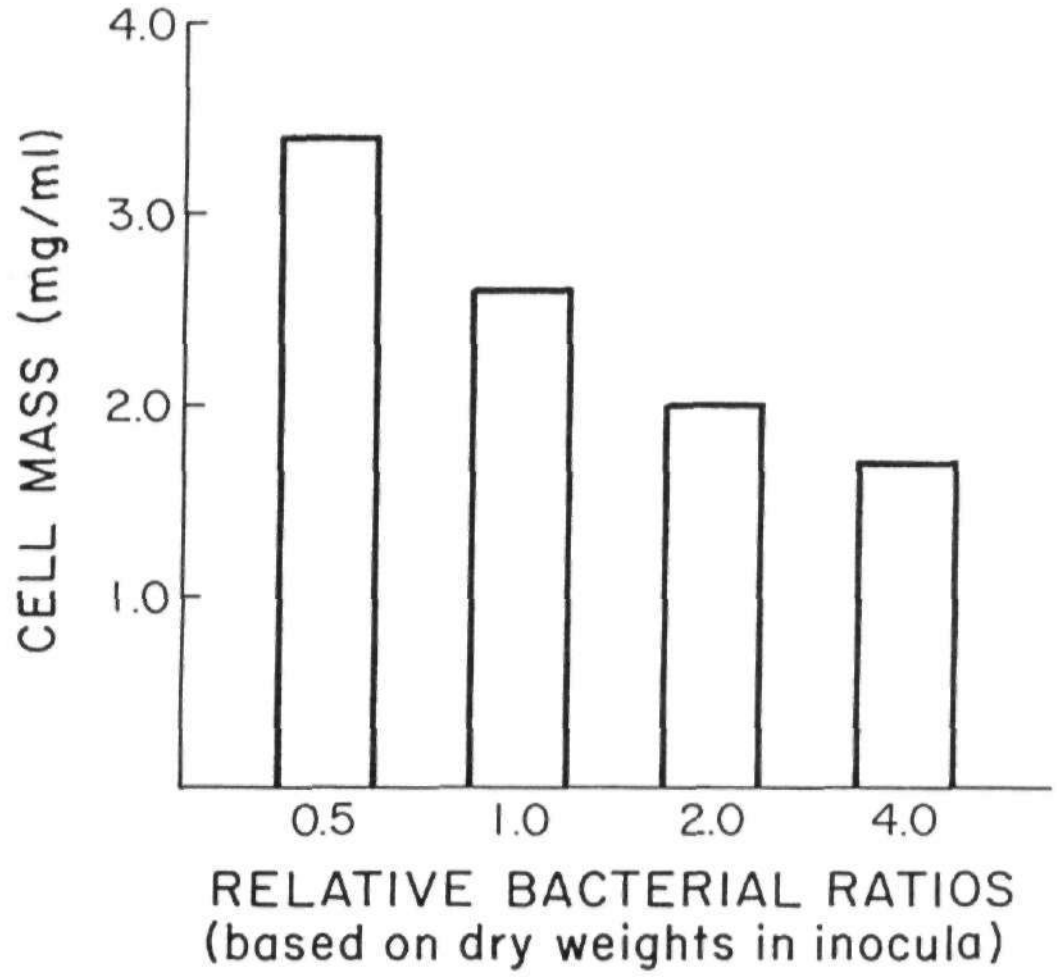

4.0
3.0
2.0
1.0
CELL MASS (mg/ml)
0.5
1.0
2.0
4.0
RELATIVE BACTERIAL RATIOS
(based on dry weights in inocula)

Fig. 18. Absorption spectrum of pigment recovered from mixed culture medium (84-90 hr of growth).

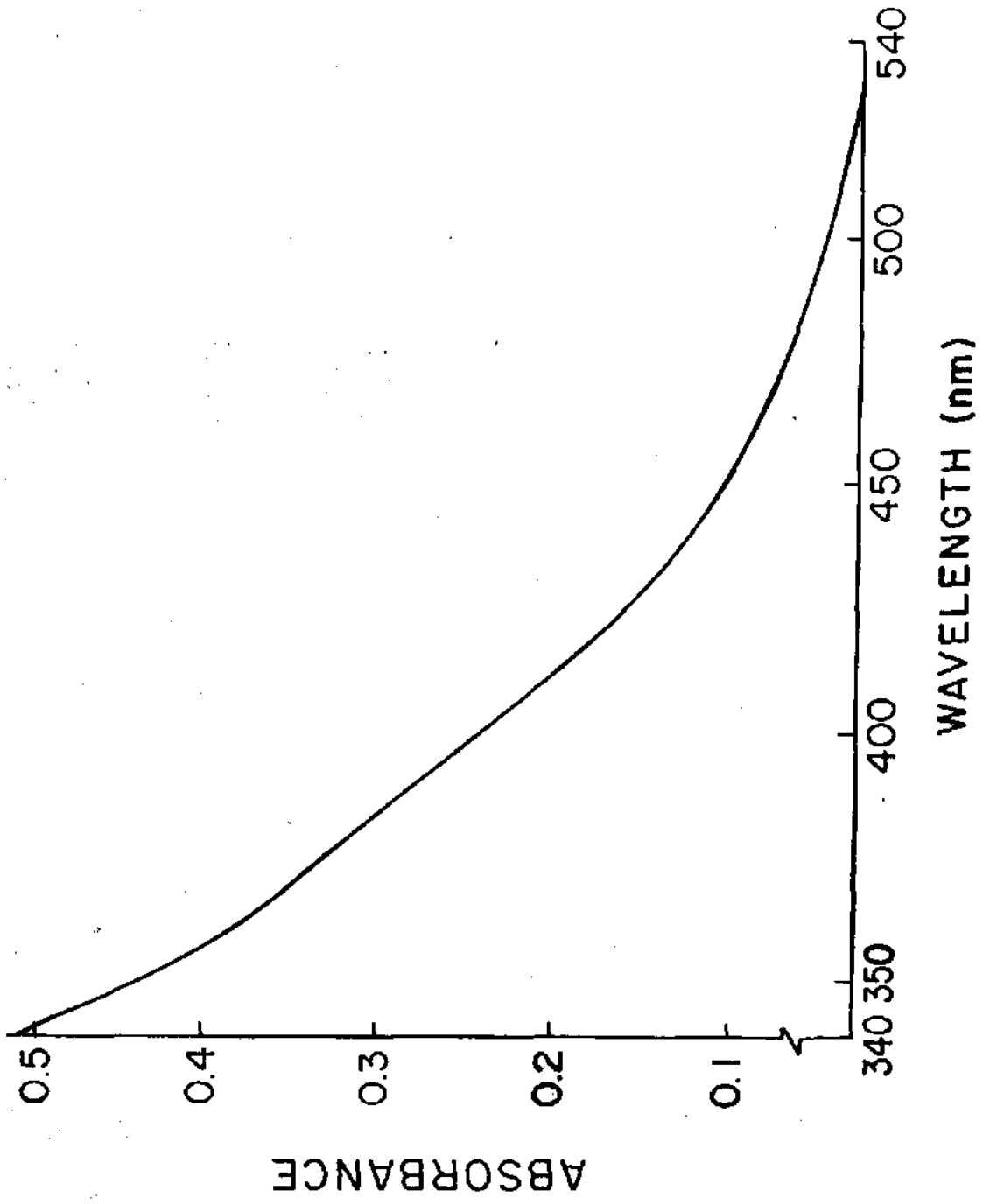

ABSORBANCE
0.5
0.4
0.3
0.2
0.1
340 350
400
450
500
540
WAVELENGTH (nm)

Table 7. Effect of varying inoculum sizes of Phaffia rhodozyma on extractability of astaxanthin.

Inoculum size employed (mg)	Total yield* of astaxanthin µg/ml	Final pH	Lytic activity Units/ml	% Extractability of astaxanthin	Cell mass mg/ml
12.00	0.69	6.58	80.80	86.40	3.30
15.00	0.73	6.62	60.50	88.00	3.60
36.00	0.75	6.65	70.50	88.50	3.60
48.00	0.87	6.65	102.40	89.30	3.10

* assayed at 48 hr. All other assays were done at 75 hr. Inoculum size of B. circulans WL-12 was 12:00 mg throughout.

of size of yeast inoculum was apparent. Extractability of astaxanthin was also comparable at all levels of yeast inoculum tested. In general increasing the inoculum size of Phaffia rhodozyma does not enhance the efficiency of the mixed culture system even in presence of high levels of Bacillus circulans.

MIXED CULTURE OF BACILLUS CIRCULANS WL-12 AND PHAFFIA RHODOZYMA IN BATCH FERMENTORS

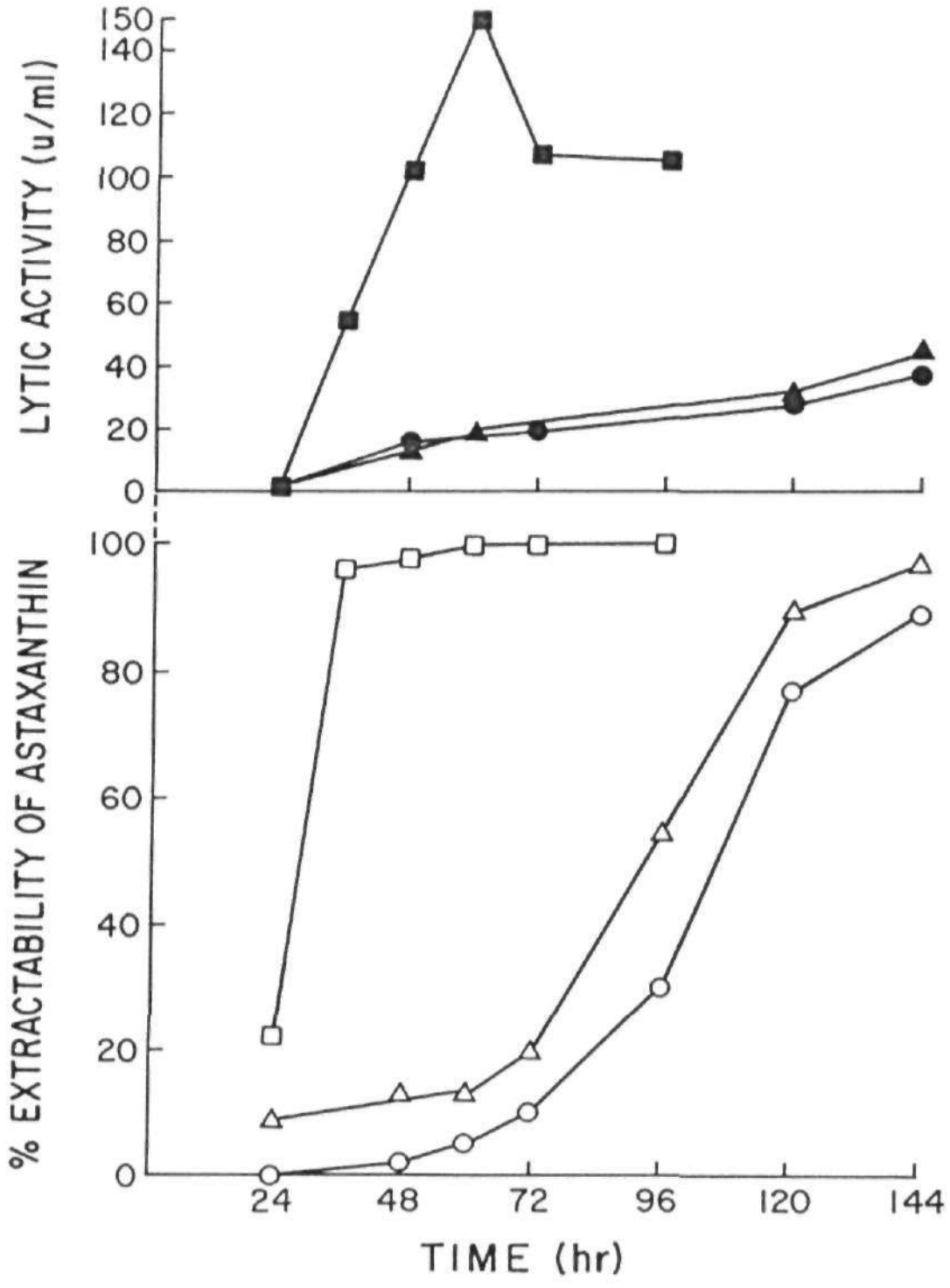
In order to examine feasibility of the mixed culture on a scale larger than shake flasks, experimentation was carried out in three 5-liter fermentors, using 3-liter working volumes. The medium, as usual, was buffered with 0.1M potassium phosphate. Initial concentration of P. rhodozyma was kept constant in all the fermentors (approximately 0.31 g on dry weight basis was used), but that of the bacillus was varied from 0.39 to 1.40 g. Details of the preparation of the inocula and of other cultural conditions employed, have been described in Materials and Methods. Astaxanthin was extractable from biomass produced in all the fermentor mixed cultures. Fig. 19a shows that bacterial inoculum size has considerable influence. At the highest inoculum employed, nearly all the pigment synthesized by P. rhodozyma by 48 hr of the fermentation, was extractable, whereas 144 hours were required to achieve comparable efficiencies at the lower inoculum levels. Yeast cell wall modification in the fermentor cultures was also associated with development of lytic activity, and proceeded most rapidly at the highest concentration of B. circulans used. At that level (1.4 g), maximum lytic activity was reached at approximately 60 hr, when 100% extractability

Fig. 19a. Extractability of astaxanthin from biomass in fermentor mixed cultures of Bacillus circulans WL-1 and Phaffia rhodozyma.

Symbols: □ ■ Inoculum size of B. circulans WL-12 was 1.40 g

△ ▲ Inoculum size of B. circulans WL-12 was 0.70 g

○ ● Inoculum size of B. circulans WL-12 was 0.39 g


LYTIC ACTIVITY (u/ml)
150
140
120
100
80
60
40
20
0
% EXTRACTABILITY OF ASTAXANTHIN
100
80
60
40
20
0
24
48
72
96
120
144
TIME (hr)

of astaxanthin was observed. In cultures which employed less Bacillus circulans, lytic activities detected at 144 hours were suprisingly small, even when approximately 90% of yield of astaxanthin was extractable. Unfortunately, maximum yield of astaxanthin was lowest when the highest bacterial inoculum was used (Fig. 19b). It was approximately 0.78 µg/ml (at 48 hr) (compared with 1.03 and 1.19 µg/ml at 96 hr at the lower inoculum sizes), and also declined rather rapidly (probably due to autolysis) to about 0.52 µg/ml by 96 hr of the fermentation. In contrast, the maximum yields of astaxanthin at the lower initial concentrations of the bacillus, were fairly constant up to 144 hr (not shown in the Fig.).

Successful development of the mixed culture in fermentors illuminate its potential commercial importance. It also reaffirms the importance of an adequate bacterial inoculum size for achieving the desired outcome as early as possible. In addition, it shows that, as bacterial inoculum is increased, time of harvest of biomass becomes very critical in order to minimize autolysis of the yeast.

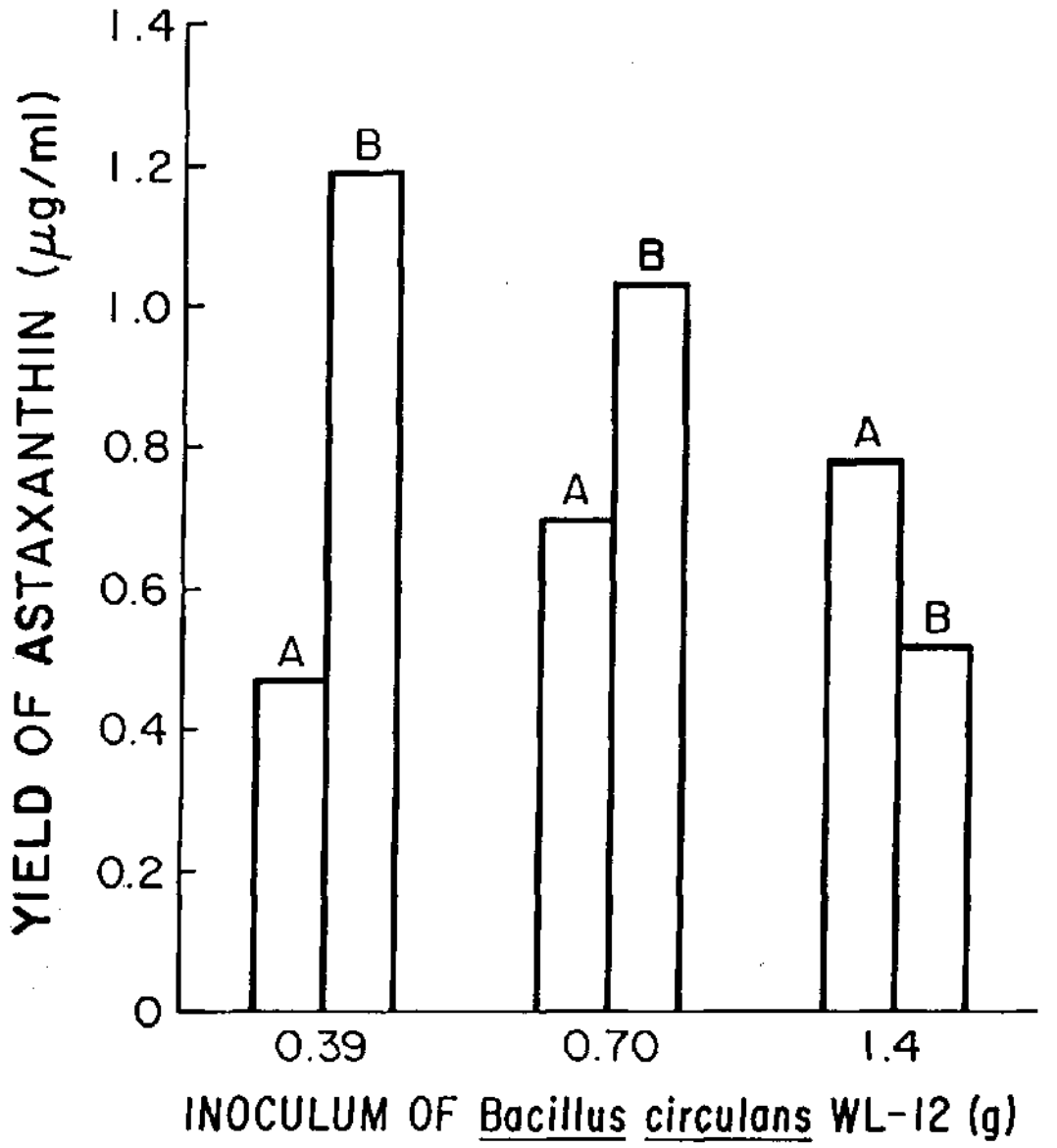
RE-USE OF CELL-FREE CULTURE FLUID DERIVED FROM THE MIXED CULTURE

After separation of biomass from the mixed culture of Bacillus circulans and Phaffia rhodozyma, the culture fluid, laden with lytic enzymes, could become a reusable resource. It could be used to treat fresh batches of Phaffia rhodozyma cell mass in order to facilitate astaxanthin recovery or absorption (if the cell mass is used in feeds). Number of recycles of the enzyme system would probably depend on the

Fig. 19b. Effect of inoculum size of Bacillus circulans WL-12 on yields of astaxanthin in fermentor mixed cultures.

Letters indicate times (hr) of sampling: A = 48 hr

B = 96 hr


YIELD OF ASTAXANTHIN (μg/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
A
B
A
B
0.39
0.70
1.4
INOCULUM OF Bacillus circulans WL-12 (g)

effect of each treatment on its lytic potency. To examine this on a laboratory scale, a cell-free lytic system was recycled on a few batches of the yeast cells. Table 8a shows that two successive treatments had appreciable efficiency in hydrolysis of the yeast cell walls. After two cycles, there was considerable reduction in lytic potency of the enzyme system.

Alternatively, the lytic enzyme (culture fluid) could be refortified with nutrients and used as a culture medium for growth of Phaffia cells. Resultant yeast mass, could contain readily extractable or biologically-available astaxanthin because the cell walls would be considerably altered or modified during growth in presence of the lytic system. In order to develop this theory, a cell-free lytic system was obtained by centrifuging twice at 13,000 rpm for 1 hr, the pooled contents of six 73-75 hr old mixed culture flasks grown as usual, with inocula containing 1:2 (dry weight basis) ratio of Phaffia rhodozyma and Bacillus circulans WL-12. Lytic activity in the system was determined to be approximately 125 lytic units/ml. 80 ml volumes of the culture fluid were dispersed aseptically into four sterile 500 ml baffled sidearm flasks. Each flask received 10 ml of a sterile aqueous stock solution of glucose, prepared to give a final concentration of 1.5% in 100 ml of medium. Another 10 ml required to make up each flask was supplied as a sterile stock solution (in 0.2M phosphate buffer, pH 7.0) of supplementary nutrients shown in Table 8b; the given concentrations of the nutrients were final values in 100 ml of medium. In short, each flask contained 80 ml of culture fluid and 20 ml of stock solutions of nutrients. An inoculum of Phaffia rhodozyma was grown up under conditions described in Materials and Methods, washed twice in sterile

Table 8a. Effect of Lytic enzyme recycle on its potency.

# of treatments/cycle	Residual lytic activity U/ml	Concentration of total astaxanthin µg/ml	Concentration of extractable astaxanthin µg/ml (after enzyme treatment)	% Extractability of astaxanthin
0	128.60*	1.80	NA	NA
1	128.20	1.82	0.92	51.10
2	104.40	1.80	0.87	48.30
3	58.00	1.80	0.31	17.20
4	42.00	1.73	0.23	13.20

* at 0 hr. NA = not applicable. The yeast cells treated are 48 hr old Phaffia cells grown in 0.1 M phosphate buffered YNB (0.7%) and glucose (1.5%) medium, pH 7.0, and washed once. The cell free lytic system was obtained from 75-80 hr old mixed culture in shake flasks. In each treatment, yeast cells were suspended in enzyme solution to give approximately 6 mg/ml. Suspensions were shaken in roller drum for 1 hr at 30°C. All data are averages of duplicate determinations.

distilled water and resuspended to a turbidity of 300 klett units. 2 ml of the suspension was inoculated into each of the four flasks and the experiment was incubated at 20°C with shaking at 200 rpm for 48 hr.

Table 8b shows that Phaffia rhodozyma was able to grow in the refortified culture fluid containing the lytic enzyme complex, and that resulting cell yields contained easily extractable astaxanthin. Of the four types of stock solutions of nutrients used in the enrichment, those containing ammonium phosphate plus peptone, and urea plus potassium phosphate plus peptone, were the most satisfactory in producing good yields of cell mass, and high proportions of extractable astaxanthin. The stocks containing entirely complex nutrients (yeast extract, malt extract and peptone (YMP)) and that containing yeast nitrogen base (YNB), were very efficient in cell mass production but did not permit appreciable cell wall modification. While the low final pH caused by YNB could partly explain the apparently limited lytic enzyme activity on Phaffia cell walls, the corresponding effect observed in medium fortified with YMP is not clear. It may be that the two supplements generally supported synthesis by the yeast of a high level of some cell wall components which was resistant to the hydrolytic action of the lytic enzyme complex of Bacillus circulans WL-12, for example, mannan-protein. What seems to be noteworthy is that the supplementation requires a relatively high level of NH_4^+ compounds, or urea, and complex nitrogen compounds in order to keep medium pH high enough for good enzymatic action on the yeast cell walls.

Table 8b. Growth and cell wall modification of Phaffia rhodozyma in fortified culture fluid containing the lytic enzyme complex of Bacillus circulans WL-12.*

Nutrient Supplementation**	Final pH	Yield of cell mass mg/ml	Yield of astaxanthin (µg/ml) Total	Extractable	% Extractability of astaxanthin
Yeast nitrogen base, 0.6%	5.94	8.30	0.81	0.31	38.30
$(NH_4)_2HPO_4$, 0.5% + Peptone, 0.5%	6.69	8.00	1.00	0.91	91.00
Urea, 0.5% + KH_2PO_4, 0.4% + Peptone, 0.5%	6.86	8.00	0.94	0.83	88.30
Yeast extract, 0.3% + malt extract, 0.3% + Peptone, 0.5%	6.46	10.50	1.50	0.54	36.00

* All assays were carried out at 48 hr and data are averages of duplicate samples.

** In addition to 1.5% glucose.

A PROPOSED SCHEME FOR BIOMODIFICATION OF PHAFFIA RHODOZYMA

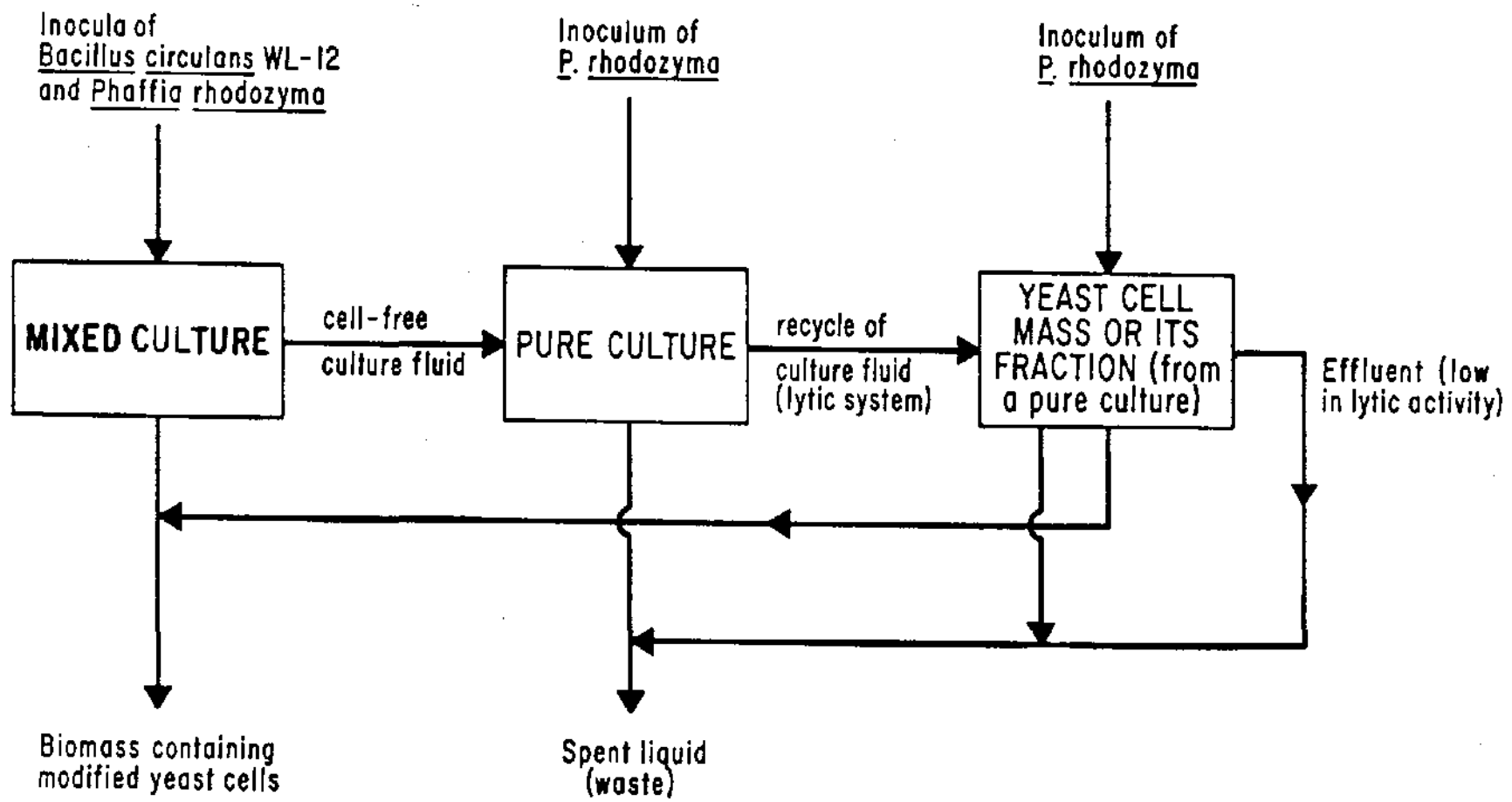
On the basis of all the results, a scheme (Fig. 20) is proposed for enzymatic treatment of Phaffia rhodozyma to enhance bio-availability of astaxanthin. In the scheme, simultaneous cultivation of yeast in pure culture and in mixed culture with Bacillus circulans WL-12 is envisaged. After about 75 hours, cell-free culture fluid from the mixed culture is recycled on some batches of the yeast cells grown in pure cultures. After a suitable number of cycles, waste liquid (low in lytic enzyme activity) is removed and all the treated cell mass (derived from both pure and mixed cultures) is pooled and channeled into desired use.

The proposed enzyme recycle would substantially improve the economic prospects of large-scale production and treatment of Phaffia rhodozyma. It is possible that a greater degree of success could be achieved in a commercial situation than is indicated in this work because longer contact periods for each cycle would be employed in industry.

In the alternative, the culture fluid derived from the mixed culture can be refortified with suitable cheap nutrients and re-used for cultivation of Phaffia rhodozyma. Resulting cell mass (after about 48 hours) would have altered cell walls and astaxanthin would be extractable and presumably biologically available. For simplicity, this option in re-use of the lytic enzyme is omitted from the scheme in Fig. 20.

Figure 20. Block diagram for a proposed scheme for enzymatic treatment of Phaffia rhodozyma.

In the scheme, modified P. rhodozyma (resulting from mixed culture with Bacillus circulans WL-12) and the unmodified yeast (pure culture) are grown concurrently. More than one pure culture vessel may be used, or the cell mass from one vessel may be divided into different portions. Cell-free culture fluid (containing lytic enzyme system) from the mixed culture is used to treat biomass from the pure culture/s. The culture fluid may be recycled if necessary.


Inocula of
Bacillus circulans WL-12
and Phaffia rhodozyma
Inoculum of
P. rhodozyma
Inoculum of
P. rhodozyma
MIXED CULTURE
cell-free
culture fluid
PURE CULTURE
recycle of
culture fluid
(lytic system)
YEAST CELL
MASS OR ITS
FRACTION (from
a pure culture)
Effluent (low
in lytic activity)
Biomass containing
modified yeast cells
Spent liquid
(waste)

AN ELECTRON MICROSCOPIC STUDY OF THE MIXED CULTURE OF BACILLUS CIRCULANS WL-12 AND PHAFFIA RHODOZYMA

This study was undertaken to obtain some evidence of physical modifications in the cell wall of Phaffia rhodozyma during its digestion in mixed culture, and to correlate such changes with extractability of astaxanthin. Samples were taken from a mixed culture containing a 1:1 inoculum (dry weight basis) of the bacterium and the yeast, and from a pure culture of the yeast (control), at 48 and 72 hr, and processed as described earlier.

Electron micrographs of 48 hr samples of the mixed culture contained few cells of Bacillus circulans; this probably explains the relatively low lytic activity and low extractable levels of astaxanthin observed in corresponding samples assayed in earlier sections of this study. In contrast, Phaffia rhodozyma cells were abundant, (Fig. 21, electron micrograph nos. 121, 122) and resembled those of the same age in the control (no. 123). In the electron micrographs, most of the spherical cells might possibly be blastospores described by Miller et al. (1976). Typical budding cells are also present. It appears that the staining technique employed in this work has not clearly revealed many of the characteristic features of Phaffia rhodozyma and other basidiomycetous yeasts described by Miller et al. (1976), and by Kreger van Rij (1971), but they can be found by close inspection. The features include a lamellar cell wall structure which shows here as a thin dark layer alternating with electron opaque layers, collar-like strands of the wall formed at the base of some buds (nos. 122 & 123), and cross-wall formation at the isthmus between bud and mother cell (no. 121). Use of ruthenium red in

this study reveals distinctly the capsule formed by Phaffia rhodozyma (no. 115); the dye is specific for anionic polymers, most of which are carbohydrates (Luft, 1971). Traces of fimbriae were also discernible at adjacent areas of yeast cell surfaces.

By 72 hr, considerable growth of the bacillus had occurred in the mixed culture (nos. 119 & 120). This stage was marked by virtual absence of budding yeast cells. Of particular interest was the occurrence in many cells of varying degrees of internal disorganization. Some of the cells, apparently, had undergone total lysis and many cell ghosts and extruded cellular materials were present. Capsular materials appeared to be considerably reduced, and only those of the relatively young cells of the population were appreciably stained by ruthenium red (nos. 130 & 131), the young cells were distinguishable by the dark-staining cytoplasm. These results indicate that intracellular modification of Phaffia rhodozyma accompanied or were induced by lytic activity on its cell walls. The electron micrographs of control samples at 72 hr (nos. 117 & 118) were not remarkably different from those of 48 hr. Unfortunately, the 72 hr samples stained with ruthenium red were unexpectedly different; the yeast cells were greatly distorted in shapes, even though cell ghosts were virtually absent as expected (nos. 128 & 129). The distortions could be attributed to faulty processing of the samples since the dye clearly revealed capsular material around most of the cells. In addition, presence of dark-staining clumps of material in most of the cells suggests that depletion of intracellular reserves which normally precede autolysis, had not occurred in the 72 hr control samples. In general, the ultrastructural study shows that extractability of astaxanthin from yeast cells grown in the mixed

Figure 21. Electron micrographs showing the digestion of Phaffia rhodozyma by wall-lytic enzymes produced by Bacillus circulans WL-12 in mixed culture.

A pure culture of P. rhodozyma was examined as a control. Each electron micrograph carries a number and can be referred to at the appropriate citation in the text.

Summary of the electron micrographs	Numbers (on the *electron micrographs*)	
Stage of culture examined (hr)	Mixed culture	Pure culture of P. rhodozyma (control)
48	121, 122	123
48*	115	-
72	119, 120	117, 118
72*	130, 131	128, 129

* Samples stained with ruthenium red as described in Materials and Methods.

All cultures were grown in YNB (0.7%) and glucose (1.5%) buffered with 0.1M phosphate buffer pH 7.0.

B. circ. = Bacillus circulans; CW = cross wall; CBW = collar-like strands of wall at base of bud; CRR = capsule stained by ruthenium red; F = fimbriae; DY = disorganized yeast. Magnification throughout: x 3000

Figure 21 (cont.): 48 hr mixed culture.



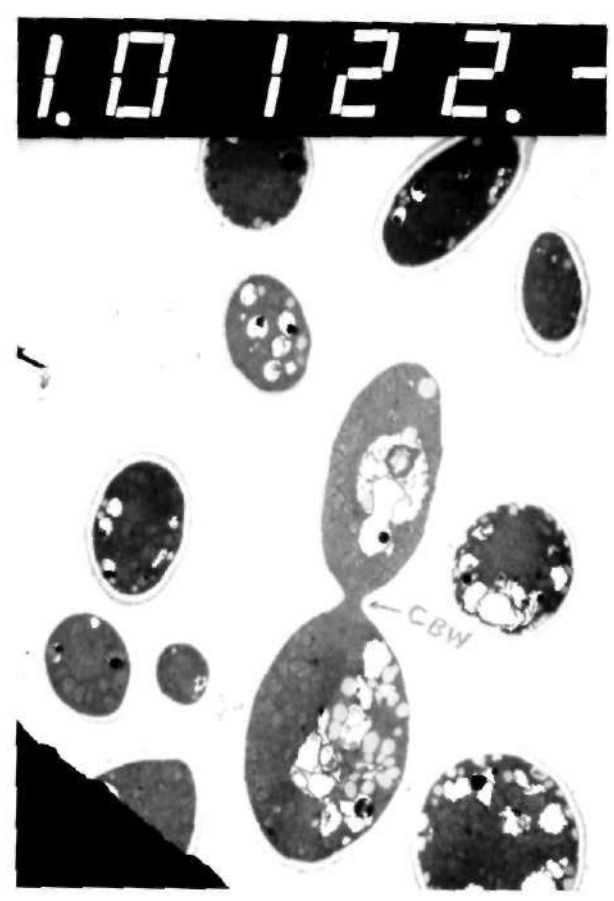
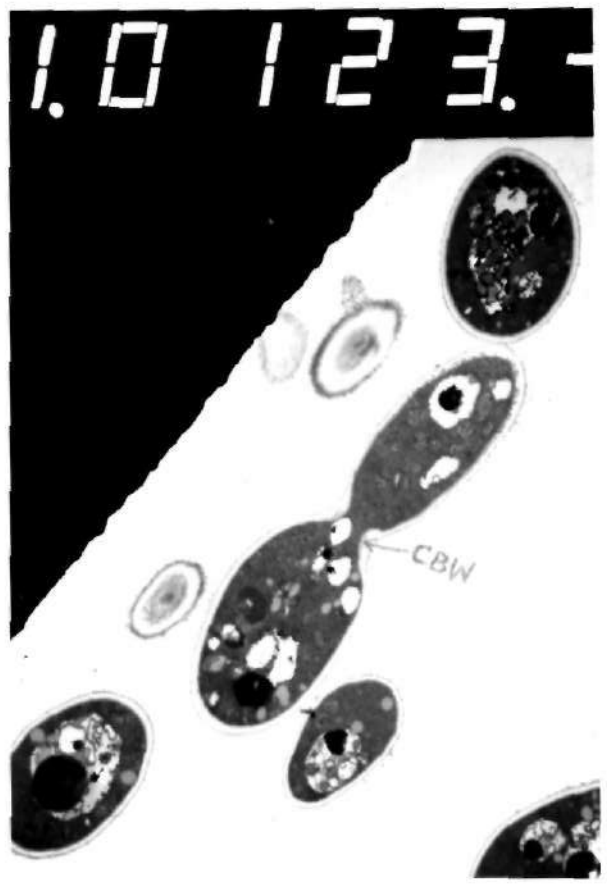


Figure 21 (cont.): 48 hr pure culture of Phaffia (123).
48 hr mixed culture stained with ruthenium red (115).



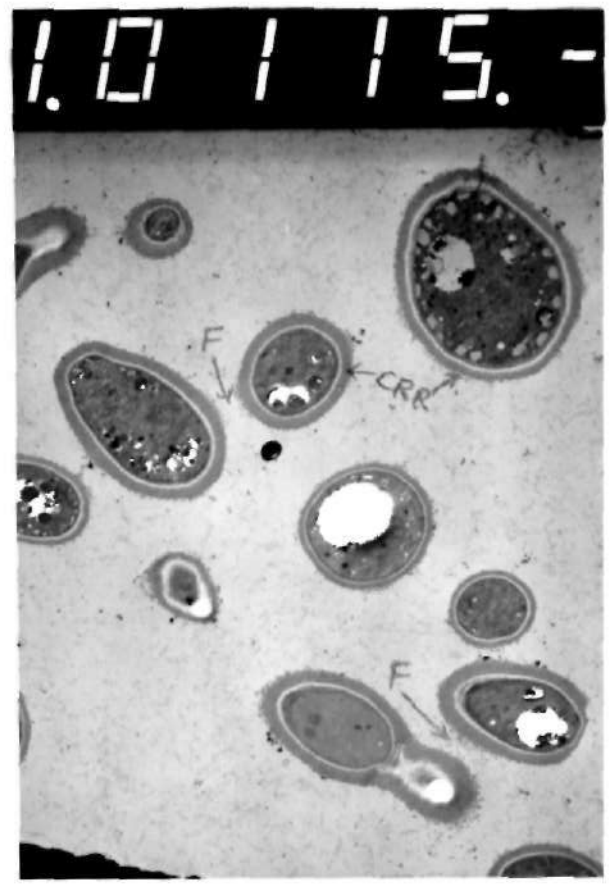


Figure 21 (cont.): 72 hr mixed culture.

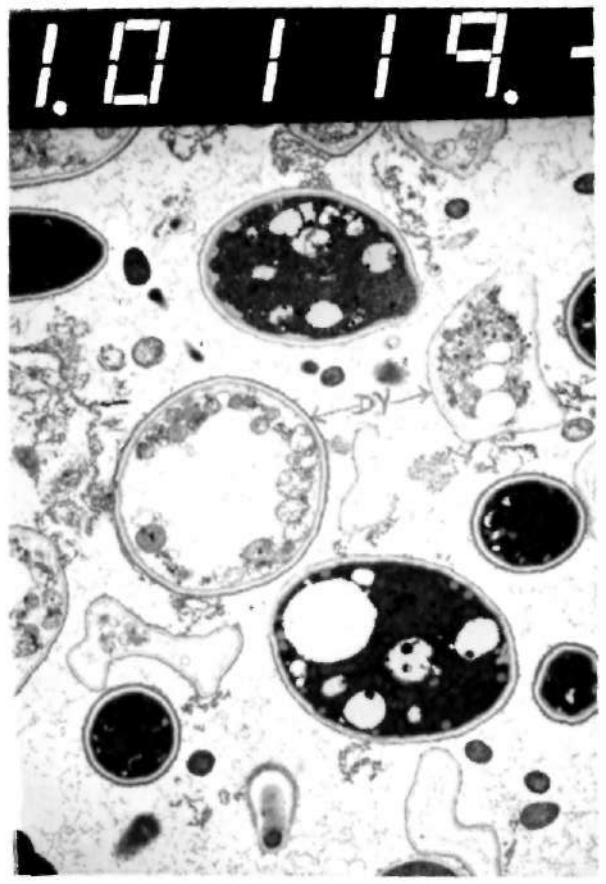




Figure 21 (cont.): 72 hr pure culture of Phaffia (control).

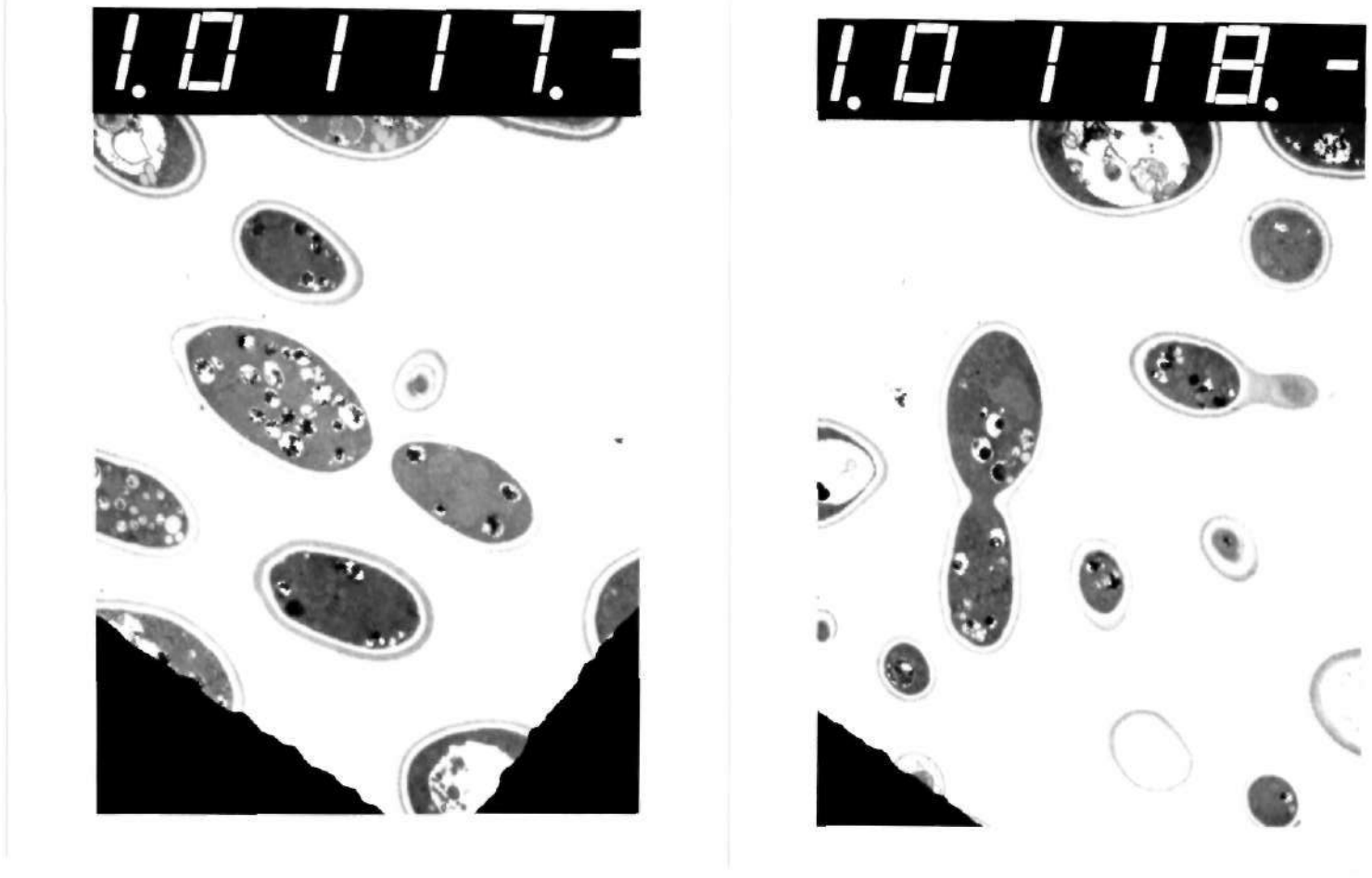
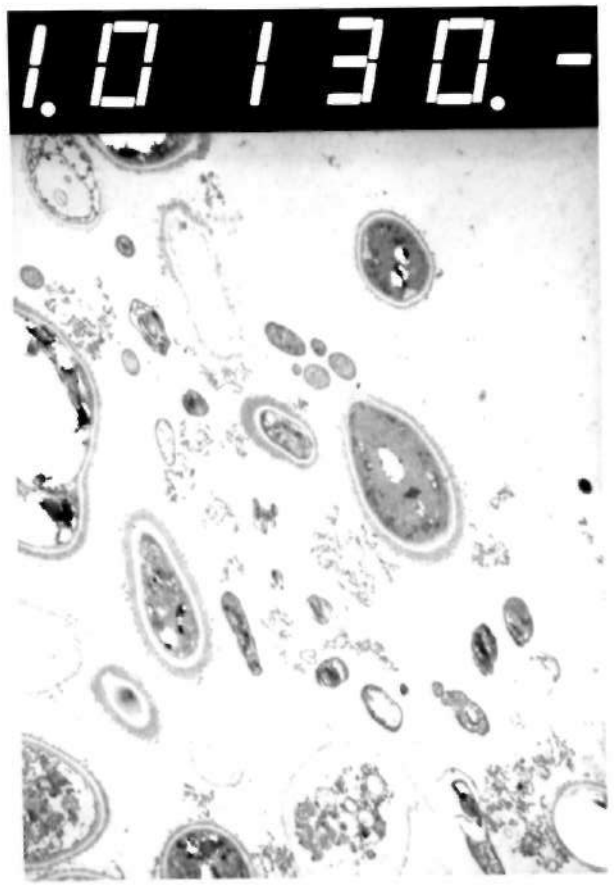


Figure 21 (cont.): 72 hr mixed culture stained with ruthenium red.



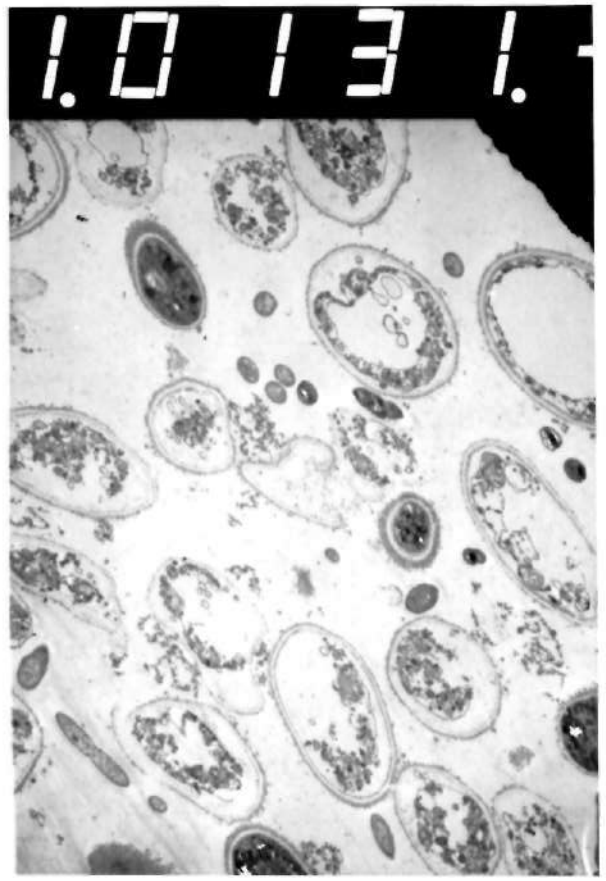
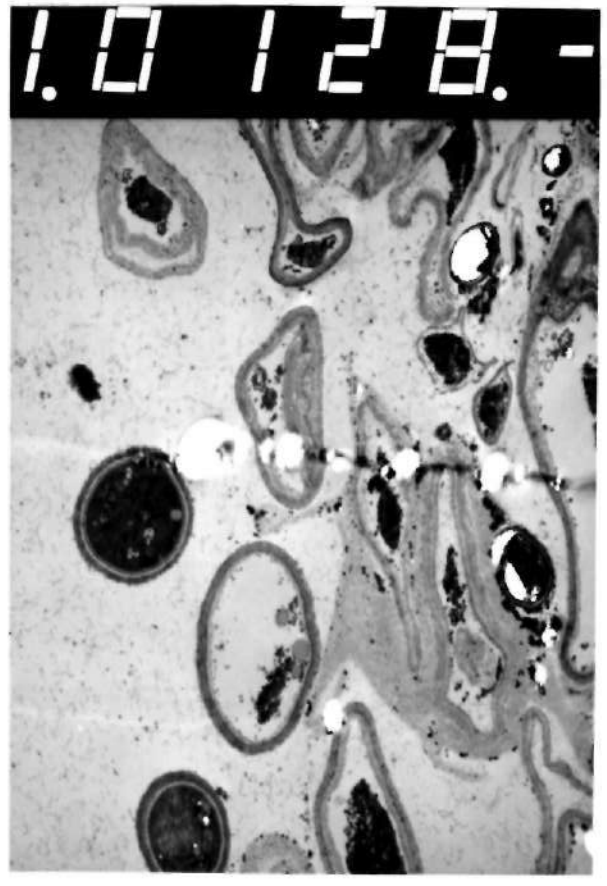


Figure 21 (cont.): 72 hr pure culture (control) stained with ruthenium red.





culture, can only be accounted for by modification of the yeast induced by presence of the bacillus.

Two other staining methods were used for monitoring the changes in the integrity of the cell walls of Phaffia rhodozyma in samples taken from a mixed culture in shake flasks. They are the Gram stain, as modified by Bianchi (1965), and the Diazonium Blue B (DBB) test procedure used by Tevayanond (1981). According to Bianchi (1965), intact yeast cells are Gram positive, whereas broken or disrupted ones are gram negative. By substituting methyl green for safranin normally used as counterstain in conventional Gram staining operations, and by using a cell wall stain, phosphomolybdic acid in addition, he achieved differential staining of intact cells (black), walls of broken cells (light green), and cytoplasm (light purple). When the technique was applied to samples of the mixed culture of Bacillus circulans and Phaffia rhodozyma, all yeast cells present in duplicate fields (light microscopy) in 48 hr samples were gram positive. By 72 hr, only 6% retained gram positivity, thereby showing that considerable wall modification or disruption had occurred at the time when most of the pigment in yeast pellets were extractable. Uptake of DBB (i.e. a positive reaction) has been shown to be typical of basidiomycetous yeasts (Hagler and Ahern, 1981). Recently, the cell wall was shown to be the site of the reaction (Tevayanond, 1981). In addition, factors such as natural or induced aging, pH, residual sugar in the medium, etc., were also shown to influence the reaction. Since Phaffia rhodozyma is a basidiomycetous yeast (Miller et al., 1976) samples taken from the mixed culture were subjected to DBB test in order to supplement previous observations on the relationship between cell wall alterations and

extractability of astaxanthin. Since the yeast is naturally pigmented, the reaction of each sample (cell pellet) was deduced by comparison with an untreated control. Samples remained negative up to 40 hr of the mixed culture, and positive (delayed) from 48 to 54 hr. From 58-72 hr, samples were intensely positive in the DBB test; this period correlated with 85-90% extractability of astaxanthin contained within cell pellets.

The results of the gram stain and the DBB test merely confirm those obtained by electron microscopy. Further since the intensity of the DBB reaction depends on culture age (Tevayanond, 1981), the test strongly indicates that a complex interrelationship may exist between yeast aging and extractability of astaxanthin in a mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma.

INFLUENCE OF AUTOLYSIS ON EXTRACTABILITY OF ASTAXANTHIN FROM PHAFFIA RHODOZYMA

Effect of culture age on susceptibility of the yeast to enzymatic cell wall modification.

Electron microscopic observations indicated that considerable disintegration of Phaffia rhodozyma had occurred during the period when astaxanthin was extractable. Apparently, lytic activity of Bacillus circulans WL-12 accelerated autolysis of the yeast. It is probable that digestion of the yeast cell walls and the associated autolytic phenomena, proceeded gradually in the mixed culture, and would depend on the concentration of the lytic enzyme at any time, as well as on susceptibility of the cell walls to enzymatic digestion. Since the latter variable would probably also depend on culture age, an experiment was

performed to test the effect of 'age' on susceptibility of Phaffia rhodozyma to the lytic enzyme complex of the bacillus.

In the experiment, culture fluid derived from a 75 hr old mixed culture after centrifugation at 12,000 rpm for 50 minutes, served as the source of lytic enzymes. The undiluted enzyme solution contained approximately 102-105 lytic units/ml. 1/10, 1/20 and 1/30 dilutions in 0.05M phosphate buffer pH 6.5 gave approximately 12.5, 7.6 and 3.4 lytic units/ml respectively. Phaffia rhodozyma cells were grown as pure cultures as described in Materials and Methods, and harvested at 24, 48 and 72 hr. After a preliminary washing with sterile distilled water, the cells were suspended in appropriate dilutions of the lytic enzyme to give concentrations of 6 mg/ml (cell dry weight basis), incubated on a roller drum at 30°C, and assayed for extractability of astaxanthin after 3 days. The results presented in Fig. 22, illustrate that digestibility of Phaffia rhodozyma cell walls depended on lytic enzyme concentration at any culture age tested. Cells harvested at 48 hr were apparently the most resistant to hydrolysis. However, with the exception of the saturated enzyme, 72 hr old cells were more susceptible to lytic activity, than those of 24 hr. These findings indicate that extractability of astaxanthin from the yeast, Phaffia rhodozyma, is at least dependent on culture age (and associated autolysis), and lytic enzyme concentration.

Effect of autolysis on extractability of astaxanthin from Phaffia rhodozyma.

It is well known that yeasts can be artificially induced to autolyse by incubation under suitable conditions of temperature, pH, buffer

Fig. 22. Influence of culture age on susceptibility of Phaffia rhodozyma to the lytic enzyme complex of Bacillus circulans WL-12.

The tested cells of Phaffia rhodozyma were grown in pure culture while the lytic enzyme complex was produced in a mixed culture of the yeast and B. circulans WL-12.

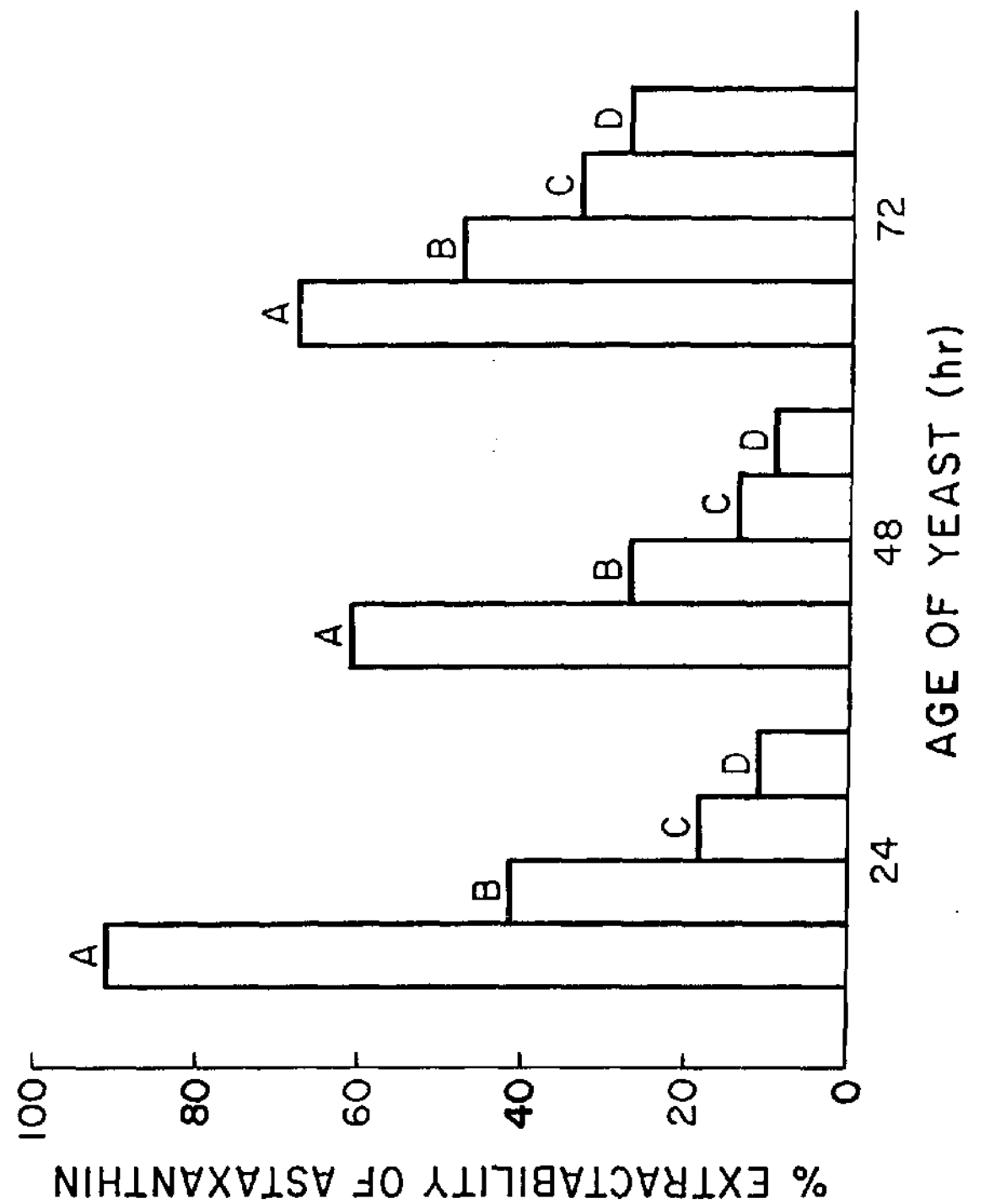
Key to letters: A = Undiluted enzyme ≅ 100 u/ml

B = 1/10 dilution of enzyme ≅ 12.5 u/ml

C = 1/20 dilution of enzyme ≅ 7.6 u/ml

D = 1/30 dilution of enzyme ≅ 3.4 u/ml

The diluent for the lytic enzyme was 0.05M phosphate buffer pH 6.5.


% EXTRACTABILITY OF ASTAXANTHIN
0
20
40
60
80
100
A
B
C
D
24
A
B
C
D
48
A
B
C
D
72
AGE OF YEAST (hr)

solutions, organic solvents and other chemicals. Since autolysis of Phaffia rhodozyma could become a useful tool for liberation of its carotenoids, efforts were made to stimulate it. Cells of the yeast were harvested after 48 hr of culture, washed once in sterile distilled water, and suspended in various solutions at concentrations of about 6 mg/ml in large test tubes. Incubation was carried out at 37°C (without any agitation of the tubes). After three days, contents of each tube was assayed for extractability of astaxanthin and visually scored for retention of characteristic salmon-pink color of Phaffia rhodozyma cells. Table 9 shows that very little autolysis occurred when the yeast was incubated in its medium of growth (which contained 0.1M phosphate buffer) with or without pH adjustment. It also seemed that the characteristic color of the yeast had deteriorated somehow. Similarly, Tris buffer, pH 8, with or without dithiothreitol (DTT), was unsuitable, but pigmentation of the yeast appeared to be protected. When distilled water containing 2% toluene was the autolysing system, considerable extractability of astaxanthin was achieved. Unfortunately, the organic solvent dissolved some of the carotenoids during the incubation period; this probably contributed to the apparent decrease in the pink color of yeast pellet. Use of toluene would probably be undesirable in a commercial autolysis of Phaffia rhodozyma meant for application to feeds. Substitution of 15% NaCl for toluene completely inhibited the autolytic system, probably because of the acidity of the salt, and also caused some loss of cell color. Distilled water (as is pH 6.6), and 0.02M sodium citrate buffer pH 7.0, promoted considerable autolysis of Phaffia rhodozyma and preservation of its pink color. Addition of DTT to citrate buffer enhanced its effectiveness. In

Table 9. Extractability of astaxanthin from autolysed Phaffia rhodozyma cells.

Autolysing medium	Final pH	Yield of total astaxanthin µg/ml in cell suspension	Yield of extractable astaxanthin µg/ml	Extractability %	Color of cell pellet
Distilled water	7.20	1.74	1.36	78.2	~~++~~
Distilled water + toluene (2% v/v)	6.53	1.46*	1.02	69.9	–
Distilled water + 15% NaCl	5.70	1.74	0.00	0	–
Medium of yeast growth (as is), pH 6.43	6.53	1.20	0.32	26.7	–
Medium of yeast growth, pH adjusted to 7.20	7.12	1.20	0.14	11.7	–
0.1M Tris buffer pH 8.0	8.11	1.60	0.47	29.4	+
0.1M Tris buffer pH 8.0 + .01M dithiothreitol	8.12	1.78	0.40	22.5	+
0.02M citrate buffer pH 7.0	8.40	1.69	1.22	72.2	~~++~~
0.02M citrate buffer pH 7.0 + .01M dithiothreitol	6.42	1.60	1.36	85.0	~~++~~

Table 9. (cont.)

++ Very good retention of pink color

\+ Good

– Appreciable loss of color

* Some pigment dissolved in the autolysing medium

general, the ability of Phaffia rhodozyma to autolyse under these conditions, is an asset from a commercial point of view. DTT would most probably be omitted in industry because of its pungent odor which might make the yeast-containing feed unacceptable to animals.

A comparison of the effects of three different methods of treatment on extractability of astaxanthin from strains of Phaffia rhodozyma.

Results of previous experiments indicated that Phaffia rhodozyma could be treated in at least three different ways to facilitate recovery of its carotenoids. The methods include: extracellular lytic enzyme digestion, autolysis in water and autolysis in 0.02M citrate buffer pH 7.0 + DTT. An attempt was made to compare the effectiveness of the treatments on different strains of the yeast. Table 10 shows that the six strains tested varied considerably in their responses to the treatments. Only strain 67-484 was remarkably susceptible to all the conditions of exposure. Strain 67-385 appeared to be very susceptible to autolysis, but not to extracellular lytic activity. For strain 67-210, lytic enzyme activity and autolysis in distilled water produced comparably good results, and were more satisfactory than autolysis in citrate buffer + DTT. All the conditions of treatment, in general, did not favor extractability of astaxanthin from 68-653C, 67-202 and 67-203, although the last one was fairly autolysed in distilled water.

It would appear from these results, that 67-484 could be the most preferred source of astaxanthin because of its susceptibility to all the methods employed above. It also seemed, in general, that the strains reacted in approximately the same order to each method of treatment.

Table 10. Effects of three methods of treatment on extractability of astaxanthin from six strains of Phaffia rhodozyma.

Strain	Methods tested								
	Digestion* by lytic enzyme (approx. 118 u/ml)			Autolysis** in 0.02M citrate buffer pH 7.0 + 0.01M DTT			Autolysis** in distilled water		
67-202	(1.13)	2.7	(6)	(1.41)	6.76	(5)	(1.46)	13.01	(5)
67-203	(1.44)	15.3	(4)	(1.60)	5.94	(6)	(1.97)	45.69	(4)
67-210	(1.17)	64.1	(2)	(1.36)	41.90	(3)	(1.17)	64.10	(3)
67-385	(1.11)	35.1	(3)	(1.13)	75.20	(2)	(1.32)	78.03	(2)
67-484	(1.09)	71.6	(1)	(1.39)	87.80	(1)	(1.41)	100.00	(1)
68-653C	(1.25)	7.2	(5)	(0.80)	17.50	(4)	(0.80)	11.88	(6)

* 1 hr at 30°C

** 26 hr at 37°C

All cultures tested were grown for 48 hr at 20°C.

Unbracketed data in each method of treatment refer to % extractability of astaxanthin.

Bracketed data on the left indicate total concentrations of astaxanthin (µg/ml) in the suspension, used for calculating % extractability, while those on the right indicate the order in which the strains responded to the treatment.

USE OF ALFALFA RESIDUAL JUICE FOR CULTIVATION OF PHAFFIA RHODOZYMA

ARJ is a relatively new waste product derived from leaf protein extraction from alfalfa. Like many food-processing waste products, its use for biomass production would help alleviate obvious disposal problems. It would be a cheap substrate for cultivation of Phaffia rhodozyma if it could support growth of the yeast and astaxanthin formation.

Substrate pretreatment.

In order to improve alfalfa residual juice (ARJ) for possible growth and astaxanthin formation by Phaffia rhodozyma, it must be diluted to a suitable consistency and sugar concentration. It has been estimated that the juice contained approximately 22% sugar (Kohler and Fremery, 1975); thus, a ten-fold dilution (to give approximately 2% sugar), would probably be adequate. In addition, ARJ must be clarified to remove plant debris and other undesirable solids.

Use of phosphate buffer pH 5.6 as diluent (0.02M-0.1M, final molarity), followed by suction filtration through Whatman no. 2 filter in presence of 10% diatomaceous earth (DE), were not satisfactory. Phosphate appeared to cause formation of a red-brown precipitate when the mediumwas pasteurized (at 145°C for 12 min) or autoclaved (at 121°C for 15 min). In addition, it (phosphate) seemed to enhance adsorption of some chromogenic components of ARJ on to Phaffia rhodozyma cells; resulting green pigment on cell 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 hr of growth of Phaffia rhodozyma; this was probably caused by presence of some alkali in DE.

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

Growth of Phaffia rhodozyma in alfalfa residual juice (ARJ).

The growth curve of Phaffia rhodozyma was determined in ARJ diluted to contain 2.0% sugar in 0.02M phthalate buffer pH 5.6. As a control, the yeast was also grown in a semi-synthetic medium (SSM) which contained 2.0% glucose and phthalate-buffered (0.02M, pH 5.6) $MgSO_4$, 0.02%, KH_2PO_4, yeast extract, peptone, each 0.5%. The curves are shown in Fig. 23. The two media supported nearly equivalent rates of growth. Their maximum yields of cell mass were also comparable, though they were attained at 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 were 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 hr (Fig. 23). Unfortunately, ARJ appeared to inhibit astaxanthin formation; cells produced throughout the 84 hr fermentation

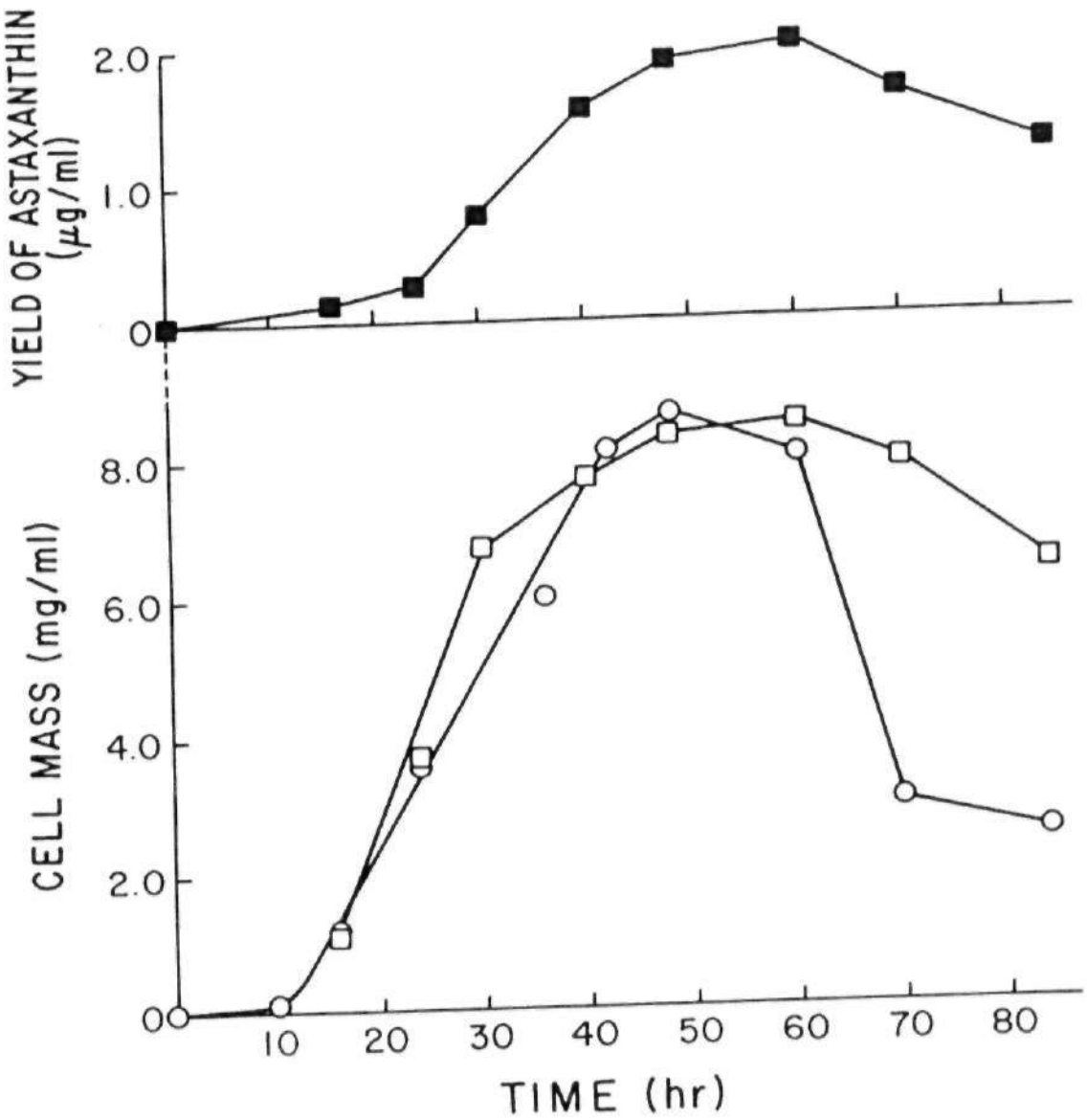
Fig. 23. Growth of Phaffia rhodozyma in alfalfa residual juice and in a semi-synthetic medium (control).

Symbols: o alfalfa residual juice (ARJ)

□ control

■ astaxanthin production in control

The semi-synthetic medium contained (in 0.02M phthalate buffer pH 5.6) $MgSO_4$ 0.02%, KH_2PO_4, yeast extract, peptone each 0.5%, glucose 2%.


YIELD OF ASTAXANTHIN (μg/ml)
2.0
1.0
0
CELL MASS (mg/ml)
8.0
6.0
4.0
2.0
0
10
20
30
40
50
60
70
80
TIME (hr)

period lacked the characteristic salmon-pink color of Phaffia rhodozyma. Hence they were not assayed for astaxanthin.

Effects of supplementary nutrients on growth and pigmentation of Phaffia rhodozyma in alfalfa residual juice (ARJ).

The last experiment had shown that pigmentation of Phaffia rhodozyma was inhibited by ARJ. The inhibition was thought to be due to deficient quantities of some essential nutrients, or to their limited availability for absorption by the yeast. Therefore, an attempt was made to enrich the ARJ. Nutrients, prepared as sterile stock solutions were added to ARJ separately and in a combination, to give final concentrations equivalent of those which previously supported astaxanthin formation in SSM. In addition, a prolonged fermentation period was adopted to determine whether astaxanthin formation might possibly develop. Table 11 shows that the objective was not realized. KH_2PO_4, alone or in combination with other supplements, appeared to enhance yield of cell mass, but pellets were dark-green in color. This phenomenon was observed earlier in this study when phosphate buffer was used as diluent for ARJ. Of the other supplements, glucose seemed to improve cell mass recovery at 105 hr; the cell pellet also showed some yellow-brown color in visual comparison with those of other supplements. Therefore, further information was desired on the ability of glucose to promote growth and pigmentation of Phaffia rhodozyma in ARJ. For the experiment, the yeast was grown in ARJ supplemented with various levels of glucose ranging from 0.5-3.0%. Samples taken at intervals were assayed for cell mass and visually inspected for pink coloration as a prelude for possible astaxanthin analysis. Fig. 24 shows that

Table 11. Effect of various supplements on the ability of ARJ to support growth and astaxanthin formation by *P. rhodoyzma*.

Additive*	Biomass (mg/ml) Time (hr) 40	70	105	Astaxanthin concentration µg/ml	Other observations on washed cell pellet
None	7.5	3.2	2.8	-**	
glucose (0.5%)	7.2	4.2	6.1	±	Some yellow-brown pigmentation apparent.
KH_2PO_4 (0.5%)	9.7	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***	7.2	7.8	6.9	1.30	

Table 11. (cont.)

* 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 the other additives.

** Cell pellet was not sufficiently pigmented to warrant analyses for astaxanthin at 105 hr.

*** A semi-defined medium similar to that of Fig. 23.

growth rate and maximum cell mass rose as the level of supplementary glucose increased to about 2%. At 1.5% and 2% supplementary glucose, yields of cell mass were maximal at 60 hr and roughly equal. Considerable autolysis occurred after attainment of maximum growth, but was apparently minimized by the glucose supplementation. Unfortunately, none of the supplementary levels of glucose caused the pink color to appear in yeast mass, at any stage of growth; astaxanthin synthesis was still inapparent and no analyses were made. From the above two series of experiments, it is concluded that the inhibitory effect of alfalfa residual juice on pigmentation of Phaffia rhodozyma is not due to deficiency in nutrients. It is obvious, however, that the juice must be supplemented with glucose or possibly other sugar, in order to enhance maximum yield of cells and minimize autolysis. These precautions would be very important if Phaffia rhodozyma is intended for use as a source of single cell protein.

Effect of ARJ on strains of Phaffia rhodozyma.

Up till now, experiments with the ARJ have tested its effects on the type strain 67-210 of Phaffia rhodozyma, and have shown that the juice is detrimental to its carotenoid formation. Since primary interest in the yeast is in its content of carotenoids (primarily astaxanthin), it was necessary to determine whether any of the other strains of the yeast could utilize ARJ for synthesis of the pigment. Accordingly, five strains, in addition to 67-210, were compared. In addition to cultivation in ARJ, each strain was grown in the usual SSM to serve as control. As expected, good yields of astaxanthin and cell mass were achieved by all the tested strains in their control cultures (Table 12).

Fig. 24. 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.

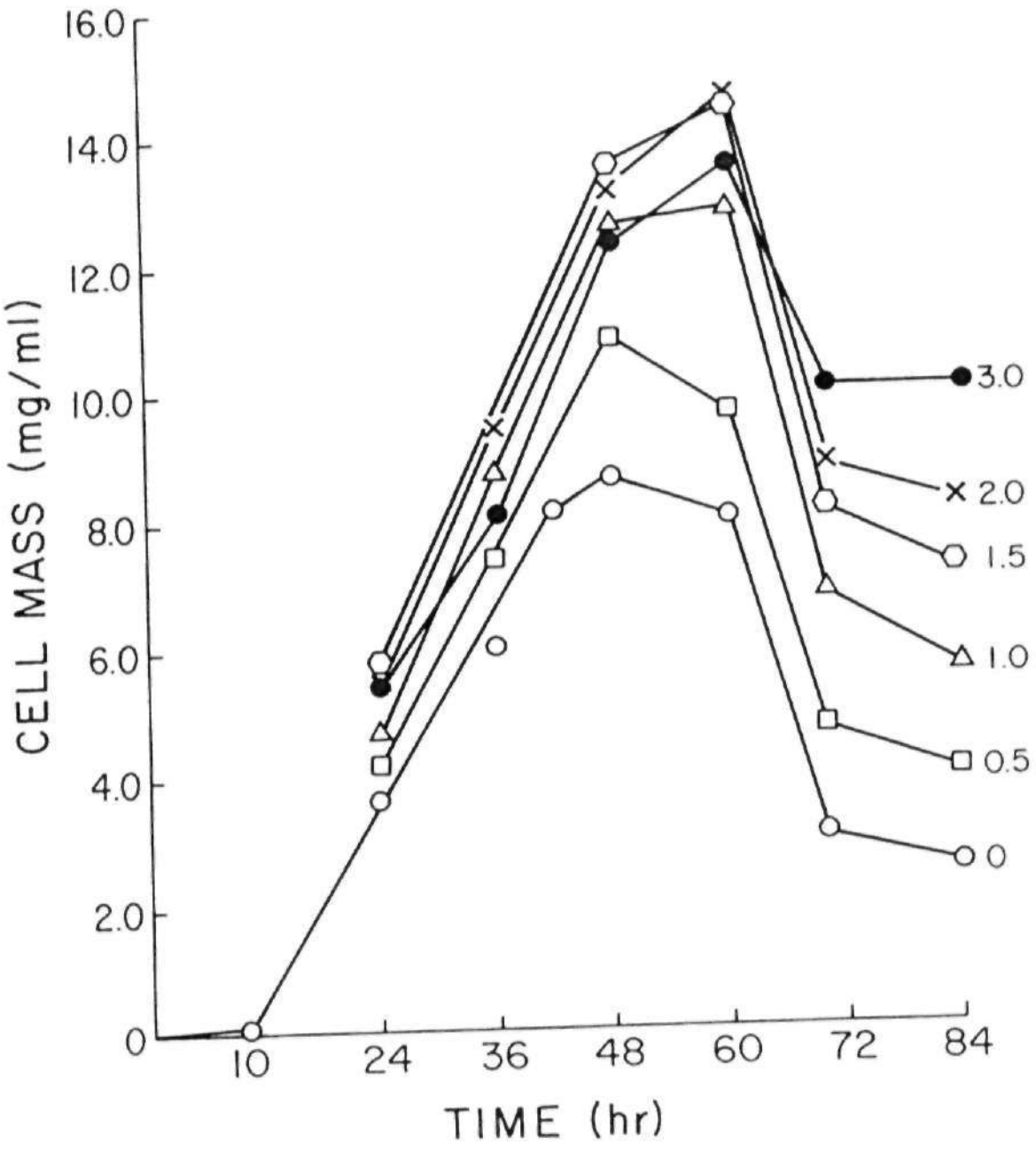

16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
CELL MASS (mg/ml)
10
24
36
48
60
72
84
TIME (hr)
3.0
2.0
1.5
1.0
0.5
0

Table 12. Growth of some strains of P. rhodoyzma in alfalfa residual juice (ARJ).

Strain	Growth conditions	Biomass (dry) mg/ml	Astaxanthin µg/ml	Astaxanthin µg/g dry weight
67-202	Control*	10.03	2.0	199.30
	Expt.**	5.88	-***	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 hr of culture. Data are averages of duplicates.

* In the controls, the strains were grown in pthalate (.02M) buffered semi-synthetic medium (SSM) pH 5.6 containing .02%$MgSO_4$, and KH_2PO_4, yeast extract, peptone, glucose, each 0.5%.

** In experimental cultures, medium was pthalate buffered alfalfa residual juice diluted ten-fold. No supplements were used.

Table 12. (cont.)

*** Cell pellets characteristically lacked pink color; hence no analyses for astaxanthin were made.

NA = not applicable.

In contrast, none of them produced astaxanthin in ARJ. Their low yields of cell mass in ARJ relative to the controls, were probably due to autolysis which has been shown in previous experiments (with the type strain only) to occur relatively rapidly in ARJ after about 60 hr of growth.

Effect of various concentrations of alfalfa residual juice (ARJ) on astaxanthin formation by Phaffia rhodozyma.

In the last series of experiments, the inability of Phaffia rhodozyma to synthesize its potentially useful, salmon-pink carotenoid, astaxanthin, was demonstrated. Apparently, one or more components of the juice inhibited biosynthesis of the pigment.

In order to examine the inhibitory effects of the juice in greater detail, various concentrations were incorporated into different batches of a standard medium known to promote good yields of astaxanthin. 80 ml (total) volumes of media were used in 500 ml baffled side-arm flasks. The standard medium (SM) was equivalent to the semi-defined medium used in earlier parts of this study and contained (as final concentrations) in 0.02M phthalate buffer pH 5.6, 0.02% $MgSO_4 \cdot 7H_2O$, KH_2PO_4, yeast extract, and peptone, each 0.5% and glucose, 2%. In control flasks, ARJ was omitted from the SM which was made up by dissolving all the components (except glucose) in 40 ml of buffer (0.04M) and then diluting to 80 ml with an equivalent volume of a sterile stock solution of glucose. In experimental flasks, the glucose was dissolved and sterilized in successively lower volumes of water, and increasing volumes of sterile juice (diluted 5-fold in water) were used to make up to 40 ml volume. In general, media in the experimental flasks were equivalent in

composition to those of the controls, and contained in addition, varying concentrations of ARJ ranging from 0-12.5 (v/v). All flasks were inoculated with Phaffia rhodozyma prepared as described in Materials and Methods, and incubated at 20°C with shaking at 200 rpm. Contents of the flasks were assayed for yields of cell mass and total astaxanthin after 48 hr.

As expected, results (Fig. 25) showed greater yield of cell mass in experimental cultures than in 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%, then less conspicuously at higher levels of the juice tested. Above approximately 3.7%, quantities of pigment detected as astaxanthin were surprisingly large and unexpected from visual estimation of colors of cell pellets, or of pigment dissolved in acetone (greenish-yellow), and from previous experience in this study. It was postulated that pigments detected at the higher levels of ARJ were probably due to adsorption of some chromogenic parts of ARJ on the surfaces of Phaffia cells; the chromogens were apparently not removed by washings with distilled water. In order to test the hypothesis, pellets were derived again by centrifugations of equivalent volumes of samples taken from all the flasks (containing various levels of ARJ). Each was washed with water until water-soluble chromogenic constituents of ARJ ceased to appear in supernatants. It was then treated directly with acetone and dissolved pigment estimated as astaxanthin. Results (Fig. 25) showed that dissolved pigments were greenish-yellow and increased in quantity with concentrations of juice in medium. The results suggested

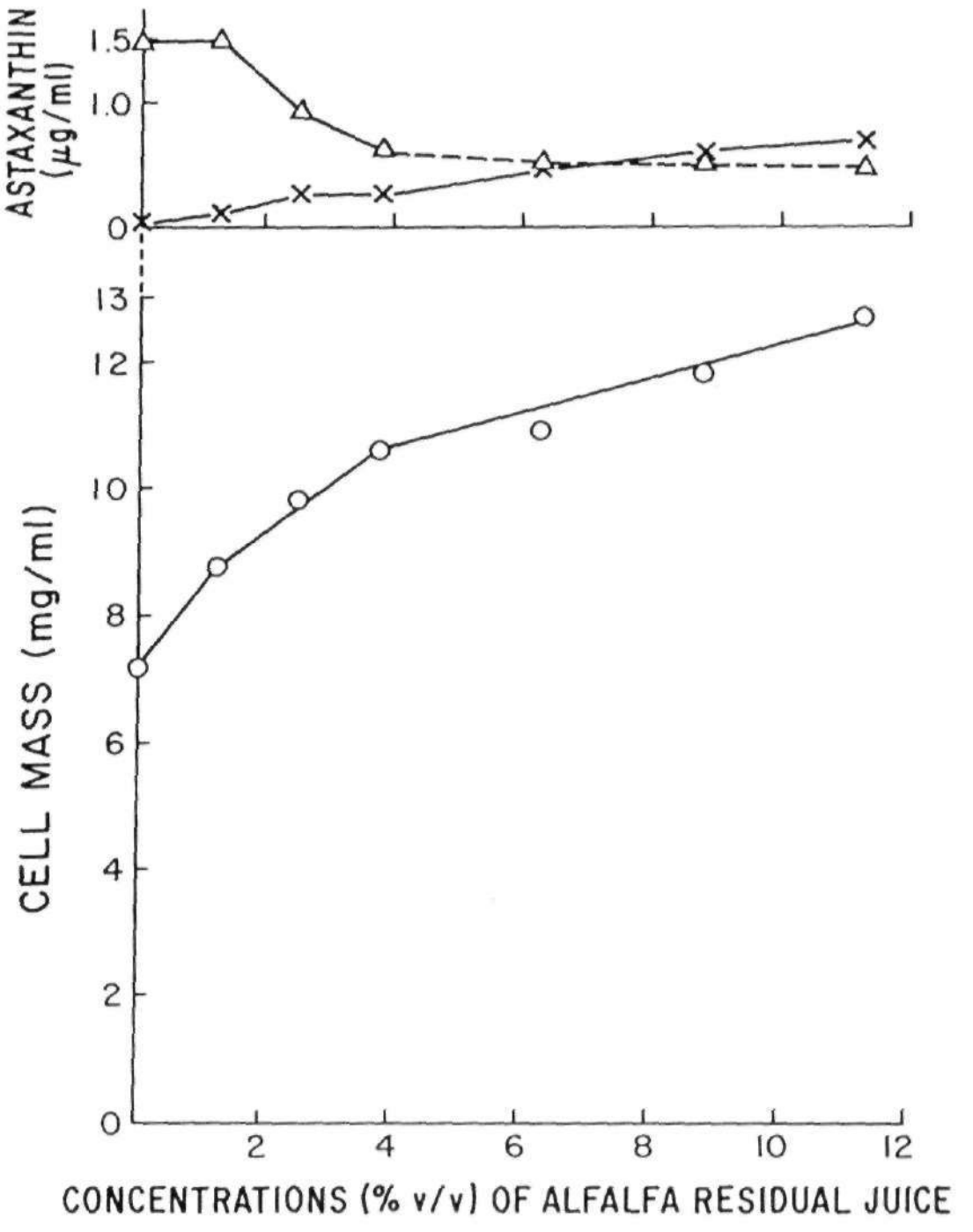
Fig 25. Effect of levels of alfalfa residual juice on growth and astaxanthin production by Phaffia rhodozyma.

Various levels of the juice were added to the semi-synthetic medium similar to that of Fig. 23.

Symbols: Δ-Δ Astaxanthin; dotted lines represent pigment possibly adsorbed on yeast cells and assayed as astaxanthin.

x-x Pigment adsorbed on yeast cells (assayed as astaxanthin).

o-o Cell mass (mg/ml).


ASTAXANTHIN (μg/ml)
1.5
1.0
0
CELL MASS (mg/ml)
13
12
10
8
6
4
2
0
2
4
6
8
10
12
CONCENTRATIONS (% v/v) OF ALFALFA RESIDUAL JUICE

that above approximately 3.7% (v/v) concentration of ARJ, there was total inhibition of astaxanthin formation. They also showed that pigments from the juice could adsorb on Phaffia rhodozyma cells and show as artifacts in assays for astaxanthin.

Two other experiments were used to demonstrate the ability of the yeast cell surface to adsorb pigments from ARJ. In the first one, pellets were again obtained from flasks containing 0%, 2.50% and 11.25% (v/v) ARJ and washed properly as described previously. They were treated with acetone with and without prior mild acid treatments in a manner used in determinations of 'total' and 'extractable' astaxanthin and described in Materials and Methods. Electronic absorption spectra of pigments dissolved in acetone, were then determined. Fig. 26 shows that, as expected, no pigment was extractable by direct 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. Though some slight differences could be observed (Fig. 26) in the breadths of the absorption spectra of samples derived from each level of juice, they were probably caused by the acid treatments given to some of the samples. Pigments from the two levels of ARJ were generally identical and their absorption spectra peaked at approximately 390 nm. The higher peak observed at the higher level of ARJ indicated that the amount of pigment adsorbed on yeast cells was related to the actual concentration in the medium. In general, this

Fig. 26. Absorption spectra (in acetone) of pigments extracted from washed cells of Phaffia rhodozyma grown in basal medium with or without alfalfa residual juice (ARJ).

The basal medium was equivalent to the semi-synthetic medium used in Figs. 23 and 25 and contained (in 0.02M phthalate buffer pH 5.6) $MgSO_4$ 0.02%, KH_2PO_4, yeast extract, peptone each 0.5%, and glucose 2%.

Key to the figure:

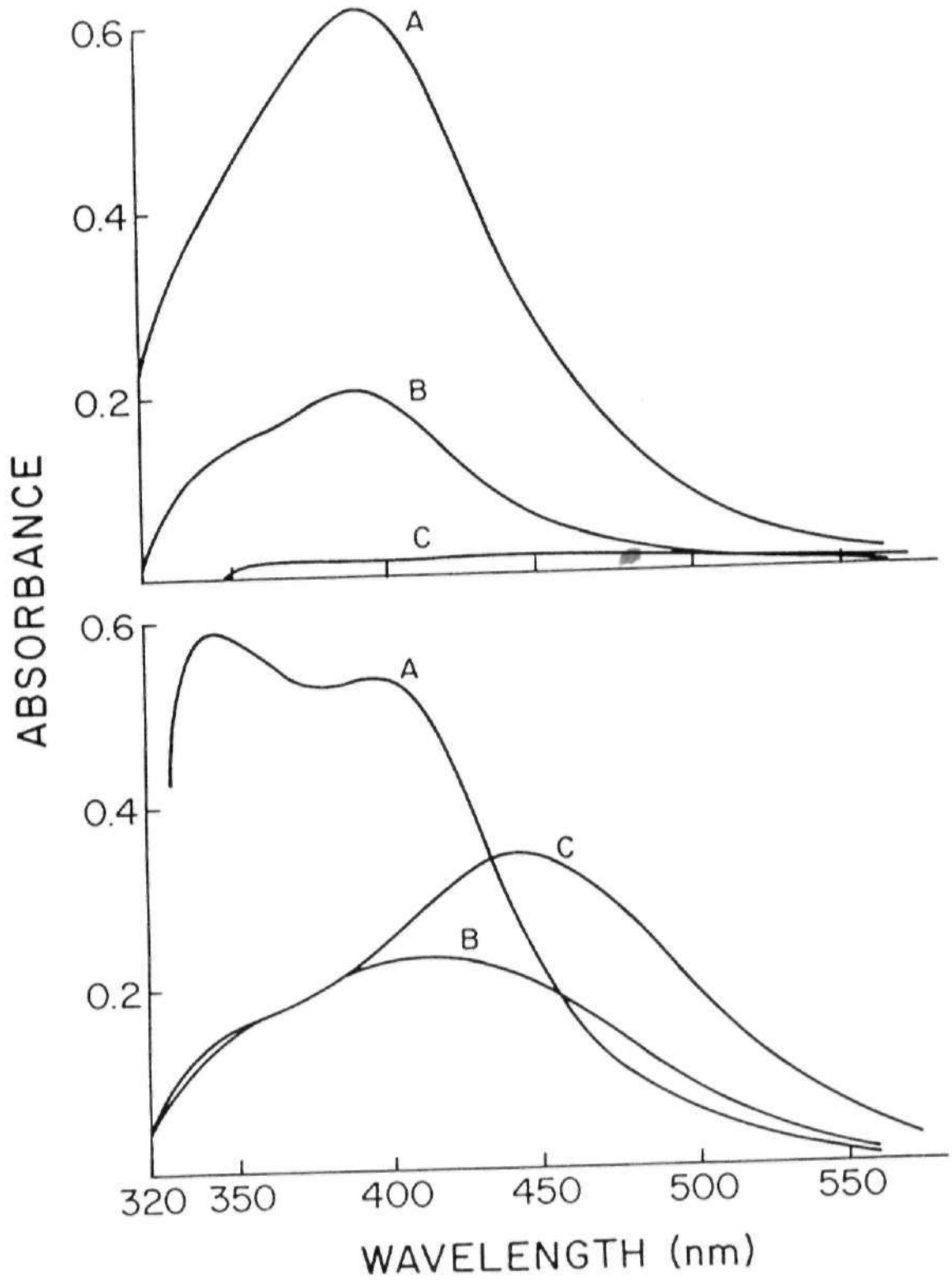
Top: washed cells not treated with HCl.

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).


0.6
0.4
0.2
A
B
C
ABSORBANCE
0.6
0.4
0.2
A
C
B
320
350
400
450
500
550
WAVELENGTH (nm)

experiment showed that high levels of ARJ completely inhibit astaxanthin synthesis and could be adsorbed on the yeast cell surface.

In the second experiment, a known non-pigmented yeast, Saccharomyces cerevisiae 71-141 (Guinness Stout yeast) was grown in media containing different concentrations of ARJ. Preparation of inocula was similar to that described previously for Phaffia rhodozyma, but incubation was at 26°C with shaking at 150 rpm. After 48 hr, the yeast cells grown in presence of ARJ were darker in color, but pigment appeared to be completely removed after three washings with water. Acetone treatment of washed cells, however, was able to detect rather low levels of adsorbed pigment (Table 13). This experiment again illustrates that components of ARJ can adsorb on to yeast cells grown in their presence. It would appear that the cell surface of Phaffia rhodozyma has greater affinity for the pigments than that of the strain of Saccharomyces cerevisiae used here; this would probably be explained by the presence of pronounced capsular material in Phaffia rhodozyma.

In the foregoing experiments, it was obvious that addition of various levels of ARJ to the standard medium increased total concentrations of sugar; final sugar concentrations varied from 2.25% to 4% (calculated amount of sugar in added quantity of juice was based on sugar content of ARJ as determined by Kohler and Fremery, 1975). It was essential to determine whether increasing sugar levels contributed to the inhibitory effect of ARJ on astaxanthin production by Phaffia rhodozyma. To accomplish this objective, an experiment was designed to maintain a constant level of sugar in presence of varying concentrations of the juice. 100 ml standard medium was prepared as a control, by combining 50 ml of a sterile phthalate-buffered (0.04M) stock solution of nutrients

Table 13. Adsorption of pigments derived from ARJ on S. cerevisiae 71-141.

Level of ARJ in medium (% v/v)	Absorbance of pigment in acetone: λ = 478 nm	Biomass mg/ml
0	0	9.0
2.50	.004	9.3
5.00	.016	10.5
7.50	.026	10.4

Assays were carried out after 48 hr of growth.

(except glucose) with 50 ml of a solution containing 2 g glucose in distilled water. Final concentrations of the components of the medium were $MgSO_4 \cdot 7H_2O$, 0.02%; KH_2PO_4, yeast extract and peptone, each 0.5%; and glucose 2%. In experimental flasks, the standard medium was similarly prepared, but volumes of the medium were progressively lowered, as were amounts of glucose. Increasing volumes of ten-fold dilution of ARJ (in 0.02M phthalate buffer pH 5.6), were then added in amounts calculated to make up 2% sugar (in 100 ml total volumes of media). Thus prepared, each medium contained its own level of ARJ plus 2% sugar, which was partly derived from the ARJ and partly from the standard medium. All media were contained in 500 ml baffled side-arm flasks. Inocula of Phaffia rhodozyma were prepared and other conditions of culture employed as described in Materials and Methods. Assays were carried out after 48 hr. Results of this experiment (not shown) were identical to those of Fig. 25. Biomass increased with increasing levels of ARJ. In contrast, astaxanthin formation was virtually absent above approximately 2.5% v/v of the juice. Thus it would appear that ARJ contains some definite inhibitors of astaxanthin formation. High sugar levels in standard media supplemented with juice could not be responsible for the inhibition.

EFFECTS OF SAPONINS ON GROWTH AND ASTAXANTHIN FORMATION BY PHAFFIA RHODOZYMA

An abundant evidence has been presented on the possible presence in alfalfa residual juice (ARJ), of inhibitor(s) of astaxanthin formation. Though the nature of the inhibitor(s) is as yet unknown, some

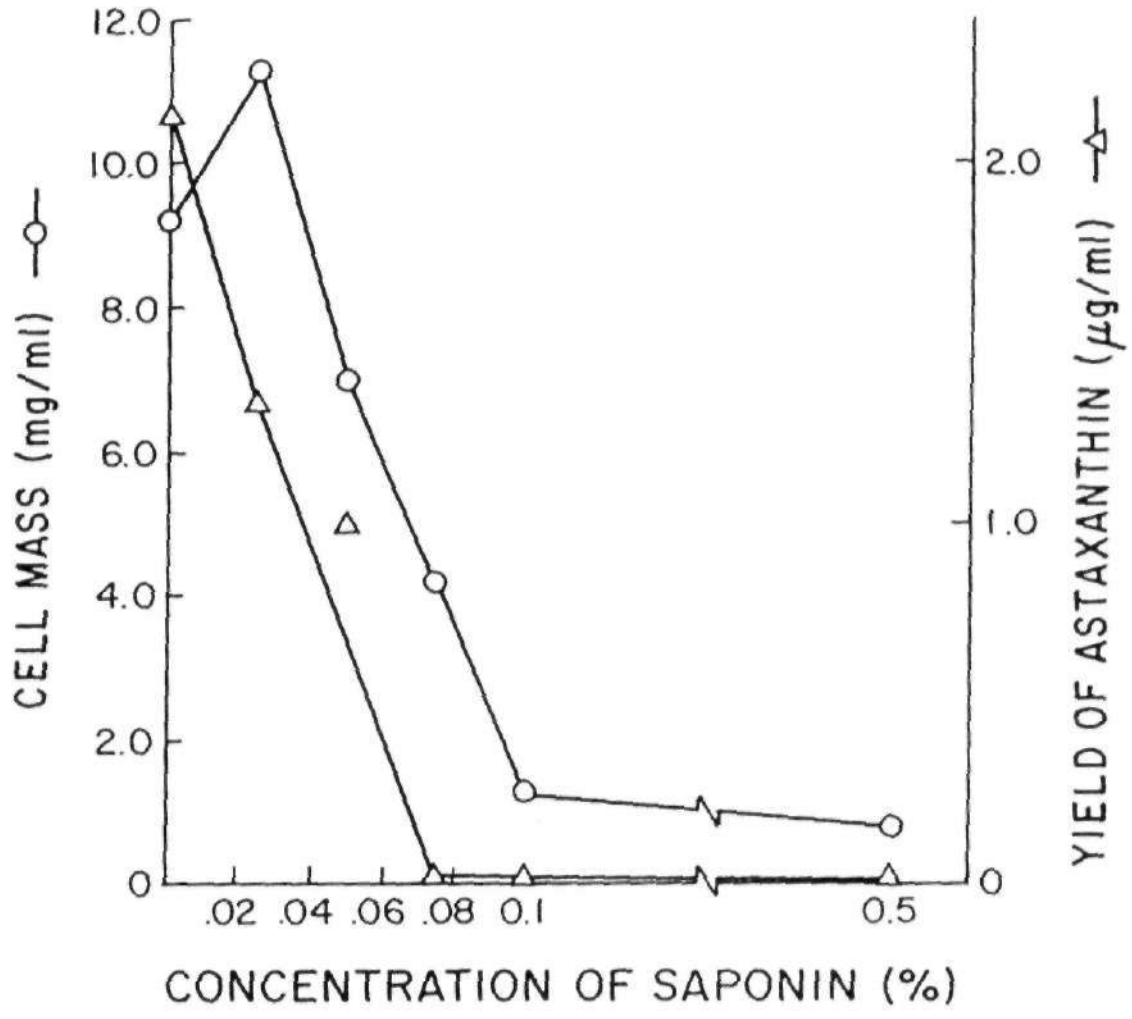
biologically-active substances such as saponins, might be involved. These compounds (the saponins), are known to be present in alfalfa and other legumes (Walter et al., 1954; Birk et al., 1963), and have been shown to accumulate in considerable amounts in alfalfa solubles (alfalfa residual juice (ARJ)) under some conditions of leaf protein extraction from alfalfa (Livingston et al., 1979). Therefore, an attempt was made to examine their effects on Phaffia rhodozyma. For the experiment, the yeast was grown in the usual standard medium (known to promote high yields of cell mass and astaxanthin) containing various concentrations of saponin (Sigma Chemical Co., St. Louis, MO, U.S.A.). 40 ml of the sterile medium (prepared so as to contain 0.02% $MgSO_4 \cdot 7H_2O$, KH_2PO_4, yeast extract, peptone and glucose, each 0.5% in 0.02M phthalate buffer pH 5.6 when diluted to 50 ml), was contained in 500 ml baffled side-arm flasks. A 2.5% v/v aqueous solution of saponin was autoclaved for 2 minutes and appropriate amounts added to the flasks containing the sterile basal medium; final concentrations of saponins in the medium ranged from 0.025% to 0.5%. The volume of medium in each flask was made up to 50 ml (where appropriate) with sterile distilled water. The saponins were omitted in control flasks. Phaffia rhodozyma was grown and prepared for inoculation into flasks as described in Materials and Methods. Cell growth and astaxanthin formation were measured after 48 hr growth of the yeast at 20°C.

Results presented in Fig. 27 show that yield of cell mass was slightly improved by 0.025% saponin. Higher concentrations of the chemical, however, inhibited growth of the yeast tremendously. At 0.1% concentration, yield of cell mass was less than 15% of the control. However, even 0.5% saponin did not completely inhibit yeast growth. In

Fig. 27. Effect of saponin on growth and astaxanthin formation by Phaffia rhodozyma.

Stock solutions of saponin were added (to give final concentrations indicated in the figure) to a standard medium containing (in 0.02M phthalate buffer pH 5.6) 0.02% $MgSO_4 \cdot 7H_2O$, KH_2PO_4, yeast extract, peptone and glucose, each 0.5%.

Cultivation of the yeast was for a period of 48 hr.


12.0
10.0
8.0
6.0
4.0
2.0
0
CELL MASS (mg/ml)
2.0
1.0
0
YIELD OF ASTAXANTHIN (μg/ml)
.02 .04 .06 .08 0.1
0.5
CONCENTRATION OF SAPONIN (%)

contrast, astaxanthin formation was more adversely affected by the saponins and total inhibition was observed at 0.075% concentration of the inhibitor. These results indicate that saponin content of ARJ could contribute to its inhibition of pigmentation in Phaffia rhodozyma. It does not seem to adequately explain the inhibition of growth observed in this study. However, the stimulatory effect of low saponin concentration (0.025%) on growth might suggest that ARJ probably contains only low concentrations of the inhibitors. Alternatively, it may be that the concentration or potency of the saponins present in ARJ could be neutralized by other complex substances present in the medium (juice).

EFFECTS OF SOME CHEMICAL AGENTS ON THE INHIBITORY EFFECTS OF SAPONINS ON GROWTH AND PIGMENTATION OF PHAFFIA RHODOZYMA

The mechanism(s) by which saponins inhibit growth and astaxanthin formation in Phaffia rhodozyma is/are unknown. However, since saponins are surface-active agents, the yeast cell membrane may be a major site of action. Contact with saponins would probably induce some alterations in cell membrane integrity as a result of possible changes in content and composition of lipids and related components of the membrane. Carotenoid biosynthesis might possibly be affected by such changes. This hypothesis would be proved if the inhibitory effects of saponins could be reversed by agents known to enrich plasma membrane lipids or enhance carotenoid biosynthesis in fungi. To test the postulate, Phaffia rhodozyma was grown in a basal medium containing 0.075% saponin and various suitable agents.

Media were contained in 500 ml baffled side-arm flasks. They contained (as final concentrations in 50 ml of 0.02M phthalate buffer pH 5.6), 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% of each of KH_2PO_4, yeast extract, peptone and glucose (autoclaved separately) and 0.075% saponin (also autoclaved separately for 2 minutes). The supplements shown in Table 14 were added from appropriate stock solutions; each concentration shown was the final in 50 ml of medium. Ergosterol and cholesterol were dispersed in tween 80 (Andreasen and Stier, 1953); the stock suspensions contained 0.05 g in 10 ml. 1 ml of 95% (v/v) ethanol was used to aid dispersion. Inoculum of Phaffia rhodozyma was grown up, processed and inoculated into the flasks as described in previous experiments. Assays for cell mass and astaxanthin production were made after 48 hr cultivation at 20°C, and the results are presented in Table 14.

The results show that only the unsaturated fatty acids (oleic and linoleic acids) and the sterols could significantly reverse some or all the inhibitions exerted by saponin on growth and astaxanthin formation. Linoleic acid appeared to support better growth at the higher concentration employed than oleic acid but the latter was probably more active at the lower level employed. None of the two unsaturated acids, however, appeared to improve astaxanthin formation. In contrast, the sterols appeared to improve both growth and pigmentation. β-ionone virtually suppressed both growth and astaxanthin formation. Ca^{++} appeared to have some advantage over Mg^{++} in promoting growth. All the other supplements tested, had virtually no adverse effects on the activity of saponin.

In general, the observed effects of the unsaturated fatty acids and the sterols may suggest that the saponins probably interfere with

Table 14. Effects of some supplements on the inhibitory effects of Saponins on Phaffia rhodozyma.

Supplement	Cell mass* mg/ml	Astaxanthin* µg/ml	µg/g
none (control)	2.0	0.12	60.00
biotin (0.22 µg/100 ml)	2.1	0.07	33.33
Ca-pantothenate (240 µg/100 ml)	1.5	0.07	46.70
inositol (1 mg/100 ml)	2.1	0.12	57.14
β-ionone (0.2%, v/v)	scanty growth	ND	NA
$CaCl_2 \cdot 2H_2O$ (2 mg/100 ml)	3.3	0.21	63.6
$MgSO_4 \cdot 7H_2O$ (2 mg/100 ml)	2.1	0.13	61.9
Oleic acid (1%)	6.0	ND	NA
Oleic acid (2%)	4.0	ND	NA
Linoleic acid (1%)	NG	NG	NA
Linoleic acid (2%)	10.6	ND	NA
Cholesterol (10 µg/ml)	6.7	1.69	238.81
Ergosterol (10 µg/ml)	7.2	1.12	155.56

Table 14. (cont.)

ND = not determined (cells white)

NA = not applicable

NG = no growth

* All data are averages from duplicate flasks, two determinations per flask.

some aspects of lipid metabolism in Phaffia rhodozyma.

CULTIVATION OF PHAFFIA RHODOZYMA ON OTHER SUBSTRATES: BREWER'S MALT WORT AND MOLASSES

Brewer's malt wort was prepared in the Brewing Lab; Dept. of Food Science & Technology, U.C. Davis, and was diluted with distilled water to contain approximately 2% soluble solids. The molasses (Red Star, Universal Foods Corp; Milwaukee, Wisconsin), was diluted twenty-fold and centrifuged twice at 13,000 rpm for one hour to remove debris. Each substrate was dispensed in 100 ml amounts into duplicate 500 ml baffled side-arm flasks and autoclaved at 121°C for 15 min. Inocula for the fermentations were prepared as described previously for experiments with alfalfa residual juice (ARJ) in Materials and Methods. Conditions of the fermentations were also similar to those of ARJ.

The results presented in Table 15a show that brewer's malt wort and molasses were good substrates for growth and production of astaxanthin by Phaffia rhodozyma. Their yields were comparable but were less satisfactory than those obtained with the peptone-yeast extracts-salts medium used as a control.

Addition of 0.25-1.00% glucose to the molasses appears to improve yields of astaxanthin but not those of cell mass (Table 15b). However, addition of some malt wort improves both parameters slightly. Peptone and other salts generally improve cell mass production but seem to depress astaxanthin production. Since malt wort might be expensive, it appears that unsupplemented molasses may be preferred as a substrate for growth of Phaffia rhodozyma and astaxanthin production.

Table 15a. Growth of *Phaffia rhodozyma* in brewer's malt wort and in molasses.*

Medium	Yield of Astaxanthin (ug/ml)	Yield of cell mass (mg/ml)
Brewer's malt wort	1.32	6.40
Molasses	1.10	6.00
Peptone-yeast extract-salts**	2.00	8.50

* Cultivation period is 60 hr. All data are averages from duplicate flasks.

** Contained phthalate-buffered (.02M, pH 5.6) $MgSO_4$, .02%, KH_2PO_4, yeast extract, peptone, each 0.5% and glucose 2.0%

Table 15b. Effect of supplementary nutrients on growth and astaxanthin formation by *Phaffia rhodozyma* grown on molasses.*

Nutrients added**	Yield of astaxanthin (ug/ml)	Yield of cell mass (mg/ml)
None	1.10	6.00
0.25% glucose	1.25	5.00
1.00% glucose	1.21	5.00
malt wort***	1.19	7.70
0.25% peptone	0.52	8.50
0.25% $(NH_4)_2SO_4$	0.90	7.50
0.25% $(NH_4)_2HPO_4$	0.46	6.70
0.25% NH_4Cl	0.42	5.00
0.25% KH_2PO_4	1.06	6.90

* Fermentation time was 60 hr.

** All nutrients were prepared as stock solutions so that final concentrations are achieved when 50ml of stock was added to 50ml of ten-fold dilution of molasses.

*** 50ml of wort diluted as described previously is added to 50ml of ten-fold dilution of molasses.

V. DISCUSSION

MODIFICATION OF THE CELL WALLS OF PHAFFIA RHODOZYMA IN MIXED CULTURE WITH BACILLUS CIRCULANS WL-12

The primary objective of this study was to determine the feasibility of using a mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma to render extractable the astaxanthin of the yeast. This would result from the lytic enzyme complex of the bacillus causing modification of the cell walls of the yeast. A preliminary attempt to obtain basic information on the nature of the interaction of the organisms in mixed culture showed that low pH, caused by growth of the yeast, was inhibitory to the bacillus. However, periodic neutralization of the medium with alkali had a beneficial effect; in consequence, extractability of astaxanthin was achieved due to improved growth of the bacillus and induction of the lytic enzyme system. The neutralization process was inefficient in this study because it was done by hand, but it seems to have great potential for industrial application because automated fermentation systems are available. Automatic pH control would efficiently maintain the medium within the pH range 6.2-6.8 determined suitable in this study. Thus, there would be no need for holding period adopted here to allow cell wall modification to occur. Since growth and pigmentation of Phaffia rhodozyma is virtually complete by 40-45 hr, it might be desirable to shift the temperature of the mixed culture to 30°C or 37°C to accelerate lytic activity, but as found in this study, time of cell harvest would be critical because of impending autolysis following completion of the modification of the yeast cell walls.

Further evidence of the feasibility of the mixed culture of Bacillus

circulans WL-12 and Phaffia rhodozyma was shown when a suitable initial pH (= 7.0) and relatively high buffer concentration (0.1M phosphate) were employed. It became possible to achieve the yeast growth and cell wall modification through a single step mixed culture operation because pH control was achieved intrinsically. This procedure is an advance over the two-step procedure of Johnson et al. (1978), which involved preliminary cultivation of Phaffia rhodozyma, followed by heat-inactivation of the culture and pH adjustment before the inoculation of B. circulans. Since the purpose of the heat treatment was to inhibit the growth of the yeast and to inactivate extracellular enzymes possibly present, the results of this study indicate that any enzymes present had negligible effects on B. circulans, or that its presence was probably repressive to the enzymes. It should be stressed that the mixed culture did not appreciably reduce the overall fermentation time required to achieve extractability of astaxanthin (approximately 65-72 hr) when compared with approximately 62 hr in the method of Johnson et al. (1978). Similarly, yields of astaxanthin in the mixed culture were generally lower (approximately 1.2 μ/ml) when compared with those of pure cultures (approximately 2.0 μ/ml) of P. rhodozyma described by Johnson and Lewis (1979). However, the single step mixed culture developed in this study is less laborious and less destructive to the carotenoids than the two-step method of Johnson et al. (1978). In addition, extractability of astaxanthin from biomass produced in the mixed culture is an obvious advantage over mono culture, since biological availability of the pigment depends on modification of the yeast cell walls.

It was shown in this study that development of lytic activity and extractability of astaxanthin were most pronounced when the glucose

concentration in the mixed culture medium approached exhaustion (level of residual glucose was approximately less than 1 mg/ml, Fig. 9a and b). This is consistent with the inducible nature of the lytic enzyme complex of Bacillus circulans WL-12 (Tanaka and Phaff, 1965). It was particularly interesting that appreciable viability of P. rhodozyma was retained during cell wall modification in presence of high lytic activity. This may indicate that B. circulans WL-12 has hydrolytic potential for living yeast; this capacity has not been hitherto demonstrated for the organism. The use of the fed-batch strategy to maintain a low level of glucose in order to circumvent catabolite repression and achieve early induction of the lytic enzyme complex was not successful. This may be explained, at least partially, by the method of glucose addition which involved periodic additions by pre-set schedules instead of by continuous feeding. Webles and Blanch (1976) found that pulse-feeding glucose to Saccharomyces cerevisiae caused a drop in cell yield and an increase in ethanol production. Since astaxanthin formation in Phaffia rhodozyma is growth-associated (Johnson and Lewis, 1979), it can be deduced (Fig. 12) from rates of astaxanthin yields that slow feeding of glucose retarded cell yields in this study (at least in initial stages of the mixed culture). Though ethanol formation was not monitored, it probably also occurred, since P. rhodozyma is fermentative, and may have affected lytic enzyme production. It must be emphasized, however, that early induction of the lytic enzyme of B. circulans would not be essential in an industrial-scale mixed culture designed to produce modified P. rhodozyma for use in feeds. The yeast requires for optimum yield of cells and astaxanthin formation, a growth period of 40-48 hr. Extensive hydrolysis of the cell walls prior to this period

would be undesirable, since (as would be discussed later), it would predispose the yeast to autolysis.

It was shown that with a suitable concentration (0.1M) of phosphate buffer, the initial pH influenced the outcome of the mixed culture through its (initial pH) effect on the final pH. The range of initial pH determined suitable was 6.8 to 7.4. The corresponding range of final pH was 6.25 to 6.83 (Table 4) and was in agreement with earlier results determined satisfactory for yeast cell modification in mixed culture held at 30°C after extrinsic (manual) pH controls.

The temperature range 20-24°C was determined to be optimal for good yield and extractability of astaxanthin in the mixed culture of Bacillus circulans and Phaffia rhodozyma. The yeast is more tolerant than the bacterium to low temperatures and its growth and pigmentation proceed very well at the temperature range 15-24°C. Above 27°C, it does not grow; this observation was previously made in pure culture by Johnson and Lewis (1979). Though the bacterium has optimal growth above 27°C, it seems to be less exacting in its temperature requirements, and its growth enables the yeast to be modified at 20°C; however, at 15°C, the viability of the bacterium is impaired with obvious consequences. In general, mixed cultivation of the two organisms, appears to have no influence on their individual growth temperature requirements. It is noteworthy that, as temperature of the mixed culture is increased from 15°C to 30°C (with consequent improvement and decline in viable counts of the bacillus and the yeast respectively, Table 5), the final pH drops; it was remarkably low at 30°C when only the bacillus grew. This may indicate that Phaffia rhodozyma could either utilize some organic acids produced by Bacillus circulans WL-12 or inhibit their formation

in mixed culture.

The initial concentration of glucose and the inoculum size of the bacterial component of the mixed culture were shown to have great influence on the extractability of astaxanthin produced by the yeast. The concentration of glucose must not exceed 1-2%. Above this level (up to 5%), yields of cell mass and astaxanthin (µ/ml) were considerably increased (as reported by Johnson and Lewis, 1979, for the monoculture of Phaffia rhodozyma), but lytic enzyme activity and extractability of astaxanthin decreased substantially. The effects of the high levels of sugar on the mixed culture can be explained in two possible ways: First, high initial levels leave high residual levels after growth of the organisms; for example when initial concentration of glucose was 5%, only three-fourths were utilized by 75 hours of mixed culture. Since lytic enzyme production is subject to catabolite repression, the effects of high levels of residual sugar are self-evident. Secondly, the adverse effects of high glucose concentrations on final pH of the mixed culture (Fig. 11) suggested that the metabolism of Phaffia rhodozyma was affected. It has been shown that in presence of high concentrations (3-5%) of glucose, metabolism in Saccharomyces carlsbergensis is not oxidative metabolism but aerobic fermentation (Rickard et al., 1971). Such a shift in metabolism of P. rhodozyma probably occurred in its mixed culture with Bacillus circulans WL-12 at high glucose levels and adversely affected lytic activity and yeast cell wall modification.

This study also showed that a high level of bacterial inoculum is required for early modification of the cell walls of P. rhodozyma in the mixed culture. This is probably because the temprature range (20-24°C) most suitable for the mixed culture is sub-optimal for growth

and lytic activity of Bacillus circulans WL-12. Consequently, low inoculum levels of the bacterium caused a prolonged lag before initiation of growth. In contrast, high levels of the bacterium shortened or eliminated the lag phase of growth and ensured quicker development of lytic activity. Thus, yeast cell wall modification was rapid and over 80% extractability of astaxanthin was possible in only 48 hr of the mixed culture fermentation. The actual level of Bacillus circulans needed to accomplish this was approximately four times that of Phaffia rhodozyma (on dry weight basis). The inoculum size of the bacillus (and associated lytic enzyme production), has further practical significance: it determines the tendency of the yeast to autolyse and the mixed culture grown with high levels of the bacillus must be harvested within 50-60 hrs (Fig. 16) to minimize autolysis. Of considerable interest is the reproducibility of these results in mixed cultures conducted in 5-liter fermentors. Successful development in this study of the mixed culture of B. circulans WL-12 and P. rhodozyma in the fermentors suggests there is some potential for large-scale application.

Electron microscopy and cytochemical assessment of mixed culture samples showed that events leading to extractability of astaxanthin were probably more complex than hitherto envisaged. The electron micrographs confirmed the role of B. circulans in yeast cell wall modifications: yeast cells in 48 hrs mixed culture samples which contained relatively low levels of the bacillus, appeared unmodified and had similar appearance to those of 48 hr monoculture samples. Conversely, the yeast cells in 72 hr mixed culture samples (in which cells of the bacillus were abundant), were considerably modified and were different from the corresponding monoculture samples. The gram reaction of the yeasts

in 72 hr mixed culture samples was negative, an evidence of loss in cell wall integrity (Bianchi, 1965). In addition, the yeasts exhibited considerable signs of aging. Among them were marked positive reaction with diazonium blue B (DBB) stain characteristic of basidiomycetous yeasts (Tevayanond, 1981), relative absence of budding cells, morphological changes, ranging from intracellular disorganization to total lysis and release of cell ghosts and cellular debris into the medium. There is further indication in this study that 72 hr cells of Phaffia rhodozyma grown in pure culture have greater susceptibility to the lytic enzyme complex of Bacillus circulans WL-12, than 47 hr cells (Fig. 22). It can be concluded from all these results (mainly electron micrographs and staining reactions) that, in the mixed culture of B. circulans and P. rhodozyma, yeast modification leading to extractability of astaxanthin was a result of a complex interaction of yeast age and lytic enzyme activity of the bacillus. Mudgett et al. (1978) showed that pretreatment of leaves with hydrolytic enzymes (cellulase and cellulase-pectinase mixture), enhanced protein recovery. Electron micrographs revealed structural degradation of plant tissue and the effects of the enzymes were attributed partially to accelerated senescence resulting from degradation of structural polysaccharides responsible for rigidity and mechanical strength of plant tissue. Knorr et al. (1979) utilized lytic enzymes including zymolase to activate the endogenous proteolytic enzymes and to effect total lysis of brewer's yeast (Saccharomyces carlsbergensis). These reports support the contention in this study, that the lytic enzymes of Bacillus circulans WL-12 probably accelerated aging and autolysis of Phaffia rhodozyma in their mixed culture.

The ability of carbon sources (other than glucose) to promote the

desired outcome of the mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma indicated the potential usefulness of cheaper substrates which contain (or can be hydrolyzed) to yield the carbon compounds utilizable by Phaffia rhodozyma. For example, molasses, a well-known fermentation substrate could be useful since it contains primarily sucrose which supported the mixed culture. Inulin, a β-fructoside, is a potential substrate because the mixed culture was successful on fructose. The polymer (inulin) is produced in large quantities by Jerusalem artichoke (Helianthus tuberosus), and is currently under intensive investigation for ethanol production by yeasts (Sachs et al., 1981). Its hydrolysis by mild acid yields primarily fructose. Ability of yeasts to ferment sucrose and β-fructosides are known to be genetically linked (Fuente and Sols, 1962). In addition, inulinase-production by yeasts have been described (Beluche et al., 1980). These facts put together indicate that the mixed culture of B. circulans WL-12 and P. rhodozyma, alone or in combination with an inulinase-producing yeast or other organism, may achieve the results of this study on inulin or its hydrolysate as the substrate. Hydrolysates of straw which contain primarily arabinose, xylose, mannose and glucose (Han et al., 1976) may be useful if the mixed culture can utilize the sugars in combination, and the repressive effect of xylose can be minimized.

The relatively poor yields of astaxanthin when the mixed culture was grown on cellobiose and in the control (pure culture of P. rhodozyma) were unexpected since the sugar served as the best carbon source for the monoculture of Phaffia rhodozyma (Johnson and Lewis, 1979). Since Bacillus circulans WL-12 does not utilize the substrate (Tanaka and Phaff, 1965), its growth was expected to depend on that of the yeast.

The amount of lytic activity achieved appeared to be commensurate with the poor growth of the yeast. It may be that the initial pH used in this study (= 7.0) was not suitable for uptake of cellobiose by the yeast. Another unexpected observation was that P. rhodozyma could not grow when starch was used as the substrate for the mixed culture. Since B. circulans WL-12 is amylolytic, it was thought that the amylolytic potential of the bacillus under the conditions employed for the mixed culture could not furnish reducing sugars in amounts sufficient to allow its growth and provide a surplus for P. rhodozyma.

Other interesting findings in this study concern the potential reusability of cell free culture fluid derived from the mixed culture of B. circulans and P. rhodozyma. At least two methods of reuse were found possible. In one of them, the culture fluid was found suitable for cultivation of Phaffia rhodozyma after suitable fortification with nutrients; resulting cell mass had modified cell walls and astaxanthin was extractable. This system in which the yeast is modified during growth in presence of the lytic system resembles one in which a unicellular alga, Cosmarium produced protoplasts in presence of cellulysin, a lytic mixture of enzymes produce by Trichoderm viride. The production of protoplasts improved the potential of the alga as a source of single cell protein for animal feeding. In the second method, the cell-free culture fluid was shown to enhance extractability of astaxanthin when recycled twice on Phaffia cells grown in pure culture. In this connection, it should be noted that cell free culture fluid derived from growth of Bacillus circulans WL-12 on heat-killed Phaffia cells was similarly potent, but Johnson et al. (1978) concentrated the enzyme by ultrafiltration, a step considered unnecessary in view of the results obtained

in this study. The results have been used to propose a scheme for enzymatic treatment of Phaffia rhodozyma. The scheme consists of simultaneous cultivation of the yeast as a pure culture and in mixture with Bacillus circulans WL-12, and use of the culture fluid derived from the latter for treating cell mass derived from pure culture. It is hoped that the scheme would enhance the potential of the mixed culture for commercial application if it (the scheme) proves to be economically and practically feasible on a large scale.

EFFECT OF AUTOLYSIS ON EXTRACTABILITY OF ASTAXANTHIN FROM PHAFFIA RHODOZYMA

Autolysis of yeast is a phenomenon in which the organism is modified by its endogenous enzymes. In the last section of this discussion, the phenomenon was shown to be accelerated in Phaffia rhodozyma by the lytic activity of Bacillus circulans WL-12 induced in mixed culture. Since yeast autolysis can be induced artificially by exposure of cells to suitable environmental conditions and by suitable treatments, it was examined as a potential tool for liberation of the carotenoids of P. rhodozyma.

It was interesting to find that astaxanthin was extractable from P. rhodozyma autolysed in various systems at 37°C (Table 9). The various degrees of extractability of pigment achieved in different autolysing systems were expected, since medium of incubation is known to influence yeast autolysis (Joslyn and Vosti, 1955). Though toluene appeared to facilitate the phenomenon its use in treating P. rhodozyma for use as a feed supplement would be undesirable. The total inhibition,

by sodium chloride, of the autolytic system of Phaffia rhodozyma, was unexpected since Kihlberg (1972) stated that up to 25% solution of salt is added to yeast to accelerate autolysis in commercial yeast extract manufacture. Sugimoto (1974) showed that up to 10% NaCl was inhibitory to some proteinases in the autolytic system of baker's yeast, but the salt had a synergistic effect with ethanol and was useful in preparation of yeast extracts. The ability of distilled water and of 0.02M citrate buffer pH 7.0 to facilitate extractability of astaxanthin from P. rhodozyma may enhance the industrial potential of the yeast. Though the pungent odor of dithiothreitol would preclude its use in commercial treatment of P. rhodozyma, its ability to enhance the autolytic effect of citrate buffer might be due to some reduction in disulfide bridges in the proteins of the cell wall. The effect of the thiol on extractability of astaxanthin would be analogous to well-documented effects on extractability of protein from yeast (Shetty and Kinsella, 1978) and in spheroplast formation from yeast (Sommer and Lewis, 1971).

Different strains of Phaffia rhodozyma were shown to vary in their susceptibility to autolysis under similar conditions. This is consistent with published studies (Joslyn and Vosti, 1955). The strains also showed some variations in their susceptibility to the lytic enzyme complex of Bacillus circulans WL-12; this was probably caused by strain differences in cell wall composition. However, when susceptibilities to autolysis and to the lytic enzyme were compared, the yeast strains examined appeared to react in approximately the same way to each method of treatment (Table 10). This may indicate that lytic activity and autolysis have some features in common; it has been shown earlier in this discussion (electron micrographs) that the ultimate effect of lytic

activity appeared to be the induction of yeast autolysis. It appears from this study that autolysis (in absence of lytic enzyme) may ultimately involve some cell wall modifications. In general, autolysis may prove to be a cheap, commercially feasible method of processing Phaffia rhodozyma for inclusion into animal feed as a source of astaxanthin. Use of distilled water seems to be particularly attractive, and, as observed in this study, at least one strain of P. rhodozyma, namely 67-484, can release all its pigment after only 26 hr autolysis in distilled water at 37°C.

Besides manufacture of yeast extracts, there seems to be very few commercial processes which utilize yeast autolysis as a routine procedure. Its potential promise in the area of single cell (yeast) protein processing seems to be widely recognized. For example, it (autolysis) has been proposed as a possible method of rupturing cells grown on hydrocarbons in order to facilitate extraction of residual hydrocarbons from protein (Tannenbaum, 1968). In addition, autolytic procedures have been used for reducing nucleic acid content of yeast (Trevelyan, 1976). The present study has shown that autolysis of Phaffia rhodozyma can enhance extractability (and presumably biological availability) of astaxanthin. Further work is required on the effects of commercially feasible conditions of culture on the susceptibility of the yeast to autolysis.

GROWTH OF PHAFFIA RHODOZYMA ON ALFALFA RESIDUAL JUICE

Phaffia rhodozyma cannot be put to commercial use as a pigment additive in salmonid and poultry diets unless it can grow and synthesize

astaxanthin on cheap substrates. One objective of this study was to examine some waste products as potential substrates for growth and pigmentation of the yeast. Many waste products of food processing industries have been used as fermentation substrates for biomass production (for a review, see Cousin, 1980). The primary focus in this study is alfalfa residual juice (ARJ) or alfalfa solubles. This is a waste product derived from a process which extracts leaf protein from alfalfa. For comparison to ARJ, molasses and brewer's malt wort have also been tested as substrates for growth and astaxanthin formation by Phaffia rhodozyma.

Unsupplemented ARJ supported high yields of cell mass of Phaffia rhodozyma; other workers had made similar observations with other yeasts (Parades-Lopez and Camargo, 1973; Mudgett et al., 1980). Yeast cell mass produced in ARJ showed greater susceptibility to autolysis after maximal growth than that grown as a control in a peptone-yeast extract-salts medium (semi-synthetic medium, SSM). Hough and Maddox (1970) stated that protein in the growth medium predisposes yeast to autolysis; ARJ is expected (from its history) to contain more proteinaceous material than the SSM. The effect of glucose supplementation in increasing yields of cell mass of Phaffia rhodozyma in ARJ (Fig. 24) is to be expected from the results of Johnson and Lewis (1979). These workers showed that the higher the glucose concentration, the higher the biomass production (mg/ml) in a semi-synthetic medium. The present study also showed that glucose supplementation minimized 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) could be useful if Phaffia rhodozyma is intended for use as a source of protein. However, further work would be required on the effects of the additions of glucose on its nutritive quality, for example, its amino acid balance.

Unfortunately, ARJ was found to be unsuitable for astaxanthin formation. Enrichment of the medium with glucose and other nutrients failed to promote pigmentation. The inhibition of astaxanthin formation, apparently, was not due to deficiency of some essential nutrients in the original juice, because addition of SSM failed to support pigmentation. Some inhibitors of astaxanthin formation are possibly present in the juice. Different strains of Phaffia rhodozyma were shown to be susceptible to the inhibition; all of them probably synthesize the pigment through the same mechanism which was adversely affected by ARJ. When different levels of ARJ were incorporated into a standard medium known to support good yields of astaxanthin, concentrations of the ARJ greater than 1.25% v/v were clearly inhibitory to astaxanthin formation. Above 3.7% v/v there was total inhibition of pigmentation (Fig. 25) but chromogenic components in the juice were adsorbed on the surfaces of the yeast cells and showed as artifacts in astaxanthin assays. That the chromogens showed greater affinity for Phaffia rhodozyma than for Saccharomyces cerevisiae 71-141, a known white yeast, indicates that there may be differences in the chemical components of the surfaces of the cells of the two yeasts. It is well known that Phaffia rhodozyma contains capsular material (Miller et al., 1976).

The inhibition of astaxanthin formation by ARJ can only be explained in terms of the activity of specific components of the medium.

Saponin comprises a class of biologically active compounds which has been described in fractions (including ARJ) derived from leaf-protein extraction from alfalfa plant (Livingston et al., 1979). Its possible role as an inhibitor of astaxanthin formation was demonstrated in this study. Surprisingly, it was also found to inhibit growth of Phaffia rhodozyma, contrary to the effect observed with the ARJ. The discrepancy may be caused by the commercial grade saponin, which apparently, was more potent on the yeast, than the ARJ. The greater sensitivity to the saponin, of astaxanthin formation in comparison with cell mass production (Fig. 27), and the stimulatory effect of low concentrations (up to 0.25%) of saponin on cell mass production but not on astaxanthin formation, indicated that the two parameters could be affected independently. It seems likely that the saponins in ARJ inhibited astaxanthin formation while the inhibition of cell mass production was somewhat attenuated or abolished by other components present in the complex medium (ARJ).

A great deal of information is available on the adverse effects of saponins (derived from alfalfa and other plants) on various groups of living organisms (for a review, see Birk, 1969). Among the recorded effects on higher plants and animals are depression of growth in poultry and other animals, toxicity to fish and amphibia, hemolysis of red blood cells, and inhibition of seed germination. Fungistatic activity of saponins is also documented. For example, lucerne saponins inhibit Sclerotium rolfsii (Gestetner et al., 1971), and activity of alfalfa saponin on Trichoderma viride has been recognized and used as a basis for a microbiological assay (Livingston et al., 1977). Information seems to be lacking on the effects of saponins on yeasts. Thus, the

activity of the compounds on Phaffia rhodozyma reported herein is important because of the specific deleterious effect on carotenoid biosynthesis, and on yeasts generally.

Many activities of saponins have been attributed to their surface-active properties. The mechanisms involved in the inhibition of growth and astaxanthin formation in Phaffia rhodozyma are not clear, but the ability of sterols (cholesterol and ergosterol) and unsaturated fatty acids (oleic and linoleic acids) to reverse some of the effects (Table 14) could be used to speculate on some probable modes of action. It is known that high levels of sterols and unsaturated fatty acids are characteristic of aerobically grown yeast, and that oxygen deficiency may lead to their requirement for growth (Andreasen and Stier, 1953, 1954). Oleic and linoleic acids are the predominant fatty acids in Phaffia rhodozyma (Johnson et al., 1980), and vigorous aeration is required for astaxanthin formation (Johnson and Lewis, 1979). It is probable that relatively high levels of saponins may limit oxygen uptake from the medium and impair with growth and astaxanthin formation in Phaffia rhodozyma. The unsaturated fatty acids, apparently, were required mainly for growth, since they could not remarkably reverse the inhibition on astaxanthin formation. A second possible mode of action is that the saponins could interfere with the integrity and biological activity of the lipid-rich plasma membrane with which it would easily come in contact in the yeast cell; permeability of that membrane might probably be affected adversely. It is recognized that unsaturated fatty acids and sterols influence the stability and biological activity of yeast lipids and membranes (Rattray et al., 1975). Further, since carotenoids in some fungi are associated with lipid globules and membranes

(Mitzka-Schnabel and Rau, 1980), any disturbance of these structures would adversely affect biosynthesis of the pigments. Though these speculations on the effects of saponins on Phaffia rhodozyma remain to be tested, it is noteworthy that other workers have interpreted the effects of saponins on other membranes in terms of permeability (Bangham and Horne, 1962).

In contrast to the observations made with ARJ, brewer's malt wort and molasses were suitable substrates for growth and pigmentation of Phaffia rhodozyma. Use of brewer's malt wort to boost astaxanthin production was reported by Johnson and Lewis (1979). Though satisfactory results were achieved when the two substrates were combined in equal proportions, their potential usefulness for large-scale production of Phaffia rhodozyma would depend on economic considerations.

VI. SUGGESTIONS FOR FURTHER STUDY

Continuous fermentation is suggested for further development of the mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma. It was conceived earlier in this study but batch culture was thought to be an essential prerequisite. Some of the conditions which have been determined suitable for the batch culture, e.g. temperature (20-24°C), pH (6.2-6.8), and relatively high initial levels of the bacillus, can be directly applied to continuous culture. However, since dilution rate is critical in continuous systems, its effects on steady state levels of the yeast and the bacillus, and on their products (astaxanthin and lytic enzyme respectively), would require extensive investigation.

Some problems might possibly limit efficiency in the proposed continuous culture. For example, Phaffia rhodozyma may produce limited levels of astaxanthin when maintained in steady state for prolonged periods of time, and susceptibility of such yeast cells to steady state levels of lytic enzyme is not known. A constant limiting level of the carbon source would be present to maintain productivity of the organisms, but it may repress or derepress lytic activity of the bacillus. If repression occurs, other carbon sources, e.g. sucrose, fructose, maltose and arabinose, which were determined as possible substitutes for glucose, may have to be tested in the continuous culture. On the other hand, derepression of lytic activity might enhance autolysis of the yeast. Despite these apparent problems, continuous culture seems to be a logical step for further evaluation.

VII. SUMMARY

A mixed culture of the red yeast Phaffia rhodozyma and Bacillus circulans WL-12, has been developed and utilized to produce a biomass containing extractable astaxanthin. This resulted from induction of the lytic enzyme complex of the bacillus and modification of the yeast cell walls. The mixed culture was feasible in shake flasks and in 5-liter fermentors and was thought to have some potential for large-scale production of modified Phaffia rhodozyma for application to animal feeds as a source of astaxanthin.

The outcome of the mixed culture depended on environmental conditions.

1.1. Control of pH is critical to circumvent possible inhibition of the bacillus by the yeast and to insure development of high levels of lytic activity. Suitable range of pH is 6.2 to 6.8 and could be maintained in the mixed culture by periodic neutralization of medium acidity with alkali or by use of suitable buffer concentration and initial pH.

1.2. A temperature range of 20-24°C must be maintained for optimal development of the mixed culture. Below this range (up to 15°C), Phaffia rhodozyma grows well without cell wall modification because the condition does not favor good growth of the bacillus. Above the range the yeast grows poorly and is totally inhibited above 27°C.

1.3. Initial concentration of glucose employed for the mixed culture must be sufficiently low to be exhausted or reduced to a negligible level during the period of active growth of the organisms.

This would suppress catabolite repression of the lytic enzyme system which would inhibit yeast cell wall modification at high residual levels of glucose. It might also minimize fermentative metabolism of the yeast which tends to increase medium acidity and inhibit the bacillus. A suitable range of glucose concentration for the mixed culture is 1.0-2.0%.

1.4. Slow-feeding of glucose by preset schedules increases the ultimate level of lytic activity attainable in the mixed culture but does not necessarily accelerate extractability of astaxanthin. It appears to reduce yields of astaxanthin and is not recommended for the mixed culture.

1.5. The inoculum of Bacillus circulans is of tremendous importance. It is essential to use a relatively high level (up to 3 or 4 times the size of Phaffia rhodozyma on dry weight basis) to shorten the lag phase of bacterial growth and thence accelerate lytic enzyme production and modification of the yeast cell walls. Since high levels of the bacterium also predispose the yeast to autolysis, the mixed culture must be harvested within 50-60 hr of the fermentation.

I.6. Carbon sources (other than glucose) such as fructose, maltose, sucrose, and to a lesser extent mannitol (all at equivalent concentrations) could be used for the mixed culture of Bacillus circulans and Phaffia rhodozyma. This indicates that cheaper, complex substrates such as molasses, inulin, grass straw, which contain those sugars or can be hydrolyzed to yield them, would probably support the mixed culture. Starch did not support growth of Phaffia rhodozyma even though Bacillus circulans WL-12 is

amylolytic and was expected to furnish reducing sugars for growth of the yeast.

2.0. Structural changes in Phaffia rhodozyma during its digestion in mixed culture, were followed by electron microscopy and cytochemical methods and related to extractability of astaxanthin. In addition to relatively high lytic activity and high proportions of extractable astaxanthin in 72 hr samples, the yeast cells showed considerable signs of aging which ranged from intracellular disorganization to total lysis. They were also gram positive and showed marked positive response to diazonium blue B (DBB) dye when compared with 48 hr samples. Since other tests showed that 72 hr Phaffia cells grown in pure culture were more susceptible to the lytic enzyme of Bacillus circulans WL-12 than 48 hr cells, it is concluded that extractability of astaxanthin from the mixed culture probably depended on lytic activity and yeast age; the former probably accelerated the latter in the mixed culture.

3.0. Cell-free culture fluid derived from the mixed culture of Bacillus circulans WL-12 and Phaffia rhodozyma is a reusable resource because it is laden with lytic enzymes. It could be recycled on unmodified Phaffia cells produced in pure culture; two recycles are possible without appreciable decrease in lytic enzyme potency. Alternatively, the lytic fluid can be refortified with nutrients and reused for cultivation of the yeast which becomes modified during growth. Fortifications with ammonium phosphate plus peptone, each 0.5% or with urea, peptone, each 0.5% plus potassium phosphate (monobasic; 0.4%), produced good yields of cell mass containing high yields of extractable astaxanthin. A

potentially feasible scheme which integrates mixed culture (with Bacillus circulans WL-12) and pure culture of Phaffia rhodozyma is presented to exploit the reusability of cell-free culture fluid derived from the mixed culture.

4.0. Autolysis of Phaffia rhodozyma under artificial conditions could be used to render astaxanthin of the yeast extractable. Distilled water, with or without 2% toluene, and 0.02M citrate buffer pH 7.0, with or without 0.01M dithiothreitol (DTT) were efficient autolysing systems at 37°C. Sodium chloride (15% v/v) was inhibitory to the autolytic system, while Tris and phosphate buffers, (at 0.1M and pH 8.0 and 7.0 respectively) were relatively inefficient even for up to three days incubation. Six strains of Phaffia (67-202, 67-203, 67-210, 67-385, 67-484, 67-653C) were susceptible in roughly the same order to autolysis and to extracellular lytic enzyme digestion. Complete extraction of astaxanthin was achieved with 67-484 after only 26 hr autolysis in distilled water. It is suggested that autolysis could become a cheap procedure for treatment of Phaffia rhodozyma to enhance extractability (and presumably bio-availability) of its carotenoids.

5.0. Alfalfa residual juice (ARJ) derived from mechanical extraction of alfalfa leaf protein and diluted ten-fold to contain approximately 2% sugar, was a suitable substrate for production of high yields of cell mass of Phaffia rhodozyma. When the medium was supplemented with up to 2.0% glucose, biomass production was improved and autolysis of the yeast minimized. The juice is potentially useful for growth of Phaffia rhodozyma as a source of single cell protein.

6.1. ARJ inhibited astaxanthin formation in Phaffia rhodozyma. Supplementary nutrients (glucose, KH_2PO_4, yeast extract, peptone each 0.5%; $MgSO_4 \cdot 7H_2O$, 0.02%) added separately or in combination, did not reverse the inhibition, and none of six strains of the yeast tested (67-202, 67-203, 67-210, 67-385, 67-484 and 67-653C) was resistant to the inhibition. When various levels (0-12.5% v/v) of the juice were added to a standard medium (SM) known to promote good yields of cell mass and astaxanthin, the former increased with concentrations of the juice, but the latter (astaxanthin) declined and was totally inhibited above 3.7%.

6.2. The cell surface of Phaffia rhodozyma could adsorb chromogens from ARJ; these showed as artifacts in estimations of astaxanthin. The amount of pigment adsorbed varied with the concentration of juice added to the SM. However a characteristic absorption peak (in acetone) is maintained at approximately 390 nm, with or without acid treatment of cell pellets, and indicates that the chromogens were not typical colored carotenoids. In contrast, pigment from the yeast grown in the SM which contained no ARJ was releasable only after acid treatment of cell pellet, and its absorption spectrum was typical of colored carotenoids (with a peak at approximately 450 nm).

7.1. Saponins have been determined to inhibit growth and pigmentation of Phaffia rhodozyma. Astaxanthin formation appears to be more adversely affected than cell mass production; 0.075% saponin totally inhibited the former, while even 0.5% did not completely inhibit cell mass production. It is suggested that saponins possibly present in ARJ, were the inhibitors of astaxanthin formation

in Phaffia rhodozyma. It is probable that other components of the juice neutralized or reduced the inhibitory effect of the saponins on cell growth.

7.2. Unsaturated fatty acids (oleic and linoleic acids) and sterols (cholesterol and ergosterol) could reverse some inhibitory effects of the saponins on Phaffia rhodozyma. Since the agents (the fatty acids and the sterols) probably enriched the yeast lipids and membranes, it is thought that the saponins probably induced some alterations in the integrity and possibly permeability of the plasma membrane of the yeast.

8.0. Brewer's malt wort and molasses were suitable substrates for growth and pigmentation of Phaffia rhodozyma. Some nutrients, for example 0.25% each of ammonium sulfate, peptone, ammonium phosphate, when added to the molasses improved cell mass but depressed astaxanthin yield. Glucose (0.25-1.0%) appeared to have an opposite effect. In general, molasses should not be supplemented with further nutrients if used for growth of Phaffia rhodozyma.

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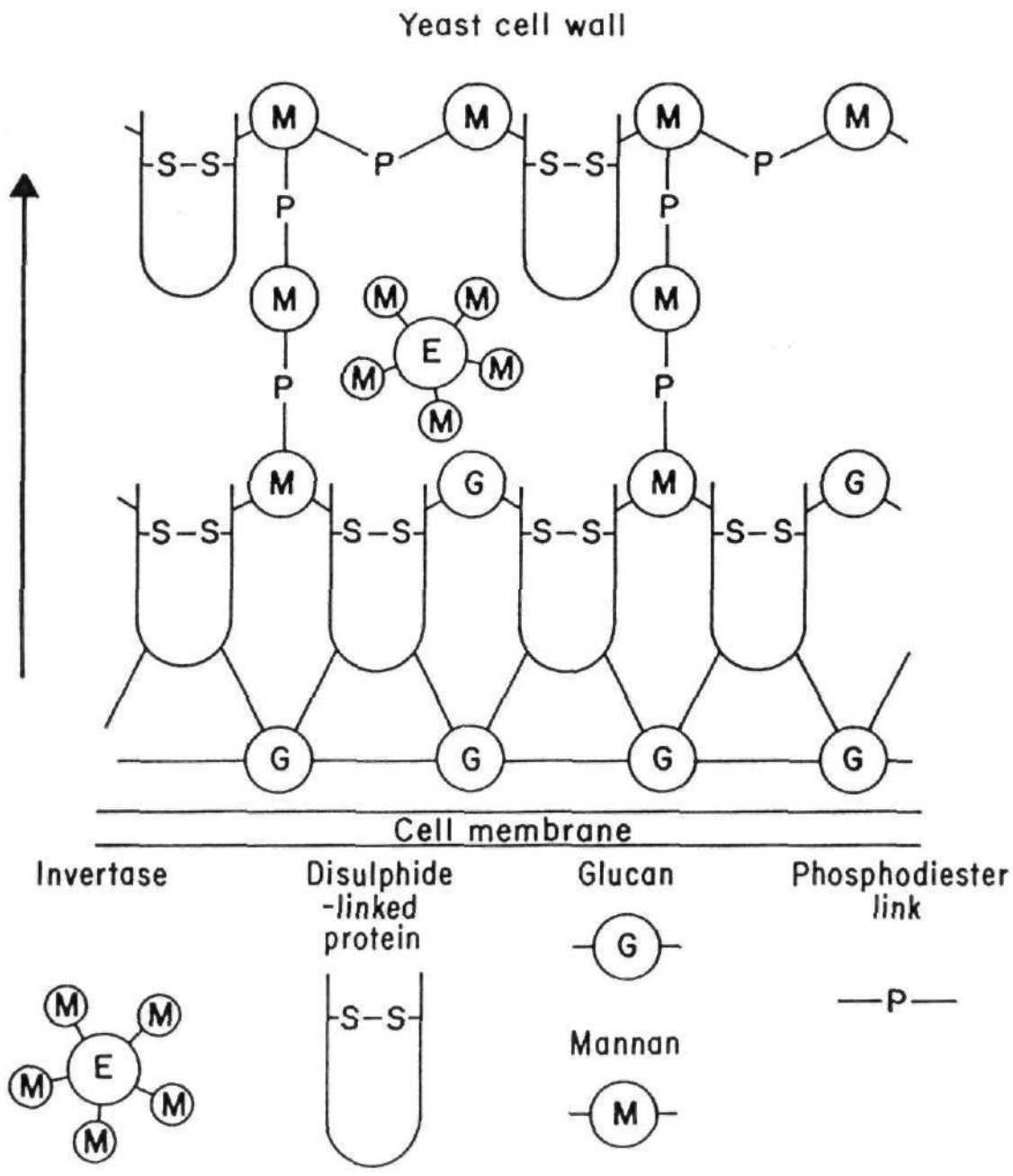
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APPENDIX I

A model for the yeast cell wall. Arrow points towards the exterior of the cell. The essential features of the model are identified at the foot of the figure. Adapted from Kidby and Davies (1970).


Yeast cell wall
M
P
S–S
E
G
Cell membrane
Invertase
Disulphide -linked protein
Glucan
Phosphodiester link
Mannan