



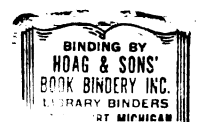
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THE AEROBIC FERMENTATION OF
SALT-STOCK PICKLE BRINE

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ABSTRACT

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By

Ratna Siri Hadioetomo

Ten species of yeasts were tested for their ability to grow in salt-stock pickle brines containing 10% salt and about 0.5 to 0.6% acid calculated as lactic acid, and having a pH of 3.4. Of those tested, Debaromyces membranaefaciens var. hollandicus (FBY-44; NFY-32) (Debaromyces nicotianae) grew and utilized the acid in the brine most rapidly. Under optimum conditions, the adapted culture oxidized all of the organic acid present within 24 to 30 hours of incubation, and this was accomplished by a pH increase from ~ 3.4 to ~ 8.0 and a reduction in the biochemical oxygen demand (BOD) of about 70%. The yeast cells and other particulate matter can be harvested by raising the pH of the fermented brine to a pH of 10.5 or higher which results in flocculation and rapid sedimentation. The salt-stock brine diluted to a 10% salt concentration supported the production of about 5 g dry weight/liter of yeast cells. On a dry weight basis, the cells were about 30% crude protein and 36% carbohydrate.

Supplementation of the brine before fermentation with lactic acid increased cell yields to some extent, and addition of ammonia nitrogen decreased the total time required to utilize all of the acid present. However, the effects observed were not great enough to indicate that such additions would be practical under commercial conditions. The data indicate that aerobic fermentation could be of great value in preparing salt-stock brine for re-use. The yeast cells produced should be valuable as animal feed and/or fertilizers.

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PICKLE BRINE

By

Ratna Siri Hadioetomo

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INTRODUCTION

About 50 percent of the total cucumbers processed annually in the United States are fermented in salt brine. After the lactic acid fermentation is complete, the salt concentration is increased to 10-15%. The resulting pickles, commonly referred to as salt-stock, may be stored in this brine for prolonged periods. When needed the pickles are removed from the brine, desalted, and manufactured into various pickle products. The salt-stock brine is high in salt, acid, and biochemical oxygen demand (BOD). Therefore, it is a difficult waste to treat.

It has been recently suggested (18, 19) that salt-stock brine might be reused to brine fresh cucumbers after treatment to insure the destruction of undesirable enzymes. One treatment proposed is to add sufficient NaOH to raise the pH to 10.0 or above, which results in the precipitation of most of the suspended solids; and then to neutralize the supernatant brine with acid. While the original authors (18, 19) stated that this treatment greatly reduced the BOD of the brine, more recent data (Palnitkar and McFeeters, unpublished data in press) indicate that it has little influence on BOD. An

additional problem with this treatment is that a very large amount of alkali would be required to neutralize the acid and raise the pH to the desired level.

There are a number of oxidative yeasts which grow on the surface of salt-stock brines if the brine surfaces are protected from sunlight (13). These yeasts oxidize lactic acid readily. Therefore, it appeared feasible to develop an aerobic fermentation process for salt-stock brine which would remove the lactic acid, reduce the BOD, and produce yeast cells which might be used for animal feed and/or fertilizer. The fermented brine might be treated further and used to brine fresh cucumbers, or diluted and discharged into a waste treatment facility.

The present investigation was designed to select a yeast culture which would grow and oxidize the acid in salt-stock brine rapidly, determine optimum conditions for growth, measure cell yields, and measure the effect of the aerobic fermentation on various organic compounds and the BOD of the brine.

LITERATURE REVIEW

Yeasts from Salt-Stock Pickle Brines. There are two groups of yeasts associated with salt-stock pickle brines. Members of one group grow below the surface of the brines and produce a gaseous fermentation causing great economic losses due to the formation of hollow cucumbers (bloaters). The other group is comprised of oxidative yeasts which produce a luxuriant, wrinkled film on the surface of the brine when sheltered from direct sunlight (13). The fermentative yeasts depend primarily on sugar for growth while the oxidative species utilize a wide variety of organic compounds including organic acids and alcohols. If the latter group are allowed to grow on salt-stock brines for a prolonged period, they may reduce the acidity to the point at which other types of spoilage organisms are not inhibited. Most yeast species in both groups are quite tolerant to high salt and acid.

Torulopsis holmii and Torulaspora rosei are two fermentative species associated with gaseous fermentations (4). The former species are found only during the first month after the cucumbers are brined (4). This may mean that this yeast is not as tolerant to salt and/or acid as

T. rosei which is found in brines for prolonged periods. The salt tolerance of T. rosei has been estimated at between 15 and 20% (13).

The yeasts most commonly found in the surface films on cucumber brines are Debaromyces membranaefaciens var. hollandicus, Endomycopsis ohmeri, Candida krusei, Pichia alcoholophila, and Zygosaccharomyces halomembranis (14). All of these yeasts except Z. halomembranis are strictly oxidative.

D. membranaefaciens var. hollandicus was found most commonly in surface films on brines of 15% salt and above. This species produces heavy films on brines containing 20% salt; has little or no ability to ferment sugar but can assimilate a large number of compounds as sources of carbon (14). This characteristic together with the high tolerance to salt and organic acids are important factors responsible for the presence of these yeasts in foods that are preserved by salting and brining (9).

Z. halomembranis is both fermentative and oxidative in its metabolism. In liquid medium without salt this yeast does not form surface films, but luxuriant films are produced on salt-stock brines or broth media with added salt. It is very salt tolerant and will grow in media with 20-24% salt (13).

E. ohmeri was found to be intermediate in salt tolerance, but produced heavy films at 15% salt (4).

C. krusei and P. alcoholophila both produced films on pickle brines of 5% salt (4) but 10% salt appeared to be about the limiting concentration in which they could grow.

The common food yeasts, Candida utilis and Saccharomyces cerevisiae have never been isolated from salt-stock brines. Neither species appears to be very salt tolerant. C. utilis has been reported to be partially inhibited from growth by 5% salt, and almost completely inhibited by 10% salt (31).

Salt-Stock Pickle Brine. Salt-stock pickles are produced by placing cucumbers in wooden vats ranging in capacity from 200 to 1,200 bushels, covering with salt brine and adding enough dry salt to equilibrate at about 5 to 6%. After 4 to 6 weeks, the salt concentration is gradually increased to 10 to 16 percent at the rate of from 1 to 6 degrees salometer per week. A lactic acid fermentation occurs during the first 3 to 4 weeks after brining. The preserving effect of the brine is due chiefly to the combined action of the acidity and the salt (15, 46).

Based on the data of the last five years, the annual production of cucumbers in the United States is about 800,000 tons. The portion that went to the fresh market was ~ 200,000 tons, and that which went into processing was ~ 600,000 tons (2). About half of those that went into processing (~ 300,000 tons) were brined to

produce salt-stock. A bushel of pickles (50 lbs) will generate approximately 4 to 6 gallons of spent brine containing 4 to 6 lbs of salt depending upon tank yard practices (Palnitkar and McFeeters, unpublished data in press). Based on simple calculations there is approximately 60 million gallons of spent brine produced annually. Spent brine contains 10 to 18% sodium chloride (19), 10,000 to 15,000 ppm BOD and has a pH of 3.3 to 3.6 (Palnitkar and McFeeters, unpublished data in press). These characteristics make salt-stock brines a serious waste disposal problem.

Since salt is corrosive in nature and non-biodegradable, the disposal of brine is costly. Geisman and Henne (18) noted that dilution is the most common means of disposal, but due to the volume of the concentrated brine produced annually, the volume of water required for dilution becomes astronomical in size. Therefore, it is apparent that a sizable saving would result if the salt could be recycled. For this purpose several methods have been developed and significant progress has been made in reuse of spent brine in the past two or three years (10, 11, 18, 19).

Based on their potential for pollution, the decreasing order of importance of various segments of the food processing industry is as follows: meats, fruits, and vegetable canning, dairy, sugar refining, fruit and

vegetable freezing, and poultry (40). Eckenfelder (12) listed the BOD value for sewage, laundry, dairy, and cannery wastes were 100-300, 300-1000, and 800-1500, and 240-6000 ppm respectively. Compared to these data, the BOD of salt-stock pickle brine is very high, 10,000-15,000 ppm (Palnitkar and McFeeters, unpublished data in press). The high acidity of the brine is another disadvantage because acid waste effluents generally require neutralization before they can be effectively treated in secondary waste treatment system such as activated sludge and trickling filters (23). Therefore, if one could use yeast to reduce the BOD, oxidize the acid, and produce protein at the same time, it should be of very significant economic value to the pickle industry.

Yeasts for Food and Feed. In a large measure, the primary food problem of the world is a protein problem. Even though it is more serious in developing countries because the population growth rate is double that of the developed world, food shortage is a world problem (6). There is considerable evidence that microbial matter can be a valuable source of food material, particularly of proteins and vitamins. The use of microbial cells as a source of food is advantageous primarily because of the rapid rate of growth which affords a means of obtaining food much more quickly than by more conventional means. Furthermore, it is independent of agricultural land and

growth is possible through utilization of inexpensive materials as energy sources that would otherwise be wasted or not readily disposed of.

The possibilities for growing yeast as a food and feed supplement material was recognized for the first time in Germany (5, 25, 47). The ability of certain yeasts to consume pentose made possible the utilization of the hardwood liquor from pulp industries to produce protein and relieve critical food and feed shortages in that country (25). A commercial scale process for producing yeast from wood sugar was developed before the end of World War I (5). During World War II, yeast was produced at an estimated annual rate of about 100,000 tons for nutritional use (47). At about the same time dried yeast was also used in Great Britain as feed for livestock and later was used to supplement the diets of certain people in colonial possessions when the food supply deteriorated (47).

In the United States the study of yeast for nutritional use started in 1913. Laboratory results indicated 65 to 75% BOD reduction of spent sulfite liquor from pulp mills. In the meantime, shortage of feed proteins, as a result of World War II and newly developed demands for vitamin products, offered definite promise of sufficient market value of yeasts. The first commercial feed yeast plant to use spent sulfite liquor in the United

States started production in Wisconsin in 1948 with an initial design capacity of 4.5 tons of dry yeast daily (25). While Candida utilis is the most commonly used species, other species have been successfully cultivated with various kinds of waste products as substrates.

Nutritional Value of Yeast. Direct analysis of single-cell proteins (SCP) for the constituent amino acids indicate that the pattern of amino acids is reasonably good when compared to high quality proteins such as those in eggs and milk (33). Amino acid assays of Candida utilis produced on sulfite liquor compare favorably with milk and meat in amino acid composition (25). Yanez reported that this yeast has a high content of lysine and threonine (48). It is well recognized that the protein of cereal grains is deficient mainly in lysine (5). Therefore, yeast protein is a very good substance to supplement cereal grains. However, yeast appears to be somewhat deficient in the sulfur containing amino acids, which are essential and represent perhaps the major amino acid deficiency of many diets (7, 25, 33, 47). According to Food and Agriculture Organization, the requirements are for about 2 g cysteine and 2.2 g methionine per 100 g protein, however, average figures for these amino acids are under 2% in yeast protein when yeast cells are grown on molasses and sulfite liquor (7). It was reported that there is an important relationship between such amino

acids and liver damage. Rats fed yeast proteins showed growth disturbance and liver injuries (47). However, the inadequacy could be corrected by the addition of synthetic amino acids (7, 25, 47, 48). Cystine corrected the deficiency to a limited extent and soybean meal was found to be an adequate supplement to the protein of yeasts (5).

Results of rat feeding test showed that when yeast was supplemented with methionine, growth characteristics were comparable with those observed when the rats were fed a casein diet; while without additional methionine the gain in weight of the rats fed yeast was only 60% of those fed the casein (25). However, the amount and composition of amino acids in yeast will vary with the strain, the substrate upon which it is grown and the conditions of propagation (35, 47). Therefore, it may be possible to manipulate these variables to produce proteins with improved amino acid patterns. Genetic make-up might well be used advantageously to produce yeast protein higher in sulfur containing amino acids (5).

Yeasts are excellent sources of vitamins of the B-complex which makes them unique among protein concentrates from vegetable origin (5, 28, 47). Kurth and Cheldelin, working with Mycotorula, Torula, and Hansenula grown on wood sugar stillage, found that the yeast cells were good sources of B vitamins, particularly riboflavin, nicotinic acid, pantothenic acid and p-aminobenzoic acid (28). They

also noticed that variations in the vitamin content among the yeasts were somewhat larger than were observed for amino acids, although for most vitamins the values were similar. Other investigators also reported that yeasts were generally capable of producing high levels of riboflavin and pantothenic acid (5, 25) but these vitamins are subject to rapid destruction under conditions of final processing. It has been reported that processing of cells is an important factor influencing the availability of certain vitamins (47). Yeasts also contain small amounts of vitamin E and vitamin D. Most yeasts do not contain vitamin A or even β -carotene except Torulopsis rubia. The chemical composition and quantities of vitamins in yeast would vary according to condition of propagation, substrates, strains and the subsequent drying process (5).

[Being rich in purines and nucleoproteins there is a possibility that yeasts have some effects upon uric acid excretion (7, 47). Yeast diets could result in high blood levels of urea and uric acids, and possibly the accumulation of uric acid kidney stones (33).]

Most SCP has low digestibility and causes gastrointestinal upsets in humans. This is particularly true with algae (33). However, there is a general agreement that yeast protein is readily digested and absorbed by the rat and is a satisfactory protein for the dog, but somewhat inferior to animal protein in human nutrition.

Therefore, a thorough study of the effects of long-continued yeast ingestion on blood constituents is an essential prerequisite for any recommendation concerning the inclusion of large amounts of yeast in human diets (47).

Biomass Production. Under optimum temperature and aeration rates, the yield of a particular strain of yeast would depend on the amount of nutrients available. Shannon and Stevenson (41) reported that the cell yields from selected brewery waste ranged from 5.02 to 8.94 g/liter dry weight for Saccharomyces cerevisiae and from 3.65 to 9.68 g/liter for Candida utilis, depending on the substrate. Among the yeasts tested, the largest dry cell mass was obtained by C. steatolytica which was 10.56 g/liter. It was noted that there was a considerable difference in the yield obtained with each of the substrates and there was a significant variation in yield among different species grown on the same substrate. Cell yield increased with nitrogen supplementation and the maximum cell yield was 12.7 g/liter for C. steatolytica (42).

Harris et al. (24) reported that wood hydrolyzates from Douglas fir contain very few requirements for C. utilis production except the sugar; supplementation with nitrogen, phosphate, and potassium was necessary to obtain optimum yield. Phosphate and carbohydrate were limiting factors for C. utilis growth on peat extract; phosphate

supplementation alone was found to be capable of increasing biomass yield considerably (two or three times) and the yield was comparable with known microbiological media (31).

Gray et al. (20) reported that the yield of yeast on synthetic medium was 8.2 g/liter dried cells. Debaromyces kloeckeri (D. hansenii) grown on soybean spent solubles gave 14.61 g/liter dry cell yields (43).

Protein and Carbohydrate Production. A review by Rose and Harrison (37) indicates that one-half of the dry weight of yeast is crude protein (calculated as N x 6.25) consisting of 80% amino acids, 12% nucleic acids and 8% ammonia. Around 7% of the total nitrogen occurs as free amino acids, and the presence of large amount of purine and pyrimidine bases lowers the true protein of yeast to 40% of the dry weight. Bressani (5) noted that only about 80% of the total nitrogen of the yeast cell is in the form of protein.

According to the published data, SCP compares well with high quality protein sources such as egg, milk, meat and fish in terms of the amount of "crude protein" (33). The composition of dried yeasts varies to some degree according to the yeast strain and conditions for growth or propagation (47). The nature of growth medium and degree of aeration are major factors influencing the

carbohydrate, protein, fat and vitamin content of yeast cells (37).

Shannon and Stevenson (41) reported that protein content of dried cells ranged from 26.77 to 32.91% for Saccharomyces cerevisiae and from 27.11 to 28.67% for Candida utilis (calculated as N x 6.25) when grown on selected brewery wastes. The highest protein content obtained from yeast cells was 32.91%. According to their data there appeared to be little difference in total protein production among the yeasts examined. In subsequent studies they observed a significant increase in protein content of yeast cells when the substrate was supplemented with nitrogen (42). The protein content of C. steatolytica was 21.30 to 23.49% in cells produced in nonsupplemented substrates, but increased to as high as 44.25% in cells produced with nitrogen supplemented substrate.

Vavanuvat and Kinsella (45) reported that Saccharomyces fragilis cells were 50% Kjeldahl protein when grown continuously under optimum condition on crude lactose. Working with yeasts grown on molasses from different sources Agarwal et al. (1) recovered 42.5 to 53.1% protein from S. cerevisiae and 43.7 to 60.6% from C. utilis. Yeasts grown on potato starch waste were approximately 55% protein (36). The protein content of Debaromyces kloeckeri (D. hansenii) grown on soybean spent solubles was 34.4% (43). Kurth and Cheldelin (28)

reported a protein content of 52.9% for yeast grown on wood sugar stillage. According to Peppler (35) the protein content was 50% for S. cerevisiae grown on molasses, 55% for Torula grown on sulfite, and 54% for S. fragilis grown on whey. Torula yeast produced commercially from spent sulfite liquor had a protein content of 47.43% (48).

Frey reported that the composition of baker's yeast dry matter was 52.4% protein, 37.1% polysaccharides, 1.7% fat, and 8.8% ash (17). According to Von Loesecke (47), T. utilis was 43.87% protein ($N \times 6.25$) and 38.55% carbohydrates; while S. cerevisiae was 39.25 to 51.46% protein and 32.04 to 42.44% carbohydrates.

Working with yeasts on soybean spent solubles, Sugimoto reported that D. klockeri removed 97.2% of total carbohydrates, 88.7% organic acids, and 56% Kjeldahl N from the medium (43).

Effect of Temperature and Aeration on Yeast Growth.

Temperature can be expected to exert a profound effect on all aspects of growth, metabolism and survival of yeasts; and the optimum growth temperature is defined, usually, as the temperature at which the growth rate is highest (38). However, an optimum temperature exists for each of the following parameters: (a) the growth rate, (b) the maximum cell density, (c) the fermentation rate, (d) the maximum formation of products, (e) the rate of cell autolysis, and (f) there is a possibility of individual optima for the

separate products of fermentations (32). A review by Rose and Harrison (38) indicates that with bacteria the optimum temperature for fermentation is lower than that for growth due to the greater availability of oxygen to the bacteria at lower temperatures, and this phenomenon is also true for yeasts. Working with distiller's yeast, Merrit (32) reported that the optimum temperature for growth rate was 35 C, whereas 30 C was optimum for maximum cell yield and glycerol and fusel oil production, and 25 C was optimum for alcohol production and maltase activity.

Aeration is one of the most important factors affecting the time needed for maximal cell production (28). In studies of yeast grown on sugar stillage, Kurth (27) found that air dispersion was an important factor in the rate of yeast growth and consumption of sugar, and was more important than the total volume of air.

The Effectiveness of Yeast in Reducing BOD. The food processing industry has been aware of pollution potential of its waste products for some time and public awareness of the current environmental crisis has prompted the industries to take action. This activity has been aimed at reducing the impact of waste products on the environment. The methods employed by food processing industries to manage their waste disposal problems have been well reviewed by Soderquist (40). One of the methods is culturing single-cell protein on the waste.

Various kinds of wastes have proved to be good substrates for fungal growth, such as wood sugar stillage, wood hydrolyzates, sulfite liquor, peat, gas oil, crude lactose, molasses, and others. Among the food processing wastes, yeasts have been successfully cultivated on corn and pea canning wastes, brewery wastes, potato starch wastes, sauerkraut, soybean spent solubles, citrus pulp and perhaps some others. Recent examples of using yeasts for BOD reduction of food processing wastes are (a) Saccharomyces fragilis on crude lactose (45), (b) Candida utilis on sauerkraut wastes (21, 22), (c) various kinds of yeasts on selected brewery wastes (41, 42), and (d) yeasts on soybean spent solubles (43).

Working with yeasts grown on selected brewery wastes, Shannon and Stevenson (41) reported BOD reduction of 25.9 to 42.4% by Saccharomyces cerevisiae and 20.0 to 45.5% by Candida utilis. The highest BOD reduction achieved was 45.5%. Their data demonstrate that the percent BOD reduction given by a particular strain of yeast depends on the kind of substrate. In a subsequent experiment (42), they reported a maximum BOD reduction of 55% given by C. steatolytica when the substrate was supplemented with nitrogen. Torulopsis utilis (Candida utilis) grown on the protein waste water from potatoes resulted in 60% BOD reduction based on 40% solid recovery (36). A chemical oxygen demand reduction of 61.2% was

achieved when S. fragilis was grown on crude lactose in a continuous culture (45). Hang et al. (22) reported BOD reduction from 12,400 ppm to 1,400 ppm (equal to 88.71% reduction) by growing C. utilis in sauerkraut waste for the production of yeast invertase. In a subsequent experiment (21), they reported that the BOD of sauerkraut waste was reduced by C. utilis, S. fragilis and S. cerevisiae from an initial value of 12,000 mg/liter to 1,550, 1,950, and 3,750 mg/liter, respectively, in 48 hours. These values correspond to 87%, 83.75%, and 68.75% reduction respectively. Inskeep et al. (41) reported that Torula utilis grown on sulfite liquor resulted in a 75% BOD removal. Working with yeast grown on wood sugar stillage, Kurth (27) reported a BOD reduction from 16,400 to 9,600 ppm (42% reduction). The results of experiments of growing yeast on soybean spent solubles by Sugimoto (43) demonstrated that BOD reduction depends on the yeast strain and the chemical composition of the waste materials. The BOD of soybean spent solubles was reduced by 83.8% through yeast cultivation.

MATERIALS AND METHODS

Cultures and Cultural Methods. With the exceptions of Candida utilis and Saccharomyces cerevisiae the yeast cultures used in this study were all isolated from cucumber fermentations by Etchells and coworkers (13, 14) and by Costilow and Fabian (8). Most of the cultures (see Table 1) were obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, Peoria, Ill. All cultures were maintained on vegetable juice agar slants (13), and transferred on a monthly schedule. All broth cultures were in 100 ml quantities in 500 ml Erlenmeyer flasks and were incubated on a rotary shaker.

Initially, various yeast cultures were screened for salt tolerance in dextrose broth (Difco) acidified to pH 5.0 with lactic acid. Slant-cultures of the yeasts used were suspended in 10 ml of sterile water and 0.2 ml of the suspensions were used for inoculation of the test media. All other growth studies were conducted in salt-stock brines prepared as outlined below. Unless otherwise indicated 5 ml of a 24-hr culture which had been subcultured daily in 10% salt brine for at least one week was

used to inoculate 100 ml of brine. Several subcultures in the 10% salt brine was necessary to fully adapt the culture to obtain the most rapid growth.

Salt-Stock Brine. The salt-stock brines used were obtained from commercial tanks of brined and fermented cucumbers at the Heifetz Pickling Co., Eaton Rapids, Michigan. The cucumbers had been fermented several months before the brines were taken for these experiments. The pH of all lots of brine used ranged from 3.4 to 3.7, the titratable acidity calculated as lactic acid varied from 0.4 to 0.65%, and the salt concentration ranged from 10 to 15%. Salt-stock brines with 5 and 10% salt were prepared by dilution with distilled water. Except for the initial experiments to determine salt tolerance, brines to be used in growth experiments were centrifuged at 10,000 x g for 20 minutes to remove suspended solids. The brines were pasteurized in the flasks before inoculation. Initially, they were held at 50 C for 20 minutes, but most were steamed for 15 minutes.

Analytical Methods. Growth was estimated by measuring the optical density (O.D.) at 600 nm using either a Spectronic 20 colorimeter (Bausch and Lomb Optical Co.) or a Gilford, Model 2000, spectrophotometer (Gilford Instrument Co.). The instrument used for individual experiments is specified since the value obtained with the

Gilford instrument for a given yeast suspension was approximately twice that measured with the Spectronic 20 colorimeter.

Cell yields were estimated by determining the dry weights of replicate samples after centrifuging and washing the cells. The samples were dried to constant weight at 60 C (~ 48 hr).

The pH was measured with a Beckman, Model G, pH meter. The titratable acidity was determined by titration with standard 0.05 NaOH to the phenolphthalein endpoint and calculated as percent lactic acid. The salt concentration was estimated by use of a salometer (hydrometer) using appropriate corrections for temperature.

The amounts of proteins in the brine was estimated by the colorimetric procedure of Lowry et al. (30), and the protein content of the yeast cells was determined by nesslerization of an acid hydrolyzate of the cells (44). Total carbohydrates in the brine was determined by the anthrone procedure (34), free amino acids by the ninhydrin reaction (39), total organic matter by chromic acid oxidation (26), and biochemical oxygen demand (BOD) according to "Standard Methods for Examination of Water and Wastewater" (3).

RESULTS

Effect of Salt Concentration on Yeast Growth. The effect of salt concentration on the growth and acid utilization by yeasts isolated from cucumber brines is shown in Table 1. Data for broth with 15% salt are not presented since little or no growth was observed with any of the cultures. All yeasts tested grew well in the medium with 5% salt, but all cultures except D. membranaefaciens var. hollandicus were inhibited to some extent by 10% salt. The response of D. membranaefaciens var. hollandicus was particularly interesting in that the culture appeared to be halophilic; there was very little growth in the medium without added salt, and growth was essentially the same in the media containing 5 and 10% salt. This yeast culture also utilized the lactic acid more completely at the 10% salt level than did the other species tested.

The four yeast species which were found to grow most extensively in the dextrose broth with 10% salt were then tested for their ability to oxidize the acid in salt-stock pickle brines containing 10% and 15% salt. All four yeasts tested reduced the acidity of the brines even with 15% salt (Table 2). However, D. membranaefaciens var.

Table 1.--Growth of various yeasts in acidified dextrose broth (Difco) with different salt concentrations.^a

Culture ^b	Salt Concentration (%)	O.D. at 600 nm	pH	% acid as lactic
<u>Torulopsis holmii</u> (FBY-3)	0	6.08	7.2	0.06
	5	3.54	6.8	0.05
	10	3.12	5.6	0.08
<u>Pichia alcoholophilia</u> (FBY-12; NRRL Y-1896)	0	3.49	5.6	0.12
	5	3.15	5.0	0.12
	10	0.00	4.3	0.19
<u>Torulaspora rosei</u> (FBY-20; RY-8)	0	6.18	7.2	0.05
	5	3.48	6.7	0.05
	10	3.08	6.4	0.06
<u>Candida krusei</u> (FBY-31; NRRL Y-301)	0	6.60	7.4	0.04
	5	3.47	6.8	0.05
	10	1.15	4.1	0.19
<u>Debaromyces membranaefaciens</u> var. <u>hollandicus</u> (<u>Debaromyces nicotianae</u>) ^c (FBY-44; NFY-32)	0	0.51	4.5	0.20
	5	2.82	7.8	0.02
	10	2.94	7.7	0.02
<u>Zygosaccharomyces</u> <u>halomembranis</u> (FBY-38; SPY-32)	0	3.33	5.0	0.16
	5	1.31	4.2	0.22
	10	0.01	4.3	0.15
<u>Endomycopsis ohmeri</u> (FBY-5; NRRL Y-1922)	0	6.31	7.3	0.04
	5	3.42	7.8	0.02
	10	0.02	4.3	0.17
<u>Endomycopsis ohmeri</u> Mixture of two mating types (FBY-76; NRRL Y-1922 +NRRL Y-2078)	0	6.25	8.1	0.01
	5	2.91	7.5	0.03
	10	3.17	5.8	0.08

Table 1.--Continued.

Culture ^b	Salt Concentration (%)	O.D. at 600 nm	pH	% acid as lactic
Control	0	0.00	4.5	0.19
	5	0.00	4.3	0.19
	10	0.00	4.3	0.19

^a Observations were performed 8 days after the broth was inoculated. The O.D. was measured using a Gilford Spectrophotometer.

^b FBY numbers are stock culture numbers used in this laboratory; other numbers given are those on the cultures at time of receipt.

^c The yeast D. membranaefaciens var. hollandicus has been renamed as D. nicotianae (29).

Table 2.--Growth of selected yeasts in salt-stock pickle brines with different salt concentrations.^a

Culture	Salt Concentration (%)	pH	% acid as lactic
<u>Torulaspora rosei</u>	10	4.2	0.12
	15	3.9	0.21
<u>Torulopsis holmii</u>	10	4.1	0.14
	15	3.6	0.36
<u>Debaromyces</u> <u>membranaefaciens</u> <u>var. hollandicus</u>	10	8.4	0.00
	15	4.0	0.22
<u>Endomycopsis ohmeri</u> Mixture of two mating types	10	4.5	0.09
	15	3.9	0.22

^aObservations were performed 6 days after the brine was inoculated. The initial lactic acid concentration in the 15% salt brine was 0.56% and the pH was 3.4, the 10% salt brine contained 0.37% acid as lactic acid and the pH was 3.5.

hollandicus was the most effective of the four species in removing the acid from brine containing 10% salt. This result was confirmed in a separate experiment using salt-stock pickle brine. With 10% salt (Table 3) D. membranaefaciens var. hollandicus not only removed more of the acid present but the culture attained an optical density almost twice that of the next best growing culture, E. ohmeri. Two common food yeasts, Candida utilis and Saccharomyces cerevisiae, showed no significant growth or acid utilization in this brine.

These data led to the selection of D. membranaefaciens var. hollandicus (FBY-44) for use in all further experiments in this study.

Effect of Temperature and Shaker Speed on Yeast Growth. The growth rates of D. membranaefaciens var. hollandicus in salt-stock pickle brine with 10% salt were essentially the same at 25 and 30 C, but no growth occurred at 35 C (Fig. 1). Similarly, the rates of acid utilization were not significantly different at the two lower temperatures, while only a relatively small amount of acid was used at 35 C (Fig. 2). It is apparent that this yeast can oxidize all the acid from such brines during a 25-30 hour incubation period. All subsequent experiments were conducted at 30 C.

Table 3.--Growth and acid utilization by various yeasts in salt-stock pickle brine with 10% salt.^a

Culture	O.D. at 600 nm	pH	% Acid as lactic
<u>Candida utilis</u> (NRRL Y-900)	0.25	3.4	0.55
<u>Saccharomyces cerevisiae</u> (NRRL Y-2572)	0.28	3.5	0.55
<u>Torulaspora rosei</u>	1.69	3.7	0.27
<u>Torulopsis holmii</u>	1.72	3.6	0.30
<u>Endomycopsis ohmeri</u> Mixture of two mating types	3.54	4.1	0.13
<u>Debaromyces</u> <u>membranefaciens</u> var. <u>hollandicus</u>	6.34	4.6	0.02

^aInitially the brine contained 0.55% lactic acid and the pH was 3.4. The inoculated brines were incubated 3 days. The O.D. was measured using a Gilford spectrophotometer.

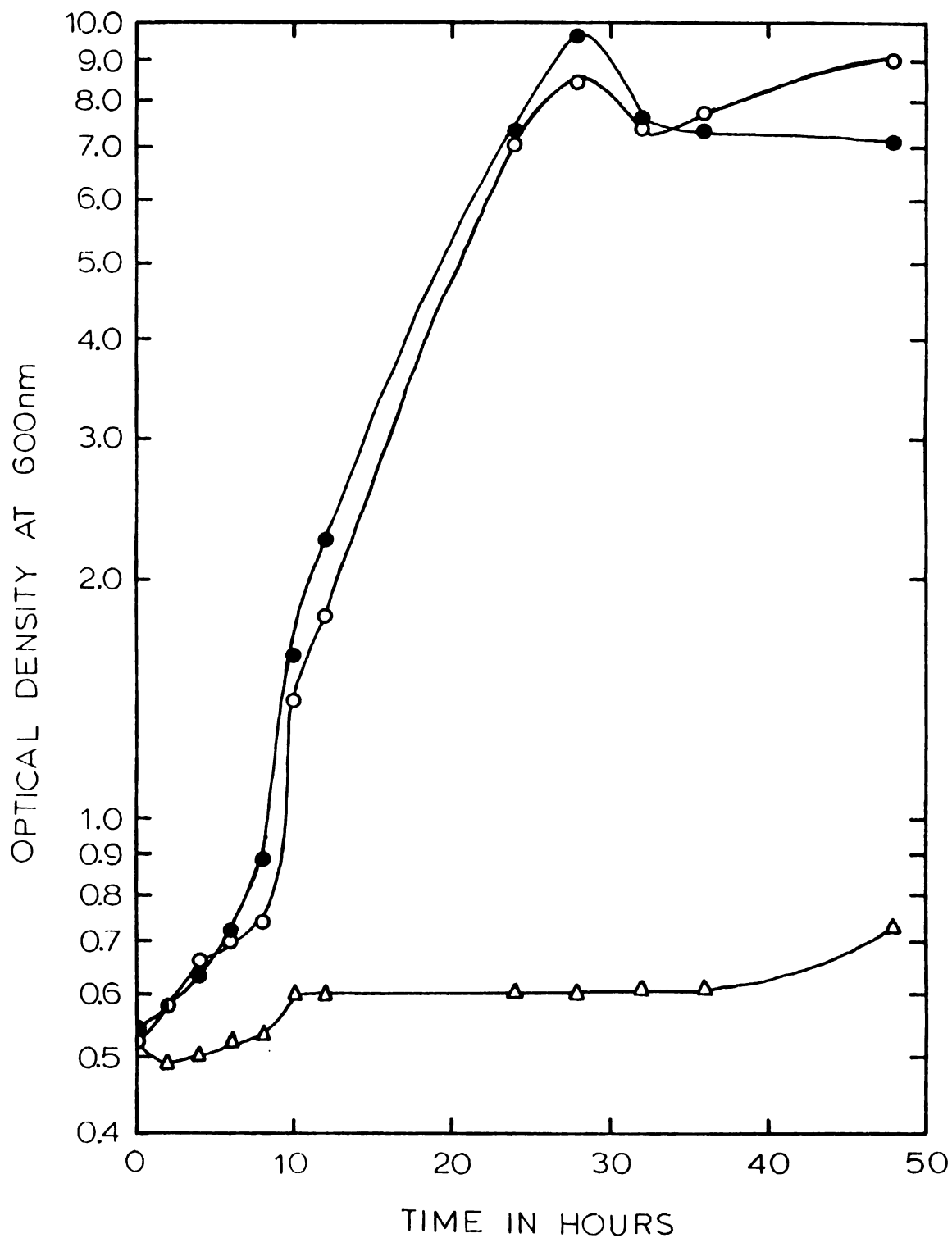


Fig. 1.--Yeast growth in salt-stock brine at various temperatures. The shaker speed was 350 rpm. Symbols: o, 25 C; ●, 30 C; Δ, 35 C. The optical density was measured using a Gilford spectrophotometer. The O.D.₆₀₀ of the inoculum was 5.8.

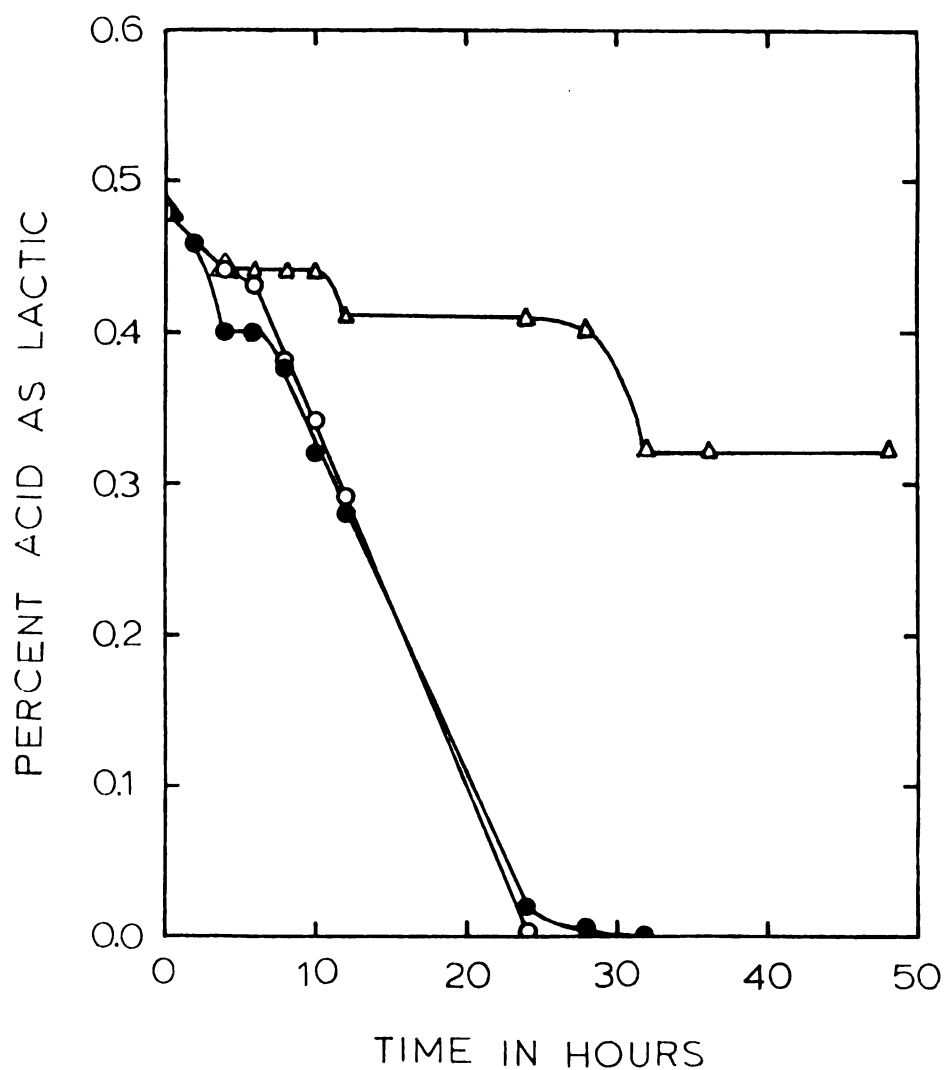


Fig. 2.--Effect of various temperatures on the rate of lactic acid utilization by *D. membranaefaciens* var. *hollandicus*. The shaker speed was 350 rpm. Symbols: o, 25 C; ●, 30 C; Δ, 35 C.

The speed of the shaker on which the cultures were incubated had a pronounced effect on growth responses (Fig. 3) and on acid utilization (Fig. 4). Obviously, oxygen was limiting at the slower shaker speeds used (125 and 210 rpm). There was no significant difference noted during early growth at the two higher shaker speeds (350 and 400 rpm); but growth ceased earlier in the culture at 400 rpm than in that at 350 rpm. This correlates with the time that all the acid had been oxidized (Fig. 4). It is possible that the data fail to reflect the true picture of growth since no optical density measurements were taken between 12 and 18 hours.

Since the differences observed at the two higher shaker speeds were of doubtful significance, a speed of 350 rpm was selected for use in subsequent experiments.

Effectiveness of *D. membranaefaciens* var. *hollandicus* in Reducing the Levels of Various Organic Compounds in Salt-Stock Brine. Sub-samples were removed at various intervals during incubation of a culture in salt-stock brine (10% salt) at 30 C on a rotary shaker operating at 350 rpm. Portions of these sub-samples were used for measuring growth, acidity, and pH. The remaining portions were centrifuged to remove yeast cells and then analyzed for various organic compounds. The typical growth of the culture and the rate of acid utilization and the changes in pH are illustrated in Fig. 5A. The levels of protein, amino acids,

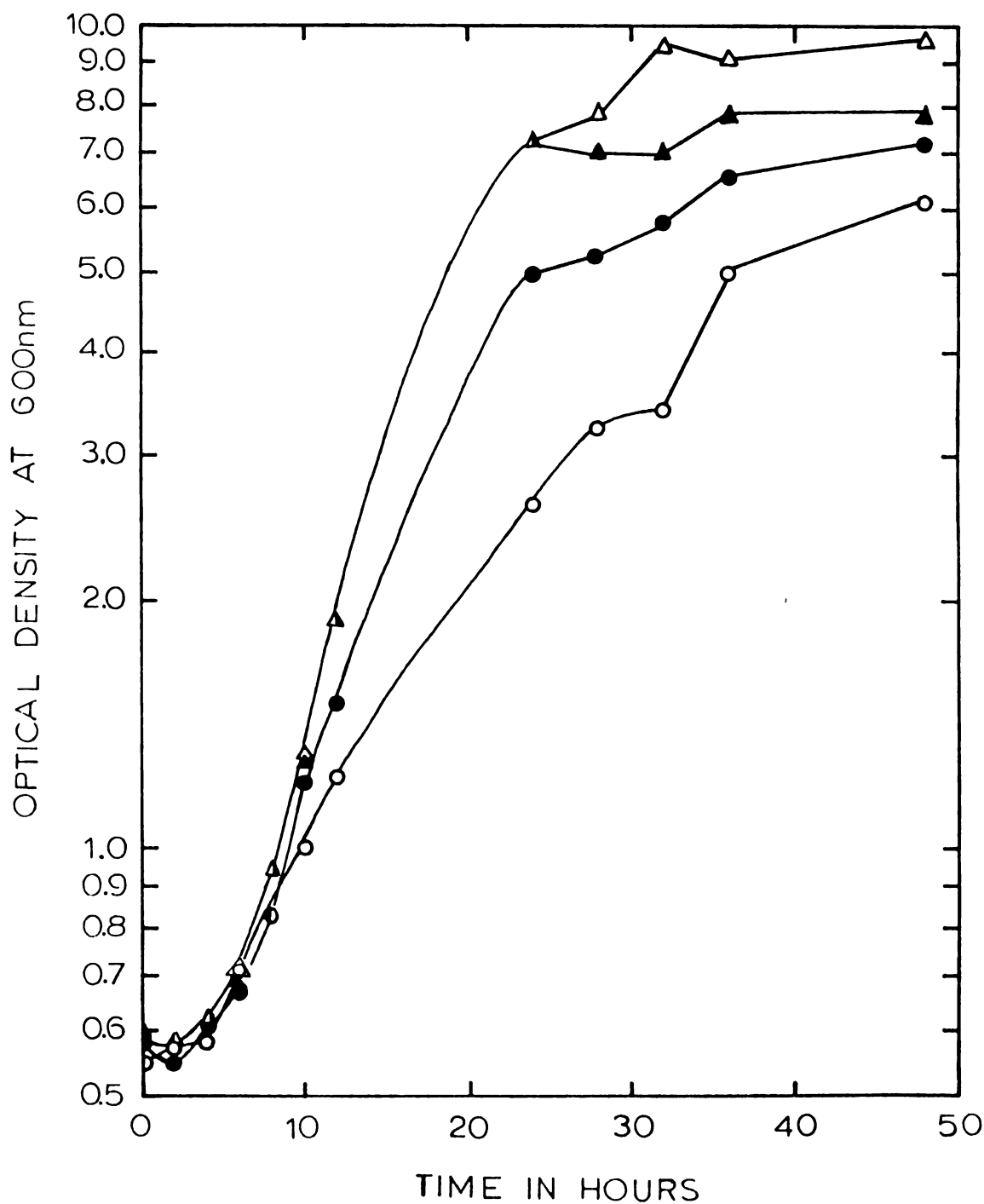


Fig. 3.--Yeast growth in salt-stock brine at various shaker speeds. The temperature was 30 C. Symbols: o, 125 rpm; ●, 210 rpm; Δ, 350 rpm; ▲, 400 rpm. The optical densities were measured using a Gilford spectrophotometer. The O.D.₆₀₀ of the inoculum was 7.10.

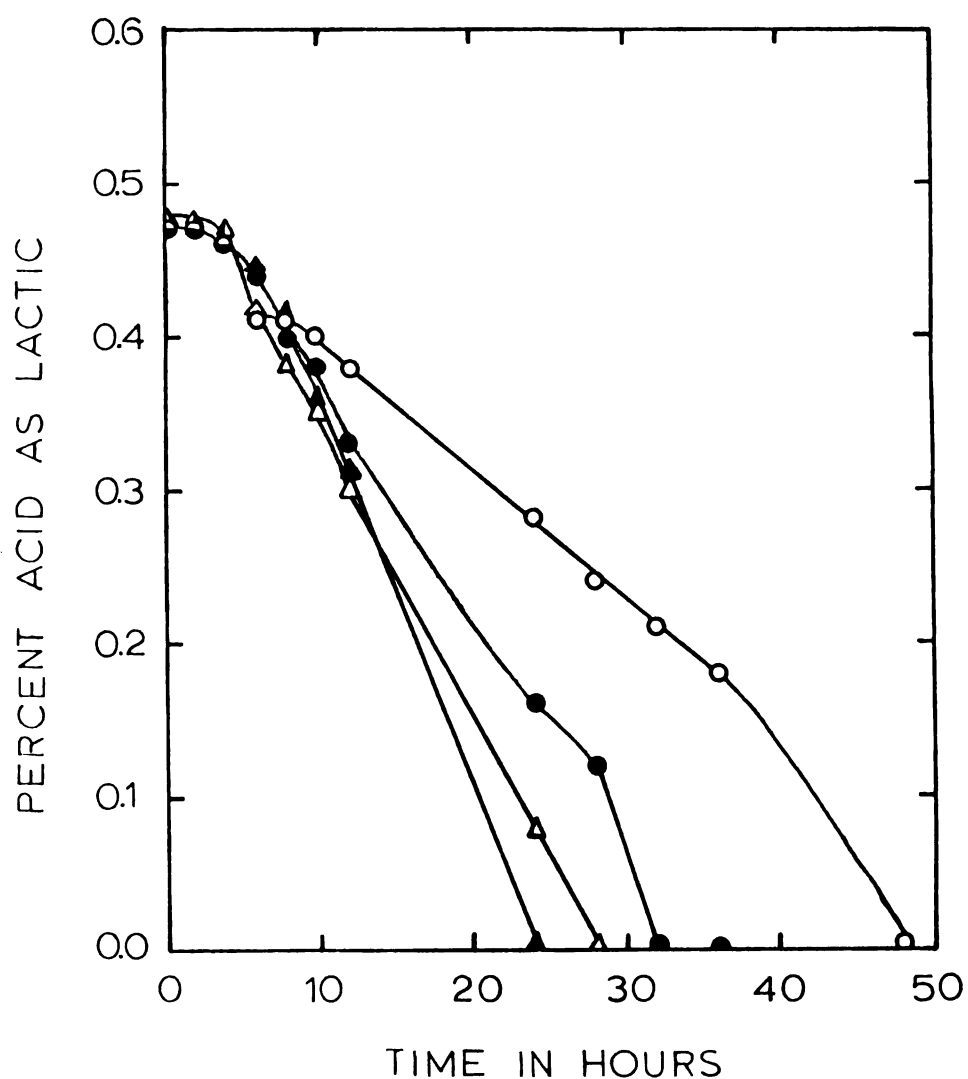


Fig. 4.--Effect of various shaker speeds on the rate of lactic acid utilization by D. membranaefaciens var. hollandicus. The temperature was 30 C. Symbols: o, 125 rpm; ●, 210 rpm; Δ, 350 rpm; ▲, 400 rpm.

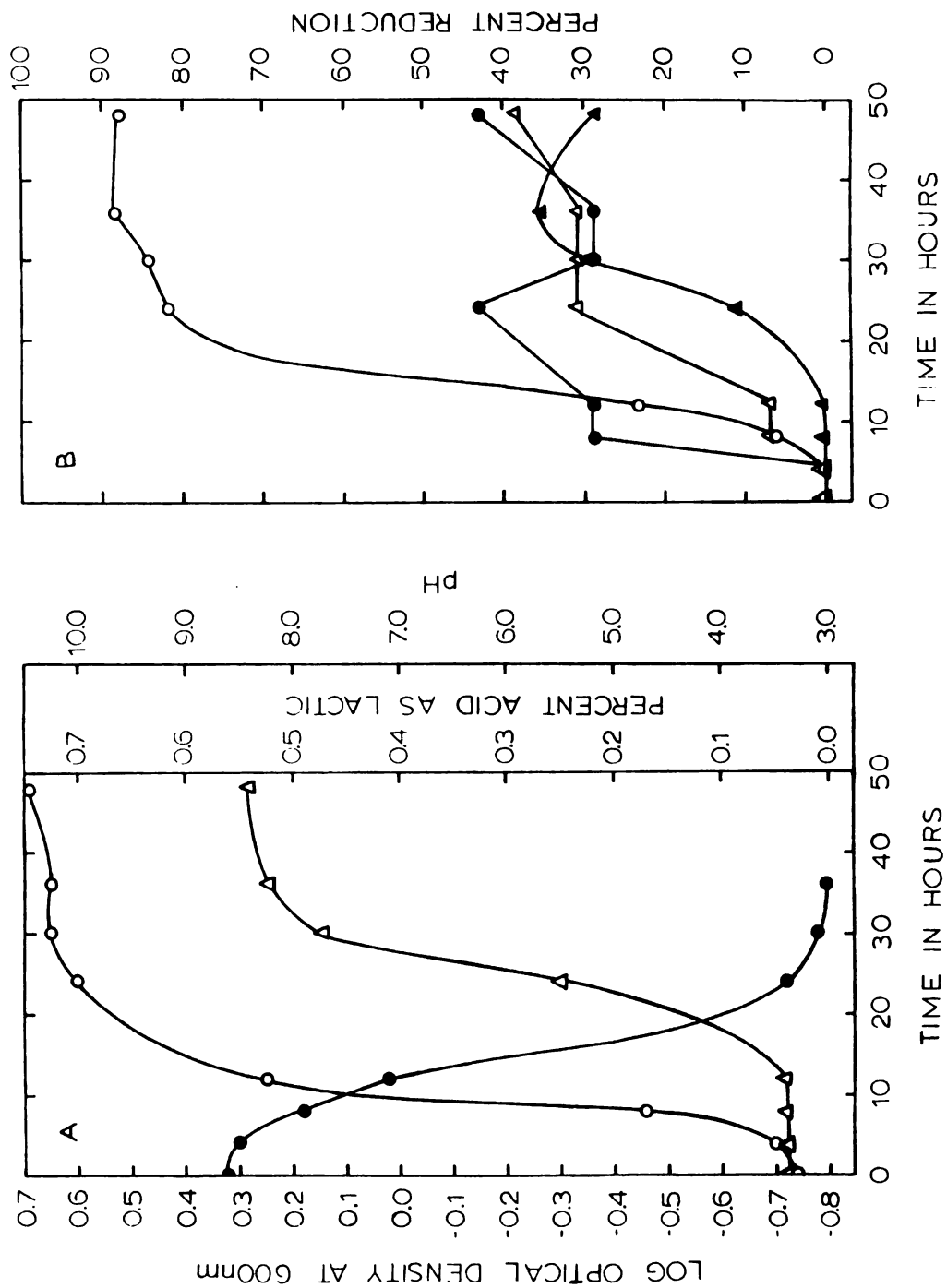


Fig. 5.--A. Changes in O.D. (o-o), titratable acidity ($\bullet$), and pH (Δ) during growth of *D. membranefaciens* var. *hollandicus* in salt-stock brine. B. Percent reduction in various organic compounds in salt-stock brine during growth of the yeast. Symbols: o, amino acid; $\bullet$, carbohydrate; Δ , protein; $\blacktriangle$, organic matter.

total carbohydrates, and organic matter are given in Table 4, and the percent reduction in these levels is illustrated in Fig. 5B.

The organic compounds used most completely were the amino acids; 80-85% of these acids were removed during the period of most rapid yeast growth. They may have become limiting for growth at some stage. Most of the carbohydrates which were utilized (~ 30% of the total present) disappeared prior to the time when the yeast was growing at the highest rate (compare Fig. 5A and 5B). It is likely that the small amount of carbohydrate used was readily oxidizable by the cells. The data indicates that about 30% of the protein in the brine was used during the period of maximal yeast growth. However, this may only reflect the utilization of amino acids, since protein was estimated by the Lowry et al. (30) colorimetric assay.

It is interesting that the greatest reduction in the level of organic matter occurred as the yeast culture approached the stationary phase of development. This time period does not correspond to the period of most rapid utilization of any of the other components measured. Therefore, it is not known what organic component(s) estimated by this assay were being utilized.

Reduction of BOD by *D. membranaefaciens* var. *hollandicus*. The effect of the yeast growing under the same conditions outlined above on the BOD of salt-stock pickle

Table 4.--Effectiveness of *D. membranaefaciens* var. *hollandicus* in reducing the levels of various organic compounds in salt-stock brine.^a

	Before Inoculation	Incubation Time (1 hr)							
		0	4	8	12	24	30	36	48
Protein	1.3	1.4	1.3	1.2	1.2	0.9	0.9	0.9	0.8
Amino acids	1.6	1.8	1.8	1.5	1.3	0.3	0.3	0.2	0.2
Carbohydrates	0.7	0.7	0.7	0.5	0.5	0.4	0.5	0.5	0.4
Organic matter	21.8	23.3	24.5	24.3	24.1	19.4	15.3	14.0	15.6

^aRefers to soluble protein, amino acids, carbohydrates and organic matter in the brine. O.D. 600 of the inoculum was 2.6.

brine is given in Table 5. It is apparent that the greatest reduction in BOD occurred during the period when yeast growth and acid utilization was maximal (see Fig. 5A). However, there was a further reduction in BOD until the time when all of the acid in the brine had been utilized.

The extent of BOD reduction observed was as high as fermented brines clarified by pH adjustment and sedimentation as in brines clarified by centrifugation (Table 5). This may be a very practical method of harvesting the yeast cells.

Biomass Production. The cell yields of D. membranaefaciens var. hollandicus from cultures in salt-stock pickle brines containing 10% salt and about 0.6% acid as lactic was about 5.0 g dry weight per liter of culture. These cells were harvested at about the time that all of the acid was exhausted from the brine. There was a very good correlation between the O.D. as measured in the spectronic 20 colorimeter and cell density. Fig. 6 shows that with this instrument there were about 1.25 g dry weight/liter/O.D.

It appeared possible that the cell yield was limited by the amount of lactic acid available. Therefore, growth studies were conducted with salt-stock brines containing 10% salt and supplemented with lactic acid to yield brines after inoculation containing 0.59, 0.70, and 0.96% acid calculated as lactic. It is apparent in Fig. 7

Table 5.--The effectiveness of *D. membranaefaciens* var. *hollandicus* in reducing the BOD of salt-stock pickle brine.^a

Sample	Expt. ^b	BOD	
		ppm	% reduction
Uninoculated	A	8,000	--
	B	6,000	--
Fermented			
24 h	A	2,950	63.8
24 h	B	3,066	48.9
48 h ^c	A	2,250	71.9
32 h ^c	B	1,875	68.7

^aThe initial salt concentration of the brines was 10% and the initial acidity of the brine used in Expt. A was 0.56% and that used in Expt. B was 0.36% calculated as lactic acid.

^bIn experiment A, the brine samples were clarified by centrifugation before BOD determinations were made. In experiment B, the brine was not centrifuged; however, the samples taken after fermentation were clarified by adjusting the pH to ~ 10.5 and allowing the flocculated yeast cells and other particulate material to settle out.

^cThere was no titratable acidity remaining in the brine at this time.

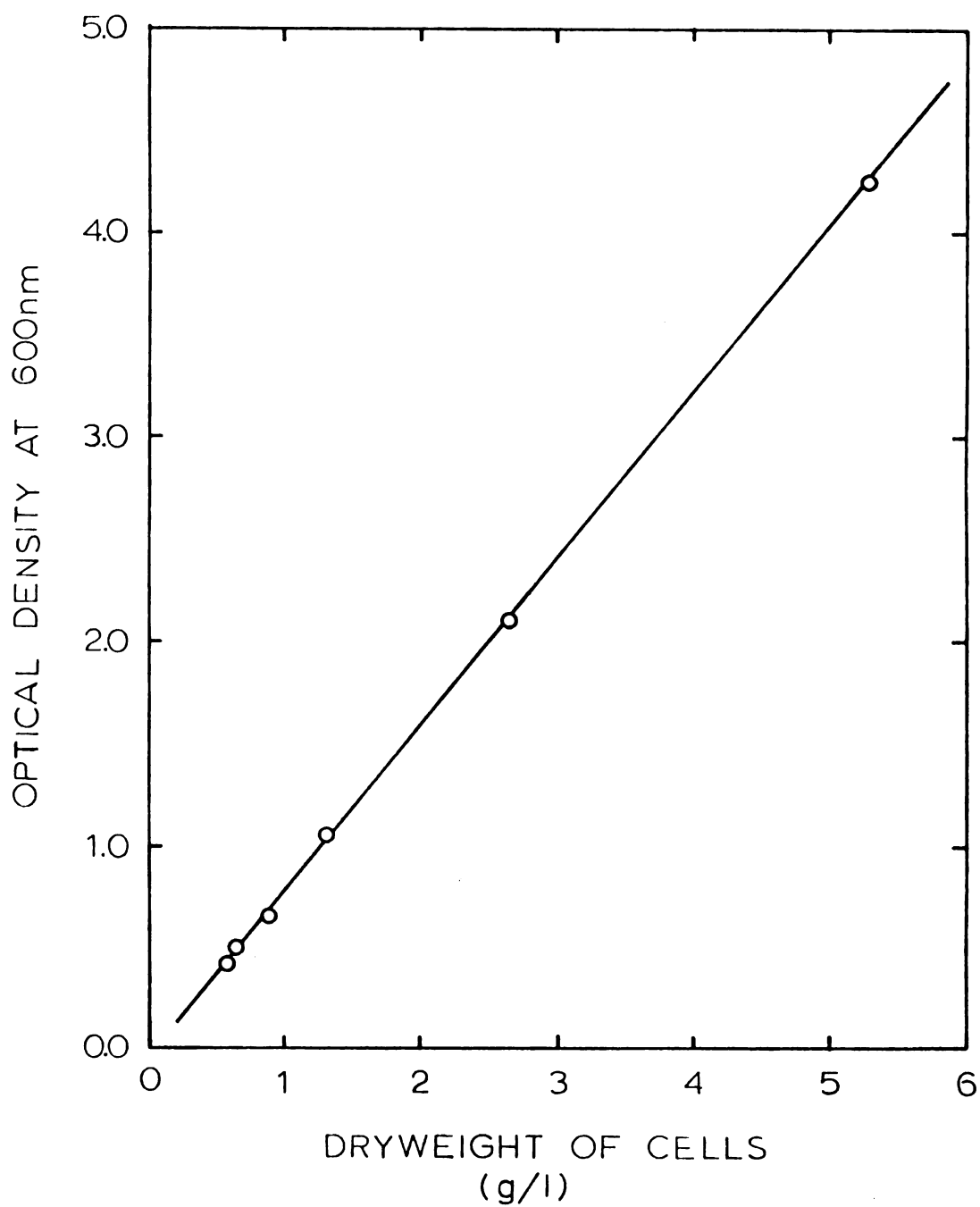


Fig. 6.--Dry weight of cells vs. their optical densities at 600 nm. The optical densities were measured using a Spectronic 20.

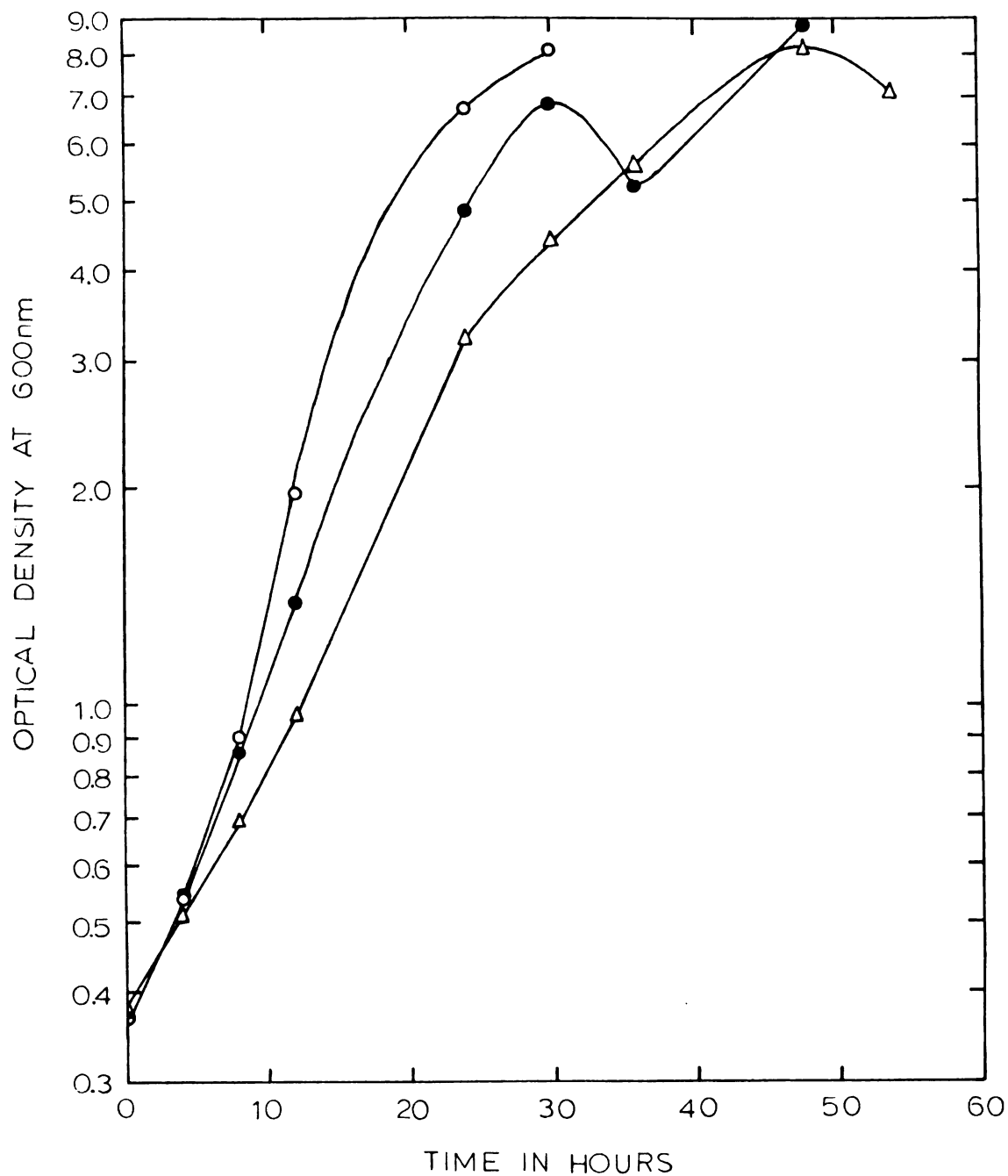


Fig. 7.--Yeast growth in brines at various concentrations of lactic acid. The initial acid concentrations were: o, 0.59%; ●, 0.70%; Δ, 0.96%. The optical densities were measured using a Gilford instrument. The O.D.₆₀₀ of the inoculum was 3.80.

that the rate of growth was decreased by increasing acid concentrations. The rate of acid utilization appeared similar in the three cultures; but longer times were required to utilize all of the acid present as the initial acid levels were increased (Fig. 8).

The optical densities recorded at the times that the acidity of each of the three cultures reached zero failed to indicate any great differences in cell yields. The dry weights of the three cultures with initial acid levels of 0.59, 0.70, and 0.96% calculated as lactic acid were 4.78, 5.16, and 5.79 respectively. These yields correspond to 0.81, 0.74, and 0.60 g dry weight of cells per g of acid (calculated as lactic acid) utilized in order of increasing lactic acid levels. Therefore, it appears that it is not the acid substrate alone which is limiting cell yields.

The correlations between dry weights and optical densities of the three cultures with different initial levels of acid are shown in Fig. 9. The optical densities recorded in this experiment were measured with a Gilford Spectrophotometer, which accounts for the difference in relative values as compared to data in Fig. 6 for which a spectronic 20 was used. With the two lower substrate levels, the correlations observed was 0.59 g dry weight of cells/liter/O.D. However, in the culture with the high initial acid level this value was found to be 0.81. Thus,

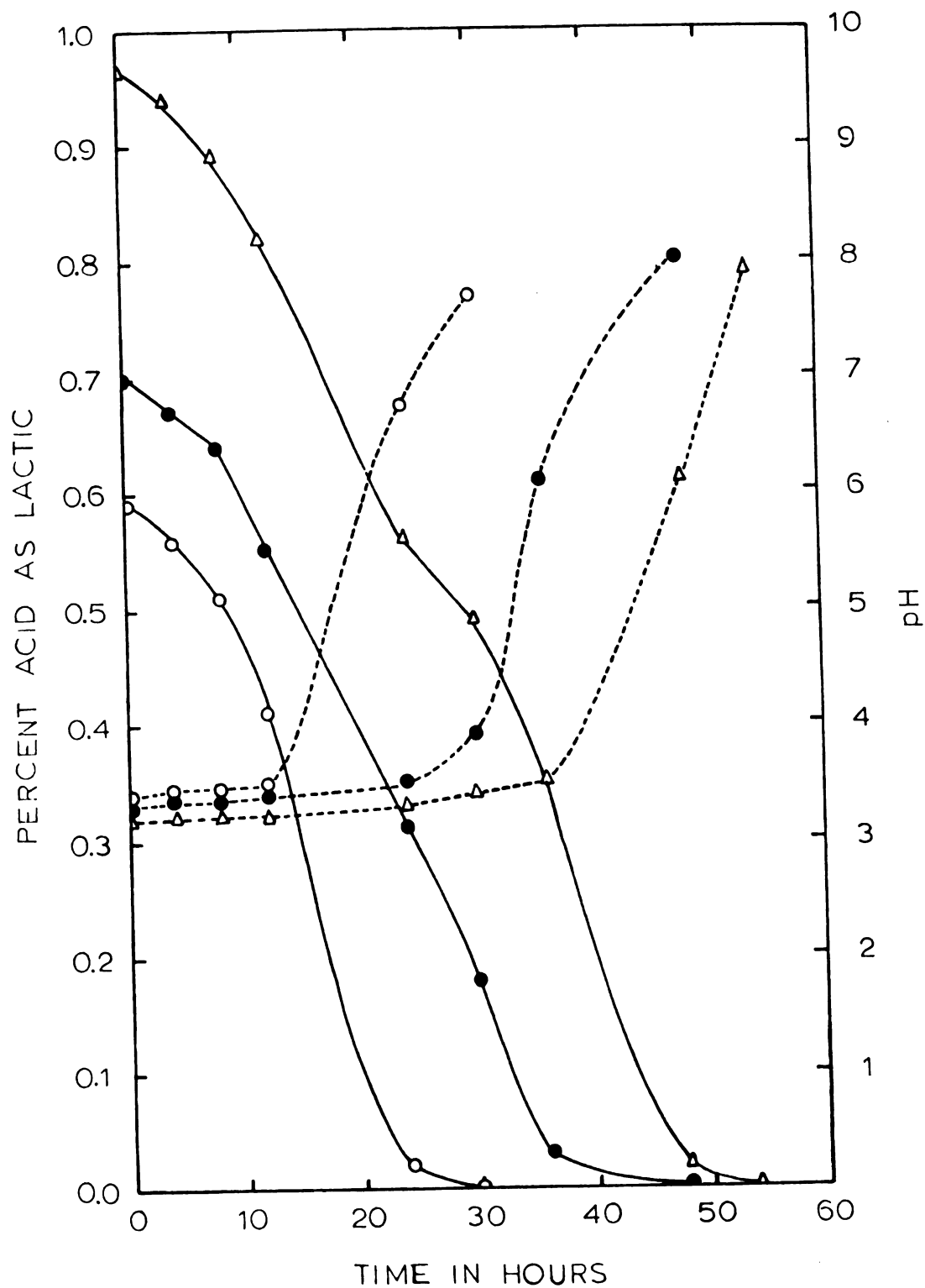


Fig. 8.--Effect of yeast growth on lactic acid reduction and pH increase of the brine with various initial lactic acid concentrations. Symbols: o, 0.59%; ●, 0.70%; Δ, 0.96%; —, percent acid; ----, pH.

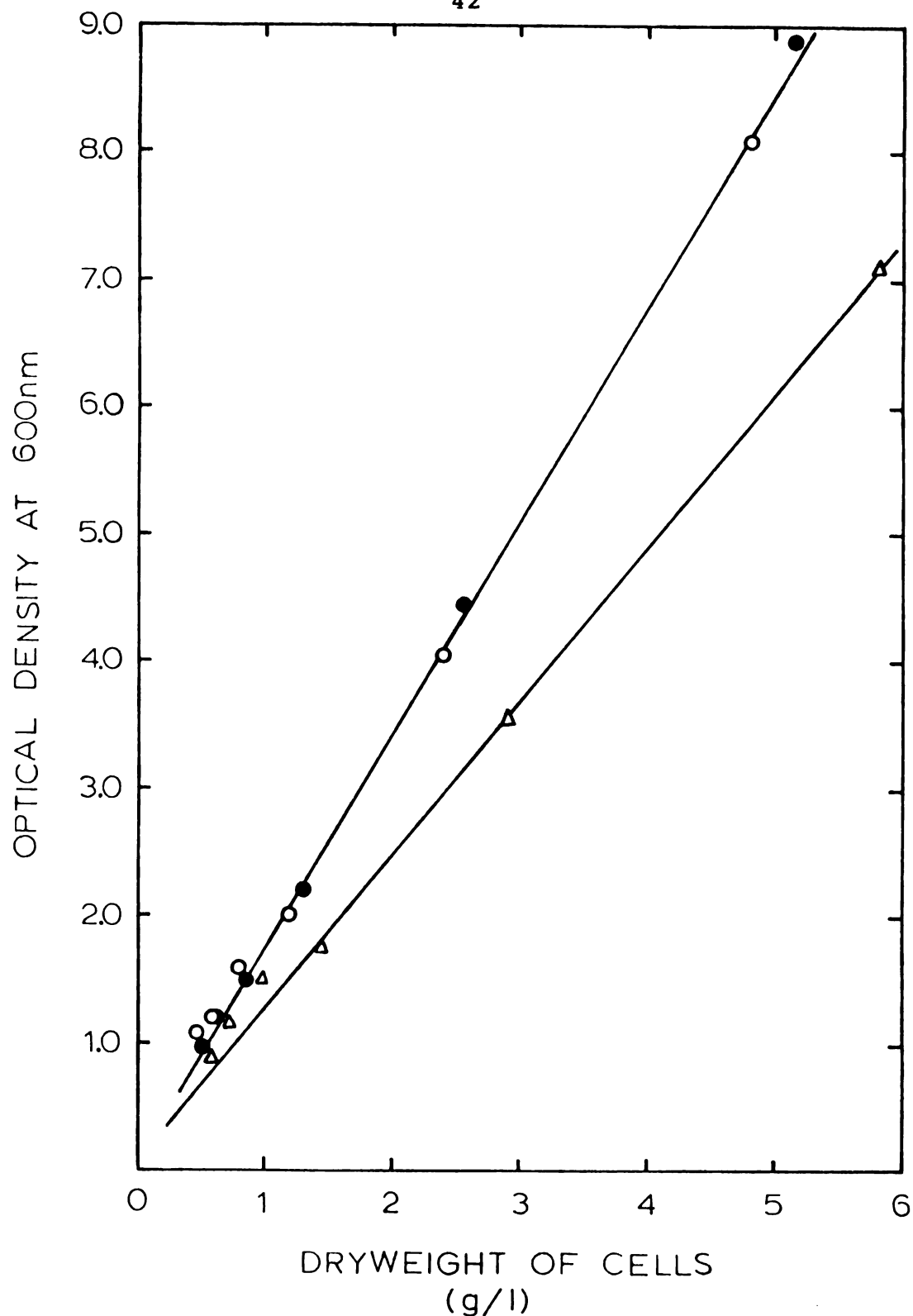


Fig. 9.--Dry weight of yeast cells grown in the brines of various lactic acid concentrations vs their optical densities at 600 nm. The initial lactic acid levels were: o, 0.59%; ●, 0.70%; Δ, 0.96%. The optical densities were measured using a Gilford instrument.

it appears that a given mass of cells produced in the high acid medium did not scatter light to the same degree as cells produced in a medium with lower initial acid levels.

The effects of nitrogen supplementation on yeast growth, pH and lactic acid content of salt-stock brine are presented on Fig. 10 and Fig. 11. Maximum growth was achieved earlier when the medium was supplemented with nitrogen. However, this was due to shortening of the lag periods rather than changes in growth rates. Supplementation with 0.5% N gave the highest optical density, the most rapid lactic acid reduction and the most rapid increase in pH of the medium. However, the differences observed were not great enough to indicate that nitrogen supplementation would be practical under commercial conditions.

Yeast cells produced in salt-stock pickle brines were analyzed for protein and carbohydrate. Crude protein as estimated by the microkjeldahl procedure constituted 30% of the dry weight, and the total carbohydrate as estimated by the anthrone method accounted for an additional 36% of the dry weight.

Role of Yeast Fermentation in Recycling of Salt Stock Brine. As mentioned earlier, there is much interest in the re-use of salt-stock brine for brining fresh cucumbers. One of the problems in doing this is the necessity to insure that pectinolytic and cellulolytic enzymes found in some brines are inactivated. These enzymes may cause

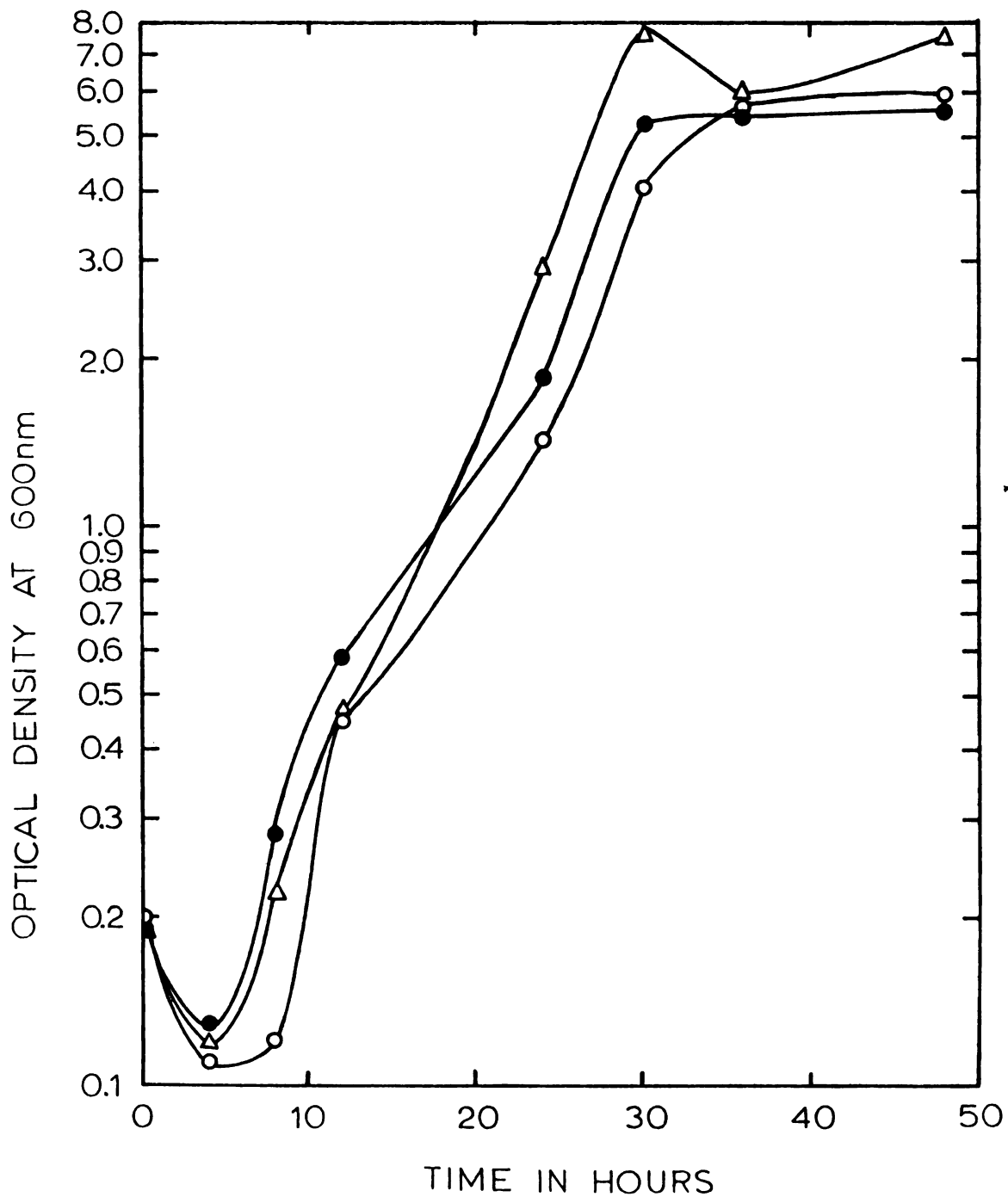


Fig. 10.--Effect of nitrogen supplementation on the growth rate of yeast. The levels of $(\text{NH}_4)_2\text{SO}_4$ supplementations were: o, none; ●, 0.2%; Δ, 0.5%. The optical densities were measured using Gilford spectrophotometer. The O.D.₆₀₀ of the inoculum was 3.82.

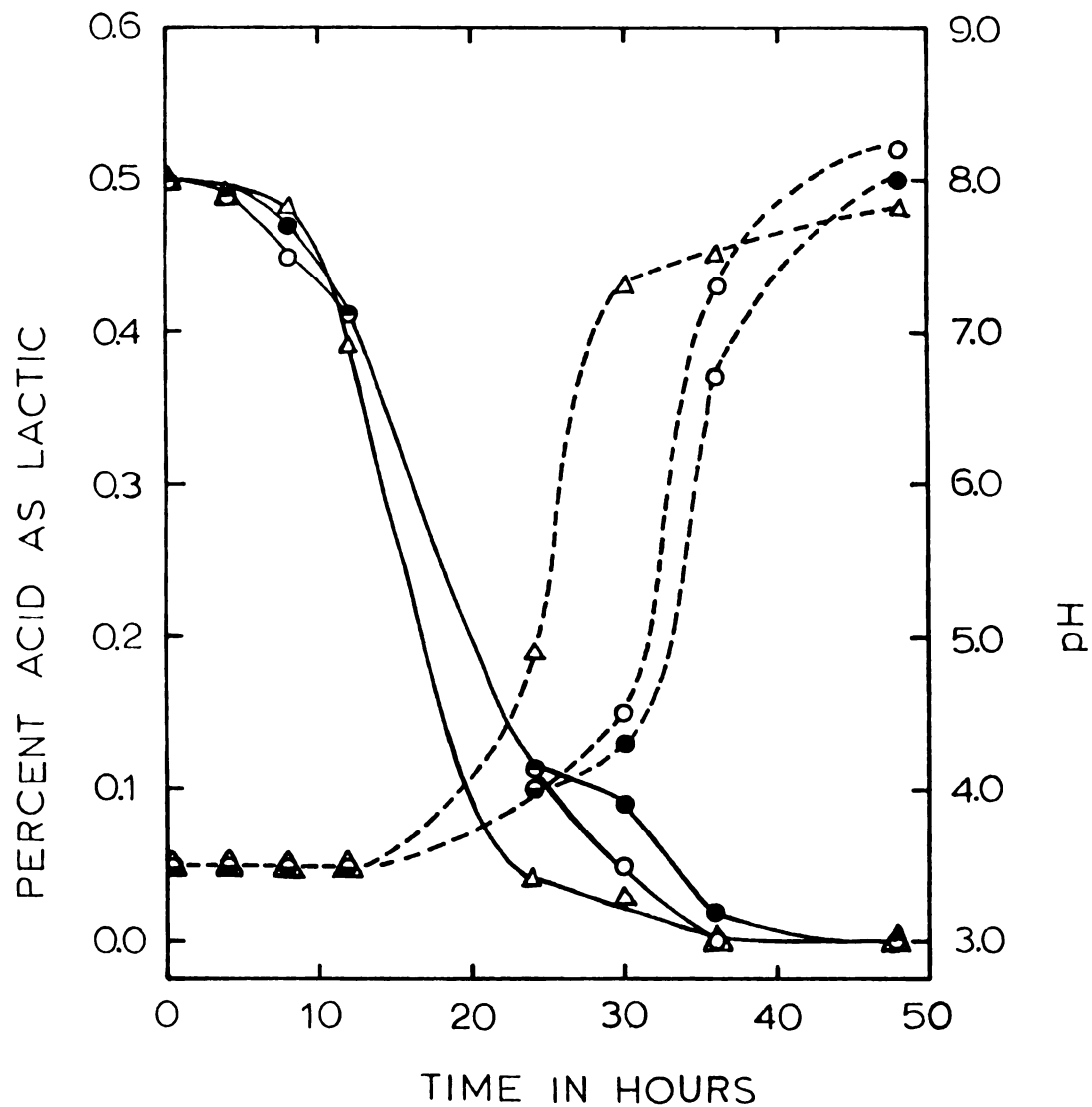


Fig. 11.--Effect of nitrogen supplementation on the capacity of yeast to reduce the lactic acid in the brine and to increase the pH. The $[(NH_4)_2SO_4]$ supplementations were: o, none; ●, 0.2%; Δ, 0.5%; —, % acid; ---, pH.

severe softening of pickles (16). It has recently been demonstrated that these enzymes are almost completely inactivated by raising the pH of the brine to 10.5-11.0 and allowing it to stand for 2 hr before neutralization (T. A. Bell, personal communication).

Possible problems in using alkali treatment to prepare used brine for re-use are: (a) the used brine has a very high BOD and this is decreased very little by pH adjustment, (b) the build-up of lactate in some brines might be high enough to interfere with the normal lactic acid fermentation of freshly brined cucumbers, and (c) the cost of the alkali and subsequent acid treatment may be excessive. The fermentation of the brines with a yeast would do much to alleviate all three of these problems. As demonstrated above, the BOD may be reduced by $\sim 70\%$ and the lactic acid eliminated from the brine. As shown in the titration curves in Fig. 12, the amount of alkali needed to raise the pH to 10.5 to 11.0 is reduced by about 50%. In addition, the rate of sedimentation of all the particles from fermented brines was more rapid than from non-fermented brine. The yeast cells and particles in 100 ml of fermented brines adjusted to pH 10.5 and placed in a 100 ml graduated cylinder settled to a volume of 20 ml in about 20 minutes while several hours were required for the particles in non-fermented brine to settle to a similar volume.

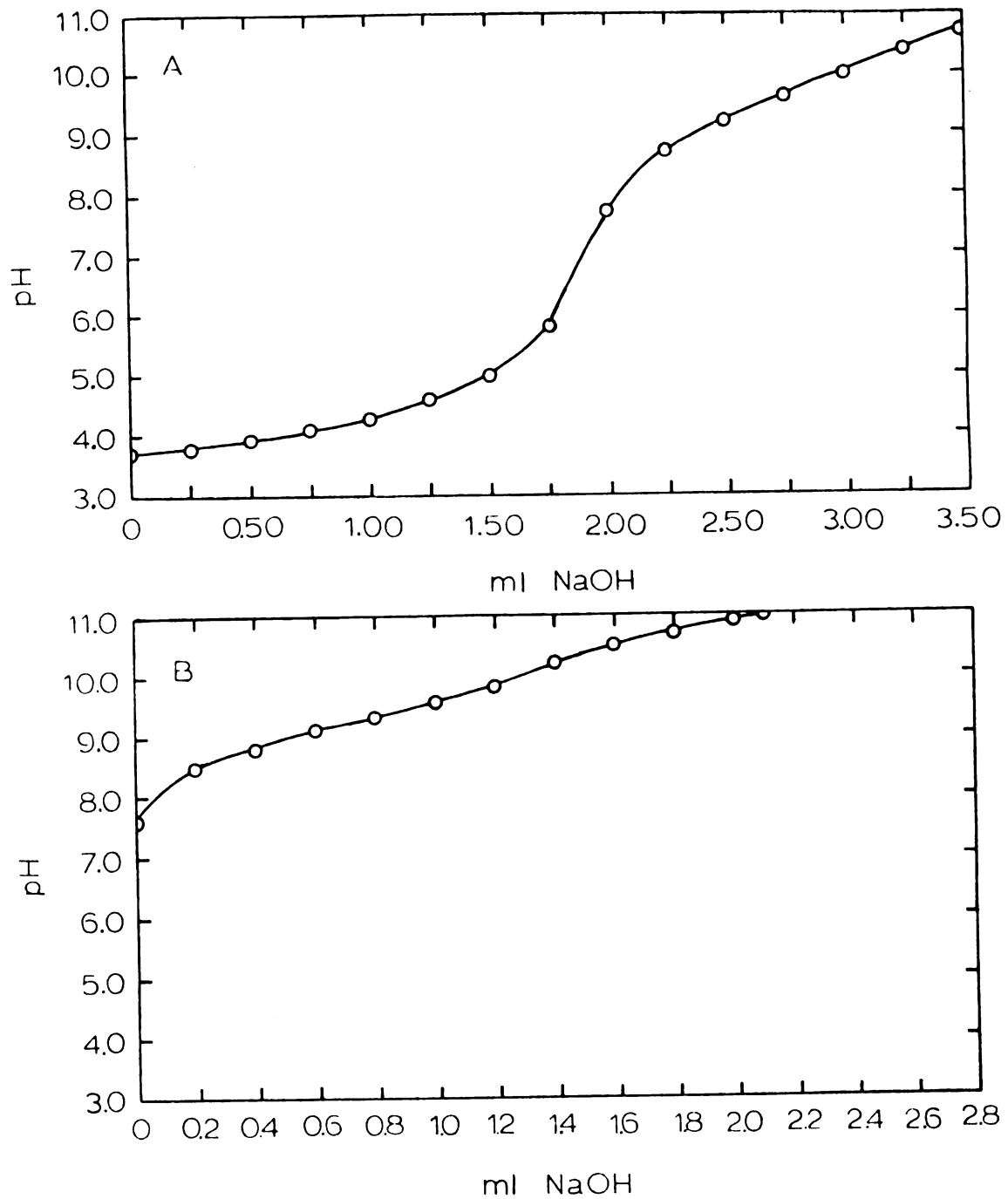


Fig. 12.--Titration of 100 ml volumes of (A) nonfermented and (B) fermented brines with 2 N NaOH.

DISCUSSION

These studies have demonstrated conclusively that it is possible to oxidize the organic acids from salt-stock brines using an adapted culture of D. membranefaciens var. hollandicus. This aerobic fermentation requires only 24-30 hr and results in a decrease about 60-70% in the BOD of the brine. The pH increases from ~ 3.4 to ~ 8.0 during the fermentation, and the yeast cells and other particulate matter in the brine can be flocculated by adding enough base to increase the pH to 10.5 or 11.0. It has been reported that this high pH will result in the inactivation of pectinase and cellulase in the brine (T. A. Bell, personal communication), and that brines treated at a high pH can be used to rebrine fresh cucumbers (11, 18, Palnitkar and McFeeters, unpublished data in press). The amount of alkali required to raise the pH to 10.5 after fermenting with D. membranefaciens var. hollandicus would be only about 50% of the amount required for non-fermented brine. This could amount to a substantial savings.

Even if the brine was not used again, the 60-70% reduction in BOD of the brine would be of great value.

Salt-stock brine has an exceedingly high BOD as compared to most wastes, 10,000-15,000 ppm vs 100-300 ppm in sewage for example (12). Yeast fermentation prior to injecting the brine into a waste disposal system would remove much of the load from the system.

The yeast fermentation of salt-stock brine would result in the production of about 5 g/liter of dried yeast cells. A salt-stock tank of 10,000 gallons capacity has about 3,500 gallons brine remaining after the cucumbers are removed. This brine would yield about 140 lbs of dried yeast cells. In the United States, there are approximately 300,000 tons of cucumbers brined as salt-stock. This would generate about 60 million gallons of salt-stock brine annually that could be used to produce 2.6 million lbs of yeast cells. These cells should be of considerable value for feeding livestock and/or fertilizer. The D. membranaefaciens var. hollandicus cells produced in these experiments were about 30% protein and 36% carbohydrate. Many studies indicate that yeasts contain high quality protein (25, 33), and that they are digestible (47).

It should not be difficult to develop a continuous aerobic fermentation for handling salt-stock brines. It appears likely that one should be able to obtain a dilution rate of about 0.1 per hr in a continuous system, since only 30 hr was required to utilize all of the lactic

acid when a rather small inoculum was used in these experiments. A continuous culture with a high cell density should reduce this time factor dramatically. The salt concentration is such that relatively few microorganisms could contaminate the culture. However, it may be desirable to adjust the continuous culture so as to maintain a relatively low pH, 4.5-5.5, to further restrict the growth of contaminants. One could control the pH by adding small amounts of mineral acid and allow the yeast to almost completely oxidize the organic acid present.

The effluent of the continuous fermentation could be continuously adjusted to pH 10.5 to bring about the flocculation of the yeast cells and other particles. Then the solids could be collected by sedimentation or screened out of the brine.

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