BASIC STUDIES ON A NEW SUGAR-TOLERANT YEAST

by

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INTRODUCTION

Until recently relatively little fundamental chemical and biological

research has been devoted to yeast organisms despite their long and wide-

spread use in brewing, wine fermentation and bread making.

However, yeasts have within the past few years become objects of

study on a much wider scale than before and substantial progress has been

made in many diverse directions as in metabolism and physiology.

It has been observed that the osmophilic yeasts grow very readily

in sugar syrup samples which are usually almost nitrogen free. This

observation suggested a study of the source of nitrogen necessary for

the growth of these organisms.

It is known that concentrated juices, dried fruits, special products

like "malt extract," honey, and sugar, sugar preserves, and sugar

confectionery, all possess two over-riding features in common--high

proportions of sugar, together with low proportions of water giving

aqueous activities* of the order of 0.75 or less. It has been shown that

it is the overall aqueous activity and not the nature and amount of the

carbohydrates present, which determines the growth of yeasts under such

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* Aqueous activity was simply measured as the relative humidity of the
  atmosphere with which the food is in moisture equilibrium and termed
  "equilibrium relative humidity," or e. r. h. (Mossel, 1955).

conditions (Schachinger and Heiss, 1951; English, 1953). This degree

of physiological dryness completely prevents the growth of the common

microorganisms, so that spoilage is effected by the "osmophilic" yeasts

(Von Schelhorn, 1951; Scarr, 1953); but even their growth is relatively

slow. Spoilage is much faster if the materials are exposed to humid

atmospheres because, being very hygroscopic, the surface layers are

rapidly moistened, and though the yeasts are called osmophilic, their

most rapid growth occurs with only about 30 per cent (W/V) of sugar

(aqueous activity about 0. 8). Further, the yeasts themselves, once

established, convert sugar into water and so accelerate the process.

There is probably a complementary effect of acidity, in raising the

aqueous activity below which growth will not occur. This has been

demonstrated for Aspergillus glaucus (Von Schelhorn, 1951); and there

are indications that the same is probably true for osmophilic yeasts in

the experience that, for example, honey ferments at higher "solid con-

tents" than more acid fruit concentrates.

In many investigations, the majority of the yeast concerned were

identified as strains of Zygosaccharomyces (Mrak and Phaff, 1948) which

have since been included in the species Saccharomyces mellis and S.

rouxii by Lodder and Kreger-Van Rij (1952).

There are indications that the flora of sugar is more varied. M. P.

Scarr (personal communication) isolated osmophilic strains of

Schizosaccharomyces and Hanseniaspora, besides Zygosaccharomyces. This may be because of the lower acidity, and it is suggested that it is the Zygosaccharomyces especially which combine ability to grow at high sugar concentrations with a high resistance to acidity. Some of these strains have been shown to produce large quantitites of acid during fermentation, leading to a pH so low as to cause hydrolysis of the sucrose, which thus becomes available to species which cannot attack sucrose per se (Scarr, 1951).

Only recently has the physiology of osmophilic yeasts been studied, and there is a paucity of information concerning their growth, their ability to fix atmospheric nitrogen and their ability to utilize amino acids. The work to be described was to investigate the possibility of nitrogen fixation and amino acid utilization by the osmophilic yeasts which have been isolated from sugar syrup samples.

The nitrogen fixation studies have been conducted using the micro-Kjeldahl method for the determination of the total nitrogen. The experiments carried out, measured the total nitrogen of yeast cultures at different intervals of time during the growth cycle to see if there is an increase in nitrogen.

The effect of several cations and anions, i.e., molybdenum, calcium, boron and phosphorus on the growth and nitrogen fixation in yeasts has been carried out using the methods of Takha, M.S. (1964).

The ability of the yeasts to utilize different amino acids as nitrogen and carbon sources and growth studies in the presence of these amino acids have been conducted using the methods of Meyer and Benziman (1959).

The composition of the biosynthesized amino acids in the pool of yeast cells, and the effect of growth media change on that pool were studied using the techniques of Halvorson and Spiegelman (1953) and of Kretovich and Krauze (1961).

LITERATURE REVIEW

I. HISTORICAL

To begin a study of yeasts one would expect to find a definition of the term "yeast." The term does not pretend to be the name of a botanical taxon; it merely designates a group of fungi which have certain outstanding characteristics in common but which may, nevertheless, belong to different classes.

As regards the literary meaning of the terms used for three organisms in different languages, it is frequently clear that they bear particularly on the property of fermentation. For example, "yeast" (in Dutch "gist") signifies foam. Its equivalent is found in the word "Gischt" still in use in modern German. Again, the French expression "Levure" is connected with the evolution of carbon dioxide which pushes upwards solid substances during fermentation.

Although yeasts had been observed under a microscope by Leeuwenhoek as far back as 1680 and Persoon had made the first recorded descriptions of them a hundred years later, it was not until the twentieth century that their life cycles were elucidated. It could hardly have been otherwise, however, for these early investigators were unaware of the significance of a pure culture. On the basis of observations on mixtures of organisms, some of the most authoritative voices of nineteenth century

biology asserted that the yeasts were merely phases in the complicated life cycles of molds, bacteria, or even algae. De Bary and Rees (1958), it is true, considered the yeasts as a group apart and recognized their similarities to other Ascomycetes, while Pasteur clearly realized that different forms of yeasts exist, although he did not succeed in isolating any pure cultures. The isolation of a single yeast cell was, in fact, first accomplished by the Danish investigator, Emil C. Hansen (1883).

Since the term "osmophilic" was given by Von Richter (1912) to the group of yeasts that can grow well in an environment of high osmotic pressures, a large number of yeasts showing sugar and salt tolerant properties have been described. Such yeasts cause spoilage of dried fruits, concentrated fruit juice, honey, maple syrup, curing brines, and salted meat. Western studies of osmophilic yeasts have been conducted primarily from the viewpoint of preventing spoilage in foodstuffs commercially preserved by drying, sugaring or salting. In Japan, soy sauce and miso paste are seasoning foods used daily, and the manufacture of soy sauce and miso paste is a major fermentation industry.

Almost all the earlier workers primarily studied the distribution and classification of these yeasts (osmophilic yeasts) and explained the origin of outbreaks of spoilage in various foodstuffs. There was less investigation of special aspects of the physiology and biochemistry of osmophilic yeasts in characteristic environments.

II. DISTRIBUTION AND TAXONOMY OF OSMOPHILIC YEASTS

A. Yeasts Found in Substrates With a High Sugar Content.

Fabian and Quinet (1928) described Zygosaccharomyces nusshaumeri as the most common yeast in honey produced in various areas of the U.S. and Canada. Lochhead and Heron (1929) found four species of Zygosaccharomyces that cause spoilage of honey. Sacchetti (1939) found Zygosaccharomyces species almost exclusively in Italian honeys. An extensive study of osmophilic yeasts prevalent on overripe berries or grapes, musts and grape concentrates was made by Krumbholz (1931) and showed that these yeasts belonged principally to the genus Zygosaccharomyces. Sacchetti (1932) also studied the yeast flora of fermenting grape concentrate in Italy and described two species of Zygosaccharomyces. Mrak et al. (1942) reviewed previous work on the spoilage of dates and showed that the ratio of Zygosaccharomyces to Saccharomyces isolated was about 15:1. Other sugar-tolerant yeasts were isolated from sugared dried prunes and figs (Baker and Mrak, 1938), fermenting citrus (lemonade) syrups (Lodder, 1932), dessert wines (Phaff and Douglas, 1944), and concentrated sugar-egg mixture undergoing spoilage (Bedford, 1942). Thus, in summary, the most ubiquitous sugar-tolerant yeasts in various substances of high sugar content belong to the genus Zygosaccharomyces.

In a monograph on the taxonomy of yeasts by Lodder and Kreger-Van Fij (1952) on the basis that no fundamental difference exists between

Saccharomyces and Zygosaccharomyces in sexual reproduction the sub-

genus, Zygosaccharomyces, is no longer recognized. According to this

classification, almost all Zygosaccharomyces, known as the osmophilic

yeasts, are included in only two species and one variety, Saccharomyces

rouxii, Saccharomyces rouxii var. polymorphus, and Saccharomyces

mellis.

B. Yeasts in Brines.

Mrak and Bonar (1939) reviewed earlier literature on brine yeast

and stated that Debaryomyces species had the greatest salt tolerance and

were the most common yeasts present. Costilow et al. (1954) isolated 89

cultures from brine meat, such as hams, beef tongues, and bacon, all of

which belonged to Debaryomyces, especially D. nicotianae. Other salt-

tolerant Debaryomyces species were isolated from rennet brine (Graham

and Hastings, 1942; Lodder, 1932) and salted beans (Hof, 1935; Lodder,

1932). A taxonomic study on the salt-tolerant yeast isolated from various

kinds of "shiokara" (fishery fermentation product) was reported by Zenitani

(1952a, b, c, 1953a). The order of frequency of isolation was as follows:

Saccharomyces rouxii, 7 strains; Debaryomyces nicotianae, 7 strains;

D. kloeckeri, 3 strains; D. hansenii, 2 strains; Hansenula anomala, 1

strain; and 1 strain each of three new species--S. kyushuensis nov. sp.,

D. oviformis nov. sp., and D. nicotianae var. salsus nov. var. Zenitani

(1953b) proposed a new classification system of the genus Debaryomyces

according to their abilities to produce acids from glucose, maltose, saccharose, and lactose. Taxonomic and ecological studies on yeasts found in the brines of cucumbers under commercial conditions have been carried out in Southern brining area by Etchells and Bell (1950) and in Northern brining area by Etchells et al. (1952). In the Southern brining area, the tiny yeast, Torulopsis caroliniana, and the very fermentative yeast, Brettanomyces versatilis, predominated in 88 per cent of the total isolates. The former species dominated the early part of the fermentation, and then the latter dominated, provoking a slow fermentation continuing to the end of the fermentation period. Observations on the population of yeasts occurring in brines of the Northern area demonstrated that two species, Torulopsis holmii and Brettanomyces versatilis, were predominant. The former yeast predominated during early fermentation and the latter yeast was most prevalent during the late stage of fermentation. Active during the intermediate period besides the above two major types of yeasts were Torulaspora rosei, Hansenula subpelliculosa, and Zygosaccharomyces halomembranis. Torulopsis holmii and Saccharomyces rosei were reported to be the predominant yeasts in cucumber fermentations (Costilow and Fabian, 1953). As for the film-forming yeasts in cucumber brines, Etchells et al. (1953) reported that Debaryomyces were the most widely distributed species, and Saccharomyces the second.

C. Soy Yeasts and Miso Yeasts.

The first report on soy yeasts appeared in 1906. Saito (1906) isolated

5 strains of salt-tolerant yeasts from soy sauce mashes in the Choshi

district and classified them into Saccharomyces soya, Zygosaccharomyces

japonicus, Pichia farinosa, Mycoderma sp. , and Torula sp. Takahashi

and Yukawa (1911) showed that Z. major and Z. soya were useful in

ripening soy mashes, giving the characteristic taste and flavor through

their fermentation. Zygosaccharomyces japonicus and Z. salsus were

shown to be harmful because they were the main cause of the formation

of pellicles leading to deterioration of the commercial quality. This

work has been believed to give the most reliable information on both

microbiological and applied aspects of soy yeasts.

Unlike soy sauce, miso pastes were commercially produced in great

variety with different raw materials and various contents of NaCl. Mogi

(1942) described many new species and varieties of miso yeasts belong-

ing to Saccharomyces, Zygosaccharomyces, Debaryomyces, Hansenula,

Pseudohansenula, Pichia, Zygopichia, Torulopsis, Pseudomycoderma,

and Pseudomonilia. Among them, the dominants were Saccharomyces,

Torulopsis, Zygosaccharomyces and Zygopichia, and the last two were

found chiefly in miso paste of relatively high salt content. Zygosaccharo-

myces major, Z. soy, Z. salsus, and Z. japonicus, known as soy yeasts,

and almost all of Z. miso Mogi were included in one species, Saccharo-
myces rouxii, by Lodder and Van Rij's system.

Recently Onishi (1960a, b) isolated many salt-tolerant yeasts from
soy mashes of the Noda Soy Sauce Co. , and made a taxonomic study for
them. Fifteen salt-tolerant yeasts, including 3 new species and two new
varieties, were found: (1) in young soy mashes, Torulopsis famata,
Pichia farinosa, Candida polymorpha, and Trichosporon behrendii, (2)
in vigorously fermenting soy mashes, Saccharomyces rouxii, S. rouxii
var. halomembranis nov. var. , S. acidifaciens, and S. acidifaciens
var. halomembranis nov. var. ; (3) in old soy mashes, Torulopsis
halophila nov. sp. , T. nodaensis nov. sp. , T. versatilis, T. etchellsii,
T. sphaerica, T. sake', and T. halonitratophila nov. sp. Saccharo-
myces rouxii is a typical yeast that plays an important role in the ripen-
ing of soy mashes. It is of interest to note that most salt-tolerant
Torulopsis spp. , found in old soy mashes, cannot form pellicles on any
liquid medium, with or without NaCl, in spite of their ability to assimilate
nitrate. These properties are considered to be a marked characteristic
of them. Only a few yeasts having these properties were described in
the monograph by Lodder and Kreger-Van Rij (1952).

III. CHARACTERISTIC PHENOMENA IN SUGAR AND

SALT TOLERANCE OF OSMOPHILIC YEASTS

A. Physiological Adaptation and Training.

The viability of osmophilic yeasts grown in a saline environment such as soy mashes was remarkably depressed in a single-step transplantation into the conventional medium used for plating count; whereas, there was no significant fall of viable counts when plating media were used containing sodium chloride in the same concentrations as in soy mashes. A fall in viability was similarly observed when, in a reverse direction, yeasts grown in a conventional medium were transferred directly to saline medium (Ōnishi, 1954). Since the salt-tolerant property of soy yeasts toward sodium chloride could be increased by culturing in media containing sodium chloride, Ōnishi has regarded this, to a certain extent, as an adaptive nature, although this property had hitherto been simply believed to be intrinsically constant and heritable.

Ziegler and Halvorson (1935) pointed out that salt agar gave a higher count than standard nutrient agar in the viable count of bacteria in curing pickles. The salt tolerance that individual cells of soy yeasts acquired by growth in saline medium was easily lost if these yeast cells were further incubated for several generations in conventional medium without sodium chloride. When the soy yeast Saccharomyces rouxii, cultured in the plain medium, was inoculated directly into a basal conventional, 18 per cent

NaCl, 50 per cent glucose, or 50 per cent galactose medium, the fall in viability was not found either in the conventional medium or in the concentrated sugar medium; but in the 18 per cent NaCl medium, a remarkable fall in viability was observed at the initial period of incubation (Ōnishi, 1957a). A possible reason for the absence of viability loss in the sugar medium might be that the sugar concentration was reduced according to utilization by the yeasts. That was not the case, however, since the tested yeast could not ferment galactose, but would assimilate it only weakly. It is apparent that the osmophilic soy yeasts exhibit characteristic tolerance toward media containing salt or sugar at equal osmotic pressures. Therefore, some limiting factors other than osmotic pressure, itself, are presumed to influence the salt tolerance of the osmophilic yeasts.

Burick (1950) found a high degree of constancy in the water requirement of several species of yeasts, and in particular, the sugar tolerance of Trichosporon pullulance could not be changed by repeated subculture on different media. Although Whalley and Scarr (1947) did increase the sugar tolerance of a commercial strain of Saccharomyces cerevisiae, the limit attained was no longer higher than that reported by other workers. Scarr (1954) and Ingran (1957) found that cultivation of facultatively osmophilic yeasts on low-sugar media reduced their tolerance toward high concentrations. The tolerance could be restored by training back

to higher concentrations, although the change was slower than in the reduction.

B. Limiting Osmotic Pressure For the Growth of Yeasts.

Limiting concentrations of sodium chloride and sugars for the growth of the or[dinary yeasts such as Saccharomyces cerevisiae and S. ellipsoideus were found to be 10 per cent in NaCl, 50 per cent in glucose, and 60 per cent in saccharose, and for osmophilic yeasts as Saccharomyces rouxii were 20 to 22 per cent in NaCl, 80 per cent in glucose, and 80 per cent in saccharose. Limiting osmotic pressures for growth were, therefore, calculated as 75 atm in NaCl, 140 atm in glucose and 90 atm in saccharose for Saccharomyces cerevisiae and S. ellipsoideus, and 150 to 165 atm in NaCl, 220 to 260 atm in glucose, and 180 to 200 atm in saccharose for S. rouxii (Onishi, 1957a).

IV. NITROGEN-FIXATION

The inability of higher plants and animals to utilize atmospheric nitrogen would be a catastrophe were it not for the fact that a few bacteria, blue-green algae, and some yeasts can do so. Only a limited amount of nitrogen is converted into the combined form utilizable by living cells by lightning and other natural means.

Biological nitrogen fixation appears to consist essentially of reduction of free nitrogen to ammonia. Glutamic acid may then be formed by amination of α-ketoglutaric acid and from it other amino acids are produced by transamination of other alpha-keto acids.

Earlier reports in the literature suggested that ordinary yeasts are able to fix atmospheric nitrogen itself. Nothing is mentioned, concerning the ability of the osmophilic yeasts to fix atmospheric nitrogen. Zikes (1909) claimed that the yeast, Torula wiesneri, and all aerobic film-forming yeasts took up nitrogen. This claim was supported by Lipmann (1911) and by Kossowicz (1912) who apparently demonstrated the incorporation of gaseous nitrogen into Saccharomyces sp. and into Monilia. However, the latter author soon reversed his claim (Kossowicz, 1914). Lindner and Naumann (1913) enunicated the view, which is now most commonly accepted, that yeasts cannot assimilate nitrogen which is free from ammonia or oxides of nitrogen. On the other hand, Ingram (1955) has commented that yeasts can exchange isotopic nitrogen with the atmosphere, thus indicating that atmospheric nitrogen can find its way into the nitrogen compounds of the yeast. However, there is presumably no net increase of the total nitrogen content of the cell population under these conditions and, hence, no net assimilation of nitrogen.

Scheffer (1950) repeated the experiments on the basis of which Schanderl (reference not available) claimed to have demonstrated fixation of atmospheric nitrogen by yeasts (Pichia farinosa, P. membrane-faciens, Mycoderma bispora No. 6) cultured on synthetic nutrient solutions and in grape must. Scheffer failed to substantiate Schanderl's claims. A confirmation of Scheffer's results was obtained by Stapp (1951). In

contrast Bakhadur (1956) using sucrose as the source of carbon and ammonium sulfate as the source of nitrogen in culture inocula, found that different values of the carbon to nitrogen ratio in the original culture inocula could affect the ability of Dkhar yeast to fix atmospheric nitrogen.

V. EFFECT OF CATIONS AND ANIONS ON GROWTH
AND NITROGEN FIXATION

The role played by mineral salts in the growth of yeasts is extremely difficult to ascertain because of the technical problems encountered in ensuring that the basal media are entirely free of the elements under examination. Consequently, it is not surprising that little precise information is available concerning this fundamentally important and interesting topic. Certain elements have been shown to be essential for growth when they are present in small amounts whereas others, while not absolutely necessary, can nevertheless exhibit stimulatory effects; further, many elements are inhibitory at concentrations only slightly in excess of that required for optimal growth. As limited as our knowledge is of the part played by cations and anions in the growth of yeast, it does nonetheless suggest a reason why defined synthetic media in general do not support growth to the same extent as do such undefined media as malt wort or corn-steep liquor. Most of the synthetic media contain relatively few elements and even supposing that the requirements for nitrogen, carbon, and vitamins are satisfied, it is almost certain that the media are unbalanced so far as

trace metals are concerned, particularly if salts of a high degree of purity are used. A large number of defined synthetic media have been devised but most of these are based on the formulae described by Fulmer et al. (1921), Devereux and Tanner (1927) and Stelling-Dekker (1931). They generally contain almost exclusively the following salts: potassium dihydrogen phosphate, dipotassium hydrogen phosphate, disodium hydrogen phosphate, calcium salts as the chloride, carbonate and/or nitrate, magnesium sulfate, and the chlorides of sodium and potassium. It has been observed on occasion that continued subculture in synthetic media eventually leads to a progressive reduction in yeast crop; such observations suggesting that the media are deficient in at least one unknown factor, which may be a trace element.

Phosphorus is essential for the growth of yeast, and among other functions this element plays an important part in carbohydrate metabolism, being concerned with the adenosine phosphate-adenosine diphosphate reaction and other metabolic reactions.

The effect of the different halogens varies considerably, but chloride appears to be essential for growth and is readily available in most media. Iodine, either as elemental iodine, or as the iodides of potassium, sodium, or calcium, stimulates growth at concentrations between 1 p.p.m. to 10 p.p.m. (Greaves et al., 1928). It is not known for certain whether bromine is absolutely essential, but Graber-Oberfeuchtner (1953) reported that

growth was stimulated by extremely small concentrations of bromine.
However, media containing bromides at a concentration of 0.5 N were
quite toxic. The inhibitory action of fluoride upon the carbohydrate
metabolism of yeasts is, of course, well known, although it appears that
its inhibitory effects may vary considerably according to the strain of
yeast and the composition of the medium used.

Unlike potassium, sodium does not appear to be necessary for growth.
The function of the latter is not fully understood, but it is known that it
is taken up early in the growth of young cultures and that at least a portion
of it is released later into the medium (Atkin and Gray, 1947). It appears
to be associated with the respiratory functions of yeast cells, but certain
yeasts have been studied in which ammonium ions can replace those of
sodium without either respiration or fermentation being affected.

Calcium does not appear to be essential for growth of yeast cells,
although it has been established that the amount of yeast ash is reduced
by approximately one-half when the medium in which the cells have been
grown is devoid of calcium. There is, however, insufficient evidence
to indicate whether yeasts grown in a calcium-deficient medium are
actually independent of calcium or whether the cells grow by virtue of
the calcium that they have previously accumulated from a calcium-rich
medium. Many workers have shown that certain amounts of calcium
definitely increase the amount of yeast growth, but opinions differ as to

the concentrations required to support maximum growth. Fulmer et al.

(1921) reported that the amount of growth was progressively stimulated as

the concentration of calcium increased from 1000 p. p. m. to 6000 p. p. m.

after which no further increase occurred; on the other hand Richards (1925)

claimed that the optimum concentration of calcium, as the sulfate was of

the order of 140 p. p. m. and that inhibitory effects were exhibited above

this amount. The evidence of Kondrat'eva (1940) indicates that the function

of calcium in cell metabolism is complex for not only does its presence in

media increase the total yeast crop, but it also stimulates sporulation and

the production of intracellular fat and metachromatin. Lasnitzki and

Szorenzi (1934) showed that calcium can be replaced to a large extent by

magnesium. It is not then surprising that the role of magnesium in yeast

growth has been found by many workers to be similar to that of calcium,

despite the earlier belief of Devereux and Tanner (1927) that magnesium

is essential for growth. There can be no doubt that magnesium is

essential for the production of high yields of yeasts and it is known that it

is associated with metabolism both of carbohydrates through the activation

of enzymes concerned with the transfer of phosphate and of nitrogenous

compounds.

Interest in the effects of copper on yeast growth is understandable

because a considerable proportion of the fermentation vessels used in

industry are made of this metal. The general concensus of opinion is

that, although it is not essential, low concentrations of copper enhance

growth. Hanson and Baldwin (1941) found that the optimum range was

as high as 35-40 p.p.m.

There is ample evidence that a small amount of iron can appreciably

enhance the yield of yeast; recently it has been established that its pres-

ence is essential for full catalase and cytochrome activity (White and

Munns, 1951). The latter authors also found that relatively large doses

of approximately 500 p.p.m. had slight inhibitory effects.

In the case of osmophilic yeasts, in contrast to the ordinary yeasts,

not much work concerning the effect of various cations and anions on

growth has been done. For example, Ōnishi (1957b) found that the growth

of Saccharomyces rouxii was inhibited by relatively low concentrations

of LiCl, such as 0.5 mole per liter. In contrast, with KCl, growth was

very good even in concentrations as high as 4 mole per liter. Magnesium

was also found by the same author to be less toxic than calcium.

The role of trace elements in nitrogen fixation was studied by

Krylova (1962). He demonstrated that the development and fixation of

molecular nitrogen by N-fixing microorganisms was stimulated by moly-

bdenum when optimal iron concentrations were present in the medium.

He found also that nitrogen fixation by yeasts was enhanced by boron.

Singh (1962) also investigated the effect of molybdenum, boron, zinc,

copper, manganese and cobalt on nitrogen fixation in liquid culture medium

and in the soil culture medium at 0.1 p.p.m. and 0.2 p.p.m. concentrations. He observed that molybdenum stimulated nitrogen fixation more than any of the other cations and anions studied.

VI. EFFECTS OF NITROGEN SOURCES ON THE GROWTH OF YEASTS

For the continued survival of yeast cells as biological and biochemical entities a great number of substances must be elaborated including structural materials, such as the cell walls, nuclei, etc., and the various vehicles for the production of energy and for reproduction, including nucleic acids, enzymes, and many other substances. Many of these compounds, e.g., the proteins and nucleic acids, are of great molecular size and complexity.

Like many other organisms, most yeast strains can use ammonium salts for aerobic growth in the presence of a suitable carbon source. Yeasts growing under conditions found in nature most probably derive at least part of their nitrogen requirements from amino acids occurring free or produced by the hydrolysis of the proteins found in the natural growth media. The ability of yeasts belonging to different strains to use amino acids for growth has, therefore, been made the subject of repeated investigations. Thorne (1946) demonstrated that, in the presence of glucose, a number of amino acids could sustain growth and that their efficiency was, on the average, about 60 per cent of that of ammonium salts. Schultz and Pomper (1948) reported similar experiments undertaken

with a considerable number of Torula and Saccharomyces strains and found that some amino acids were used as both nitrogen and carbon donors, whereas others were not capable of utilization in the absence of an additional carbon source. The availability of a given amino acid for yeast growth was generally assumed to be determined by the presence of suitable mechanisms leading to its deamination; whereas the availability of its nitrogen-free carbon residue was less thoroughly investigated, owing to the fact that in most experiments glucose was supplied in adequate quantities. Meyer and Benziman (1959) studied the utilization of amino acids as nitrogen and carbon sources for the aerobic growth of yeast. They found that Torulopsis yeast utilized all the amino acids for growth both in the absence and in the presence of glucose, but isoleucine, valine and phenylalanine were utilized very poorly without the addition of glucose. The Saccharomyces strain could use all of the amino acids for growth when glucose was present, but in its absence only alanine, glutamic acid and aspartic acid produced growth.

As mentioned above, it is well known that, if the supply of other nutrients is adequate, ammonium nitrogen such as ammonium sulfate can be utilized by yeasts as the sole source of nitrogen in a conventional medium. Takahashi (1954) investigated the requirement of vitamins in the assimilation of amino acids by yeasts and pointed out that in such experiments the inoculum size should be controlled below 5×10^3 cells

per ml of culture medium. Besides these, reports concerning the nitrogen

nutrition of yeasts were made by Thorne (1946), Wickerham (1946), Atkin

and Gray (1947), and Swanson and Lifton (1948). However, a comparative

study has not yet been carried out on the requirement of nitrogen sources

in two contrasting conditions--one conventional and the other a 30 per

cent sucrose medium. In spite of that, Ōnishi in 1957 made some studies

comparing the growth of osmophilic yeasts in conventional medium and

in 18 per cent sodium chloride medium. The author found that in con-

ventional media a salt-tolerant soy yeast, Saccharomyces rouxii, could

fully utilize all of three nitrogen sources--ammonium sulfate, ammonium

lactate, and casein hydrolysate, but that in the presence of 18 per cent

NaCl, assimilation of ammonium sulfate was apparently very poor.

However, this poor growth cannot be attributed to the low assimilability

of ammonium nitrogen under this condition, because of the good growth

with ammonium lactate in the saline medium. In this experiment, the

initial pH was 5.0 in the conventional medium and 4.4 to 4.6 in the saline

medium, being independent of the kind of nitrogen source; and the final

pH was 2.8 to 3.0 in the ammonium sulfate medium under both normal

and saline conditions. The latter pH values were much lower than 3.4 to

3.8 in the media of ammonium lactate and casein hydrolysate. Therefore,

it was considered that in the saline medium, inhibition of growth might

be caused by the lowering of pH below 3.0. This idea was confirmed by

the fact that if potassium citrate-citric acid buffer was initially added to the ammonium sulfate medium, growth was sufficient even in the 18 per cent NaCl medium. In this case, the initial pH of the medium was 5.0 but the final pH remained at 3.6 to 3.8. From these results, it is quite clear that the growth of osmophilic yeasts in high concentrations of sodium chloride depends largely on the pH value of the medium.

Stuart (1940) observed that the presence of cysteine and a high concentration of gelatin favored growth of a red obligatory halophilic sarcina in a medium of 3 $\underline{M}$ NaCl concentration. Castell and Mapplebeck (1952) noticed a similar stimulating effect of protein on the growth of halophiles at high salt concentrations. The stimulating effect of sulfur-containing amino acids such as methionine on the growth of soy yeasts, Saccharo-myces rouxii, in saline medium, was shown by Sato and Uemura (1958). On the whole, it is said that tolerance to high salt concentration is greater in rich organic media like foods than in simple synthetic media; for example, complex media containing milk or fish extract (Dussault and Lachance, 1952) and synthetic media containing protein hydrolysate (Katznelson and Lochhead, 1952) are recommended for halophilic bacteria.

VII. EFFECT OF HYDROGEN ION CONCENTRATION

Sherman and Holm (1922) studying the pH-growth-rate curve of Escherichia and Alcaligen faecalis, observed that a concentration of

0. 2 M̲ NaCl was optimal in 1 per cent peptone medium and expanded the
pH range that allowed rapid growth. The growth of salt-tolerant Sac-
charomyces rouxii in various concentrations of sodium chloride and
hydrogen ion was observed (O̅nishi, 1957b). The results showed that
the growth of the yeast was uniformly good in the pH range of 3. 0 to 6. 6
in a 1 M̲ concentration of sodium chloride, but that growth was limited
in the pH range 4. 0 to 5. 0 in media of more than 2 M̲ sodium chloride.
English (1954) stated that a sugar-tolerant Saccharomyces rouxii was
able to grow in a wide pH range of 1. 8 to 8. 0 in a high glucose concen-
tration of 46 per cent and a similar phenomenon in a medium of high
sugar concentration was also observed with a salt-tolerant soy yeast,
Saccharomyces rouxii (O̅nishi, 1961). In contrast, in a high-saline
medium the growth of the same yeast was limited to within pH 4. 0 to
5. 0. The different nature of salt tolerance and sugar tolerance in
osmophilic yeasts was apparent in this pH dependence.

VIII. EFFECT OF CULTURE TEMPERATURE

The effect of culture temperature on the growth of yeasts has been
investigated numerous times. Van Halteren (1950) showed that yeast is
protected against the effect of heat by a concentrated solution of salts or
sugars. Anderson et al. (1949) found that glucose and sucrose increased
the heat resistance of Bacillus coagulans, and that NaCl and several
organic acids decreased it. It appears that the effect of salts is variable,

depending on concentration, kind of suspension medium, and test organism

used. Onishi (1959) found that Saccharomyces rouxii was able to multiply

at 40° C in the presence of sodium chloride at concentrations exceeding

3 to 4 per cent. Sugars at concentrations higher than 30 per cent were

also effective in supporting growth. He also found that the thermal death

time of S. rouxii cells were extended about tenfold in saline media over

that in conventional media. A similar phenomenon was found in that the

higher the temperature, the faster the growth of the osmophilic yeasts

in high-sugar media (Kroemer and Krumbholz, 1931; Scarr, 1951). The

high resistance of dry proteins to heat denaturation is well known. Thus,

the greater thermal resistance of cells or spores of microorganisms in

concentrated solutions has hitherto been attributed principally to the

difficulty of heat denaturation of enzyme proteins through actual dehydra-

tion of cell contents in solutions of high osmotic pressure.

IX. COMPOSITION OF FREE AMINO ACIDS
BIOSYNTHESIZED BY YEAST CELLS

It was found that in many microorganisms, the induction of enzyme

synthesis was carried out in the absence of an external nitrogen source.

In such inductions the nitrogen employed by the cell in fabricating the

new enzyme molecule must derive from some preexisting nitrogenous

compounds in the cell. One is immediately faced with the obvious

necessity of identifying the components thus employed. Ability to form

enzyme in the absence of an external supply of nitrogen is far more widespread among the yeasts than among the bacteria. The work of Taylor (1947) suggests a reasonable explanation for this apparent independence of the yeast enzyme-synthesizing mechanism. Employing the amino acid decarboxylase procedure Taylor (1947) surveyed a variety of yeasts and bacteria for the presence of free amino acids in their internal environment. Analyses were made for arginine, glutamic acid, histidine, lysine, and tyrosine. Of the three yeast types examined, all possessed detectable quantities of these five amino acids. The observations of Taylor (1947) on the existence of free amino acid pools in yeast were readily confirmed with strain K of Saccharomyces cere-visiae. Examination (Halvorson and Spiegelman, 1953b) of the free pool content of exponential phase cells, grown in complete medium, revealed that of 16 amino acids analyzed for by microbiological methods, all could be found in detectable amounts. The major components were glutamic acid, aspartic acid, and serine. A survey was made by Halvorson and Spiegelman (1953) of different yeast species as well as of different representatives of S. cerevisiae. They found that all of the strains examined possess considerable amounts of free amino acids. They did not detect strikingly unique patterns of pool composition.

Concerning the effect of growth medium on pool composition, Nagai as quoted by Spiegelman and Halvorson (1955) has found that growth of

yeast in media containing NaCl at hypertonic levels led to the disappear-ance of many free amino acid pool components. Only glutamic acid, alanine, histidine and small amounts of aspartic acid are found in such cells. Nagai found also that pools of somewhat modified composition can be obtained by growing the organisms in media with differing carbon sources. Relatively little difference was observed between cells grown in complete or synthetic medium. However, several of the amino acids almost disappeared from the pools of cells grown on either pyruvate, lactate, or glycerol. He explained the almost complete absence of serine and glycine in these cases, in terms of the aerobic mechanism, involved in the utilization of these compounds.

One of the most successful methods found for varying both pool level and pool composition was the employment of cycles of nitrogen depletion and replenishment (Halvorson and Spiegelman, 1953a). They found that pool levels were readily lowered by exposing cells to a nitrogen-free medium containing glucose, but this would be insufficient for the depletion of the free amino acid pool.

Concerning the biosynthesis of the free amino acids, yeast cells can synthesize all their amino acids from certain inorganic sources of nitrogen, primarily ammonia, and that among the most important mechanisms for the incorporation of this nitrogen into organic structures are those pro-viced by the enzyme systems, glutamic acid dehydrogenase and aspartase.

Of these two systems the former is probably the more important.   The
formation of glutamic acid and aspartic acid in this way occurs at the
expense of alpha-ketoglutaric acid and oxaloacetic acid which are them-
selves participants in the respiratory cycle, known as the Krebs cycle
or the citric acid cycle.   It is known that yeasts contain a considerable
pool of free amino acids among which glutamic acid, aspartic acid, and
also alanine predominate (Gale, 1947; Gale and Freeland, 1947; Bair et
al., 1952).   The latter amino acid may possibly arise from pyruvic acid
in a manner analogous to the formation of glutamic acid and aspartic acid.
Thus a dynamic state may be visualized in which the citric acid cycle
generates the alpha-keto acids which in turn combine with ammonia (or
undergo transamination).   When the metabolism of the yeast occurs
largely through the Krebs cycle, for example under aerobic conditions of
growth, the production of energy is increased and protein synthesis is
enhanced largely via glutamic acid and aspartic acid (Bair et al., 1952).

This view of the connection between carbohydrate and nitrogen metabo-
lism of yeast has been justified by establishing that the Krebs cycle exists
in yeasts and, as has long been known, in mammals as well.   All the
reaction steps of the cycle have been demonstrated in baker's yeast
(Nossal, 1954a, b; Foulkes, 1951; Lipmann, 1954) and respiring particles
regarded as mitochondria have been isolated from the cells (Linnane and
Still, 1955).   Indeed, even before the Krebs cycle existence in yeast was

established with certainty a number of investigators were able to explain

various aspects of the biosynthesis of amino acids by invoking the reactions

of this cycle (Abelson and Vogel, 1955; Ehrensvärd, 1955, Vavra and

Johnson, 1956).

In general the amino acids, consituting the proteins of yeasts or

other microorganisms after growth on a radioactive carbon source, fall

into several distinct families according to their degree of labeling with

the radioactive isotope (Abelson and Vogel, 1955; Abelson et al., 1953;

McQuillen and Roberts, 1954). These groups comprise: (1) the glutamic

acid family, consisting of glutamic acid, proline and arginine, (2) the

aspartic acid family, containing aspartic acid, methionine, threonine,

and isoleucine; (3) lysine; (4) the pyruvic acid family, including alpha-

alanine, valine, and leucine; (5) histidine (Levy and Coon, 1954; Ames et

al., 1953); (6) the serine family, namely serine, glycine and cysteine;

and (7) the aromatic family consisting of phenylalanine, tyrosine, and

tryptophan (Tatum and Perkins, 1950). Thus, Ehrensvärd (1955) and

Abelson and Vogel (1955) found that this classification applied to the

organisms, Torulopsis utilis and Neurospora crassa. Abelson and Vogel

commented that their results were in conformity with the existence of

the Krebs cycle in both T. utilis and N. crassa.

Publications of the past few years concerning the biosynthesis of the

amino acids are reviewed in the following pages:

A. <u>Serine and Glycine Biosynthesis.</u>

The pathway for serine biosynthesis (Eq. 1) proposed by Ichihara

and Greenberg (1957) is concluded by Umbarger et al. (1963, 1962) to

be the only significant one operating in <u>E</u>. <u>coli</u> and <u>Salmonella typhimurium</u>.

$$\text{3-phosphoglycerate} \xrightarrow{\text{+NAD*}} \text{phosphohydroxypyruvate} \xrightarrow[\text{or alanine}]{\text{+glutamate}} \text{phosphoserine}$$

$$\xrightarrow{\text{-PO}_4} \text{serine}$$
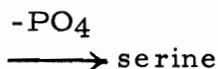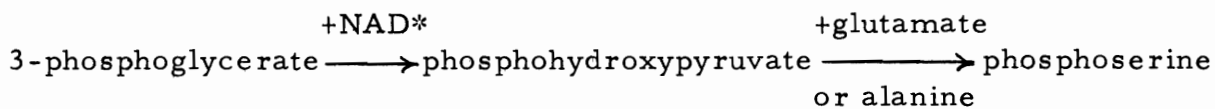
Serine biosynthesis was first studied by competition experiments

with bacterial cell extracts, since the whole cells are impermeable to 3-

phosphoglycerate. In these experiments with glucose-U-C^{14}, it was

observed that labeled phosphohydroxypyruvate and phosphoglycerate

greatly diminished the concentration of the label in serine. Further proof

was obtained by showing the existence in the cell extracts of each of the

three enzymes required for the pathway: i.e., 3-D-phosphoglycerate dehy-

drogenase, phosphoserine transaminase, and phosphoserine phosphotase

(Umbarger, 1962). Studies with two serine or glycine auxotrophs of <u>E</u>.

<u>coli</u> showed that 3-phosphoglycerate dehydrogenase was the missing

enzyme (Umbarger, 1962). In two serine-glycine auxotrophs of <u>S</u>.

<u>typhimurium,</u> one lacked the dehydrogenase, a second the phosphoserine

phosphotase.

---

* NAD is Nicotinamide adenosine dinucleotide.

The serine-synthesizing enzyme system conforms to the common pattern of the control of metabolite flow by end-product inhibition. In accord with this pattern, L-serine inhibits the first enzyme in the pathway, 3-phosphoglycerate dehydrogenase.

Glycine is generally considered to be mainly formed from serine through the action of hydroxymethyltransferase. A second possibly significant pathway would be transamination of glyoxylic acid. Evidence that this may occur in the silk worm has been reported by Muramatsu and Shimura (1962).

B. Arginine Biosynthesis.

The pathway of arginine biosynthesis in microorganisms is well established. Vogel and Vogel (1963) demonstrated the presence of acetyl-ornithinase in B. subtilis, thereby establishing that this organism has the same arginine synthesis pathway as E. coli. Formation of N-acetylglutamate has been shown to occur in the green algae Chlamydomonas reinhardi (Hudock, 1962). This differentiates this mold from Neurospora, in which the acetylated intermediates appear not to be involved in arginine synthesis. For the formation of acetylglutamate synthetase in a particular strain of E. coli, both glucose and glutamate were required in the culture medium to secure maximum enzyme activity. The glucose presumably is needed to furnish the acetyl moiety (Vyas, 1963). Arginine inhibits acetylglutamate synthetase by enzyme repression.

C.   Threonine Biosynthesis.

The synthesis of threonine is initiated by aspartokinase through the formation of aspartyl phosphate.   In the yeast Saccharomyces cerevisiae, but not in E. coli, this enzyme is repressed by threonine or homoserine (De Robichon-Szulmajster, 1963).   The third enzyme in the biosynthetic chain leading to threonine, homoserine dehydrogenase, is repressed by methionine or ethionine, but not by threonine (Karassevitch, 1963). The findings indicate an independent genetic regulation of aspartokinase and threonine dehydrogenase.   Homoserine dehydrogenase was purified to a considerable degree from S. cerevisiae and the Michaelis-Menton constants of the several substrates evaluated by the latter author.

D.   Leucine Biosynthesis.

The steps in the biosynthesis of leucine were proposed several years ago by Strassman (1956) and Abelson (1955).   The first condensation product in the proposed scheme, β-hydroxy-β-carboxyisocaproic acid was recently isolated from the culture media of leucine auxotrophs of S. typhimurium by Jungwirth (1963), N. crassa (the same author), and baker's yeast (Strassman, 1963).   A second intermediate, alpha-hydroxy-β-carboxyisocaproic acid, has been isolated by Gross (1962) from N. crassa and S. typhimurium.

The enzymatic synthesis of β-hydroxy-β-carboxyisocaproate was accomplished by incubating alpha-ketoisovalerate and acetate or acetyl

CoA with extracts of baker's yeast (Strassman, 1963) of or $\underline{S}$. $\underline{typhimurium}$ (Jungwirth, 1963). The enzymatic isomerization of β -hydroxy-β - carboxyisocaproate to alpha-hydroxy-β-carboxyisocaproate with dimethyl- citraconic acid as an intermediate is reversible (Gross, 1963) and analo- gous in many respects to the reaction catalyzed by aconitase. However, a separate enzyme, β -hydroxy-β-carboxyisocaproate isomerase is required which has been purified 25- to 50-fold from mycelial extracts of the leu-1 mutants of $\underline{N}$. $\underline{crassa}$.

A dehydrogenase which catalyzes the oxidation and decarboxylation of alpha-hydroxy-β-carboxyisocaproate to alpha-ketoisocaproate has been partially purified from $\underline{S}$. $\underline{typhimurium}$ by Burns (1963). The dehydro- genase is NAD-dependent and requires both K^+ ions and Mn^{++} or Mg^{++}. The optimum activity is at pH 9. 5. The very high affinity of alpha- hydroxy-β -carboxyisocaproate for the enzyme is shown by its K_m value of 3. 8 x 10^{-6} M. The condensation of acetyl CoA with alpha-ketoiso- valerate is analogous to the condensation of oxaloacetate with acetyl CoA to form citrate in the citric acid cycle. This type of condensation appears to be quite general and has been shown by Ingraham (1961) to occur with a variety of alpha-keto acids and acetyl CoA.

E. Isoleucine and Valine Biosynthesis.

The dihydroxy acid dehydratase that catalyzes the removal of water from alpha, β -dihydroxyisovaleric acid and alpha, β -dihydroxy-β -

methyl-n-valeric acid to yield the alpha-keto acid precursors of valine

and isoleucine has been purifed about 120-fold from acetone powder

extracts of spinach with a final yield of less than 1 per cent (Kanamori,

1963). The optimal pH was at 8.0 to 8.2. There was a requirement

for Mg^{++}, although Mn^{++} and Co^{++} stimulated the enzyme activity to a

lesser degree.

F. Lysine Biosynthesis.

A key step in the alpha-aminoadipic acid pathway for the biosynthesis

of lysine, which is characteristic of yeast and molds, is the reduction of

alpha-aminoadipic acid to alpha-aminoadipate-δ-semialdehyde, which

exists in equilibrium with Δ' - piperidine-6-carboxylic acid. A soluble

crude enzyme system from baker's yeast that carries out the above

reduction requires ATP, Mg^{++}, and a reduced pyridine-adenine nucleo-

tide (Larson, 1963). Upon addition of o-aminobenzaldehyde, an orange-

colored derivative of Δ'-piperidine-6-carboxylic acid was obtained

identical with that from the authentic synthetic compound.

Sagisaka and Shimura (1962) obtained about a tenfold enrichment of

this enzyme, named alpha-aminoadipate reductase, by acid precipitation

and repeated ammonium sulfate fractionation. The cofactor requirements

agree with those found by Larson et al. (1963), but a marked stimulation

by reduced glutathione was also observed. Optimum activity was obtained

at pH 7.6 to 8.0. The enzyme was inhibited by EDTA, iodoacetate, and

PCMB.

To obtain some insight into the mechanism of this enzymatic reaction, Sagisaka and Shimura (1963) studied the exchange of pyrophosphate with ATP catalyzed by the enzyme extract. This exchange was dependent on the presence of alpha-amminoadipate, Mg^{++}, ATP, and PP. It was concluded that δ -adenyl-alpha-aminoadipic acid was formed and that this was the substrate for reduction to the semialdehyde. Support for this was the reported isolation of adenylated alpha-aminoadipate in yeast by Mattoon in 1961.

Alpha-aminoadipic acid-6-C^{14} and pipecolic acid-6-C^{14} were converted to lysine in Euglena gracilis by a direct route, indicating that pipecolic acid was an intermediate in the aminoadipic acid pathway of lysine biosynthesis (Rothstein and Saffron, 1963).

G.   Hydroxyproline and Hydroxylysine Biosynthesis.

The evidence now appears to be conclusive that the oxygen of the hydroxyl group of hydroxyproline is derived from atmospheric oxygen (Paik, 1963). Hydroxyproline in the cell wall protein of higher plants is formed in a similar way (Lamport, 1963).

It has been known since 1949 (Stetten) that proline, but not free hydroxyproline, is incorporated into collagen. This suggested, at first, that protein-bound proline was hydroxylated. Evidence continues to be obtained, however, that some comparatively low molecular weight proline-containing peptide is the actual substrate for the hydroxylation. Puromycin,

which inhibits incorporation of both proline and hydroxyproline into collagen, does not prevent the conversion of proline to hydroxyproline, as judged by the radioactivity in the free hydroxyproline (Manner, 1963). The same study reports isolation of traces of radioactive hydroxyproline-s-RNA, and suggests that proline-s-RNA might be the hydroxylation substrate. In similar experiments, Peterkofsky and Udenfriend in 1963 obtained results which were inconsistent with this view, but which indicated that the hydroxylation substrate was a microsomal RNA-proline polypeptide adduct.

Formation of hydroxylysine parallels that of hydroxyproline and is completed during incorporation into collagen (Popenoe, 1962). When lysine-C^{14} is injected into young rats the specific activities of the lysine and hydroxylysine isolated from gelatin prepared from the insoluble skin collagen become equal in 4 to 15 hours after the injection.

H. Cysteine-Cystine Biosynthesis.

It has long been known that microorganisms and green plants utilize inorganic sulfur compounds for the synthesis of the sulfur-containing amino acids, although the reactions involved in the synthetic pathway have, up to now, remained obscure. Recently, an enzyme was discovered (Brugyemann, 1962) that catalyzes the condensation of L-serine and H_2S to form L-cysteine. This enzyme, serine sulfhydrase (cysteine synthetase), occurs in a variety of bacteria molds, yeasts, and green plants,

and in various mammalian tissues. The level of activity in chicken liver was about the same as in yeast extract. Enzyme activity was enriched 50-fold by ammonium sulfate and Al(OH)3-gel fractionation from baker's yeast (Schlossmann, 1962). The enzyme required pyridoxal-P, utilized only L-serine, and formed L-cysteine. The enzyme from yeast showed optimum activity at pH 8.0 to 8.6 and was highly stable to storage and heating at 85° (Schlossmann, 1962).

I. <u>Aromatic Amino Acids Biosynthesis.</u>

Recent studies of the biosynthesis of the aromatic amino acids from shikimate have led to the development of the partial scheme shown below (Scrinivasan, 1963 and Rivera, 1963).
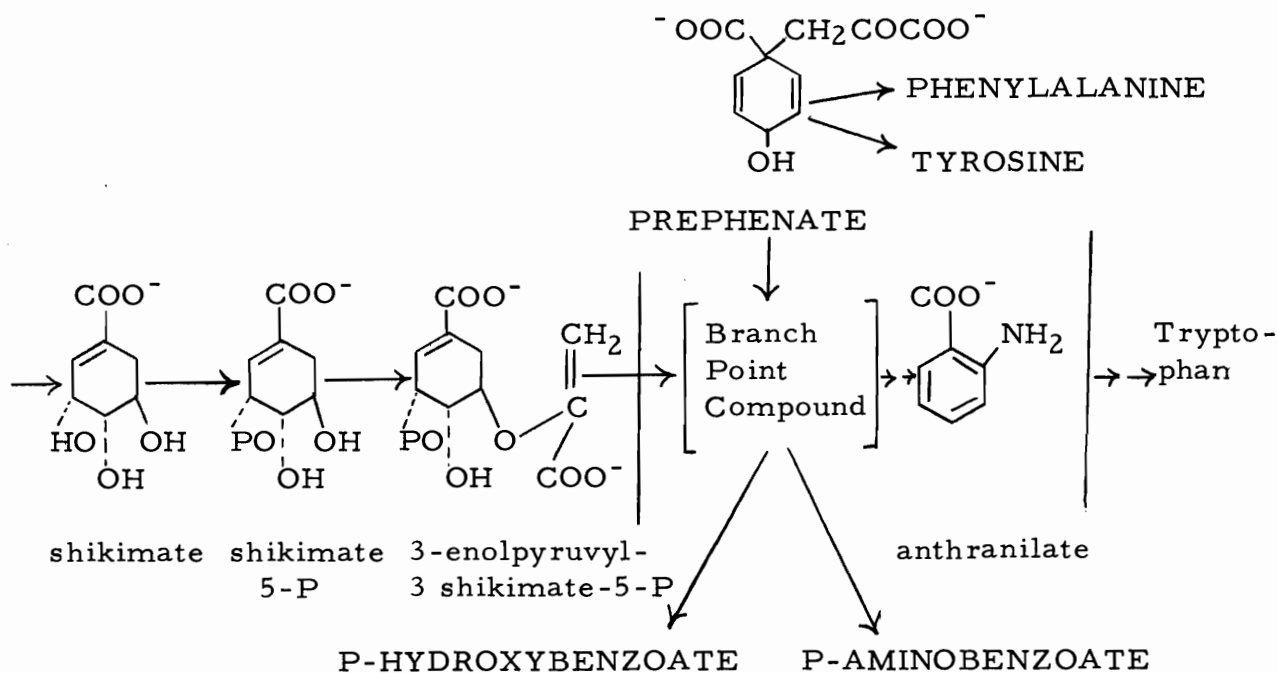
Earlier studies had established that 5-dihydroquinate and 5-dihydroshiki-
mate were precursors of shikimate. The pathway has now been established
to the branch point compound of the last scheme.

Using cell-free extracts of $\underline{E}$. $\underline{coli}$ B-37, Srinivasan and Rivera (1963)
showed that anthranilate could be formed from shikimate-5-P and gluta-
mine. This reaction required an NADH-generating system, and was
promoted by Mg^{++} or Fe^{++}, and less effectively by Mn^{++}. That the
amide nitrogen of glutamine was incorporated into anthranilate was shown
by use of N^{15} (amide) glutamine. The reaction was strongly inhibited by
aza-L-serine and 6-diazo-5-oxo-L-norleucine.

Fractionation of the bacterial extract with protamine sulfate and
ammonium sulfate yielded fraction I, containing 3-enolpyruvylshikimate-
5-P-synthetase, and II, the anthranilate-forming enzyme system (Rivera,
1963). Neither fraction alone could synthesize anthranilate from shikimate-
5-P, nor could the two combined unless phosphoenolpyruvate was present
in addition to glutamine, glutathione, and an NADH-regenerating system.
In the presence of 3-enolpyruvylshikimate, phosphoenolypruvate was
unnecessary. Enzyme fraction I catalyzed synthesis of 3-enolpyruvyl-
shikimate from shikimate-5-P and phosphoenolpyruvate. Three-
enolpyruvylshikimate is converted to an unidentified compound (branch
point compound) which is a precursor of anthranilate (Rivera, 1963).
This has been termed compound x by Gibson et al. (1962), who,

independently, obtained results paralleled to those described above

with extracts from mutant strains of Aerobacter aerogenes.

MATERIALS AND METHODS

I. CULTIVATION OF YEAST CELLS

The osmophilic yeasts used in these studies have been isolated from sugar syrup samples by inoculating Mycophil agar* slants acidified with lactic acid (15 ml of 10 per cent lactic acid/liter). The tubes were incubated at room temperature for four days and then the yeast cells streaked on Mycophil agar plates to study their purity. The Mycophil agar slants were maintained in the refrigerator as stock cultures.

A. Growth Media.

The growth medium used in these studies was variaties of Mycophil broth containing some salts and growth factors. This medium was prepared as follows:

peptone	10 gm
sucrose	20 gm
water	1000 ml

Salts in gm/liter (Meyer and Benziman, 1959).

0.5 gm K_2HPO_4	0.01 gm NaCl
0.5 gm KH_2PO_4	0.008 gm $FeSO_4$
0.2 gm $MgSO_4 \cdot 7H_2O$	0.01 gm $MnSO_4 \cdot H_2O$

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* Mycophil agar manufactured by Baltimore Biological Laboratory (B. B. L.), Baltimore, Maryland.

<u>Growth factors (in ug/ml, final concentration).</u>

0.2 ug Riboflavin	0.1 ug Ca Pantothenate
0.1 ug P-aminobenzoic acid	0.1 ug Pyridoxine
0.05 ug Biotin	0.2 ug Nicotinic acid
0.1 ug Thiamine	

B. Culturing Methods.

A volume of 500 ml of the growth medium, containing the salts and growth factors in a wide flat-bottomed flask, were seeded with yeast cells and placed on a shaker at room temperature for four days. The yeast cells were centrifuged at 2500 rpm for 15 minutes, washed three times with deionized distilled water by centrifugation at the same speed and for the same time; and then the yeast cells were suspended in deionized distilled water. The optical density of the yeast suspension was adjusted to a specific reading according to the different experiments using a spectronic 20 Spectrophotometer (Bausch and Lomb) at 700 mu applying the red filter.

II. CLASSIFICATION OF OSMOPHILIC SUGAR YEAST

The yeast cells isolated from sugar syrup samples of high count were the basis of study in this investigation. As for the osmophilic yeasts, many new species and varieties have been described in the literature. An attempt was made to classify those isolated in this laboratory.

The methods employed and the classification system referred to here were essentially the same as those outlined in the monographs of Wickerham (1951) and Lodder and Kreger-Van Rij (1952). These methods were:

A. Morphology.

Cornmeal agar petri dishes were used for studies of morphology. The yeast cells were streaked across the plate near one side. The amount of inoculum used was small, and enough pressure exerted on the needle to scarify the agar. On the same plate but opposite to the streaks two point inoculations, again using small amounts of inoculum, were made. A 22 mm square coverglass was removed from absolute alcohol, with sterile forceps, drained, flamed, and cooled for a few seconds and then placed over the point streak. The plates were incubated at 25° C for 6 to 8 days. The general appearance of the resulting culture and colony morphology were noticed. Yeast cells were observed microscopically using methylene blue stained smears.

B. Sporulation.

Yeast cultures were continuously shaken in solutions containing 1 to 3 mg of glucose plus 10 to 30 mg of potassium acetate per milliliter for two weeks. After that, the cells were stained as follows: Heat fixed smears were stained in hot (80° C) 5 per cent aqueous solution of malachite green for 3 to 5 minutes. Decolorized by water for 30 seconds and

counter stained by 0.5 per cent aqueous solution of safranin for 10 seconds (if there was any spore formation, spores were stained blue green and vegetative cells red).

C. Carbon Assimilation Tests.

A ten-fold concentration of stock medium was prepared by dissolving 3.4 g of the dehydrated carbon assimilation medium (Difco) and an appropriate carbon source in 50 ml of deionized distilled water. (If the latter was an acid or acid salt, the pH was adjusted to 5.2 by the addition of NaOH pellets). The solution was sterilized by passage through a Seitz filter and then kept in the refrigerator until needed. Five-tenths milliliter portions were pipetted into clear 16 mm tubes containing 4.5 ml of sterile deionized distilled water. The tubes after thorough mixing were ready for inoculation. The final concentration of carbon source was equivalent in assimilable carbon to a 0.5 per cent glucose solution.

An inoculation medium was prepared which contained the usual concentration of other ingredients but only 0.1 per cent glucose. Each tube contained 10 ml of inoculation medium. One milliliter was removed and used to suspend the growth from a 24 or 48 hour slant of yeast. Two to for-tenths ml of the suspension, depending upon the abundance of the growth on the slants, was returned to the inoculation medium and the culture was then diluted with the base medium which contained no glucose. Five milliliter of inoculum in 16 mm tubes were diluted until black lines

drawn on a white card became visible as dark bands. Generally, about

two volumes of diluent were required per volume of culture. Each tube

in the set of test media received 0.1 ml of the diluted inoculum. The

tubes containing the various carbon sources were then incubated at 25° C

and read at approximately 7 days and again at 20 to 24 days. The

cultures were observed for pellicles and the presence of visible amounts

of riboflavin (practically the only soluble yellow compound produced by

yeast). They were then shaken and placed against a white card bearing

lines approximately 3/4 mm wide, drawn with India ink. If the growth

in the tubes completely obliterated the lines, it was recorded as 3+; if

the lines appeared as a diffuse band, the growth was recorded as 2+;

and if the lines were distinguishable as such but had indistinct edges,

it was recorded as 1+. The absence of growth was recorded as -

(negative). A 3+ or 2+ reaction at 24 days was considered as positive,

and a 1+ reaction as weak (partially assimilated). Glucose, galactose,

maltose, sucrose and lactose were used in the carbon assimilation tests.

D. Nitrate Assimilation Test.

The test medium was similar to that for tests on carbon assimilation,

except that it contained, when completed with water, 10 g of glucose, and

0.78 g of KNO_3, per liter, and smaller amounts of each of the 3 amino

acids (histidine, methionine, tryptophan). It contained no asparagine or

other source of nitrogen in amounts large enough to interfere seriously

with a demonstration of the assimilation of nitrate. A tube of nitrate medium received 0.1 ml of the same inoculum used for the carbon assimilation tests. After the nitrate culture had been incubated about seven days, even though nitrate had not been assimilated, it usually showed growth, probably due both to the soluble nitrogenous compounds excreted by the cells of the inoculum and to the small amount of ammonium sulfate in the inoculation medium. Hence, a second nitrate tube was inoculated with one loop from the first. After a similar period of incubation the second tube was observed. If it showed a 2+ or 3+ reaction, the yeast was considered capable of assimilating nitrate; if there was no growth or a 1+ reaction, the yeast was considered incapable of assimilating nitrate.

E. Vitamin Deficiency Test.

The vitamin deficiency medium was similar to that used in the carbon assimilation tests except that 10 g of glucose per liter were included, and the vitamins were excluded. Sixteen and seven tenths gm of the vitamin free yeast base were dissolved in 100 ml deionized distilled water and filtered through a Seitz filter. The test was run in exactly the same manner as the nitrate assimilation tests. The first tube usually showed growth at 7 days, owing to the carry-over of vitamins in the inoculum. The yeasts which produced a 2+ or 3+ reaction in the second tube were capable of synthesizing all the vitamins they require

for growth; those which produced no growth or a 1+ reaction were considered to be incapable of synthesizing all of the vitamins required for growth.

F.   Fermentation Tests.

The medium used consisted of 4. 5 g of powdered yeast extract, 7. 5 g of peptone, 1, 000 ml of deionized distilled water, and enough bromothymol blue to give a sufficiently dense green color.   Two-milliliter quantities were placed in 150 x 12 mm tubes.   A smaller tube measuring 50 x 6 mm was put inverted into the medium to detect the formation of gas.   After sterilization, a 1 ml quantity of an individual sugar solution was added to each tube to make the final sugar concentration 4 per cent.   Aqueous solutions of glucose, galactose, maltose, sucrose and lactose were prepared in 12 per cent concentration.   The solutions were sterilized by Seitz filtration and kept in the refrigerator. The fermentation media were inoculated with 0. 1 ml of a suspension of cells prepared by suspending the growth of a 24 or 48 hour malt extract-yeast extract agar slant culture of yeast in 4. 5 ml of sterile deionized distilled water.   The cultures were incubated at 28° C.   The tubes were shaken and observed for the amount of gas in the inverted gas tubes and a change of color of the indicator after 1, 2, 4, 6, 8, 12, 16, 20 and 24 days of incubation.

G. Growth at Moderate Osmotic Pressure.

The medium used consisted of 10 g NaCl, 5 g glucose and 100 ml deionized distilled water.   The solution was pipetted into 16 mm tubes in 4. 5 ml quantities.   The tubes were stoppered with cotton and autoclaved for 20 minutes.   Each tube received 0. 5 ml of sterile glucose-free base medium prepared at a 10X concentration, and 0. 1 ml of the same inoculum as used in all the assimilation tests.   Judgment of growth was as described above.   A 1+ amount of growth after 20 to 24 days of incu-bation at 25° C was recorded as weak, a 2+ amount of growth as moderate, and 3+ as good.

H. Ability to Liquify Gelatin.

Ten grams of gelatin were dissolved in 90 ml of hot deionized distilled water.   The solution was pipetted in 4. 5 ml quantities into 16 mm test tubes stoppered with cotton, and autoclaved for 15 minutes.   After cool-ing to somewhat above room temperature, 0. 5 ml quantities of ten fold glucose concentrate, as used in the assimilation tests, were added.   The final medium contained approximately 10 per cent gelatin, 0. 5 per cent glucose, and 0. 5 per cent ammonium sulfate in addition to the usual vitamins, trace elements, and salts.   The medium in the tubes was then allowed to solidify.   Each tube was inoculated with the same amount of inoculum as employed in the assimilation tests and was spread over the solid surface of the medium.   Incubation was at 25° C, at which temperature

the medium remained solid unless liquified through proteolysis by the
yeasts. In approximately 7 to 24 days the depth of the liquid layer,
if any, was measured in millimeters.

I. Ability to Grow at 37° C.

A tube of the usual glucose assimilation medium, inoculated in the
manner described above was incubated at 37° C. It was observed and
read in the same manner at approximately 7 and 24 days. The results
were recorded in the same manner as in the test for the ability to grow
in the moderate osmotic pressure medium.

J. Production of Starch.

Inoculated and incubated tubes of the 24 day carbon assimilation
test cultures which contained the various sugars were examined for the
presence of starch. One drop of approximately 0.02 N iodine solution
was added to each tube and the tube shaken. Those strains producing
starch would show reactions varying in intensity from blue to purple to
green; the color may reside in the medium, in the cells, or in both.

In some genera the relatively large amounts of glycogen synthesized
may interfere with the test, especially if stronger iodine solutions are
used. The brown color typical of glycogen may mask a weak starch
reaction. Where doubt arose, the tubes were allowed to stand at room
temperature for a few hours or overnight. The brown color indicating
glycogen disappeared but the blue color indicating starch remained. The

ability to produce starch has interesting applications in the taxonomy

of yeasts.

K. Ethanol as Sole Source of Carbon.

The basic medium used contained $(NH_4)_2 SO_4$ 0.1%, KH_2PO_4 0.1%

$MgSO_4 \cdot 7H_2O$ 0.05% in distilled water. It was sterilized in tubes for

15 minutes at 120° C, then 3% ethanol and a drop of sterile yeast extract

or a mixture of vitamins were added. Tubes filled with 5 ml of medium

were inoculated with 0.1 ml of the yeast suspension or was used to

inoculate the assimilation tests. They were incubated at 25° C. The

results were read after 1 week and at 3 weeks.

III. NITROGEN-FIXATION STUDIES

A. Biological Nitrogen Determination.

It was observed that osmophilic yeast cells grow very readily in

sugar syrup samples. This observation suggested a study of the

possible ability of these yeast cells to fix atmospheric nitrogen.

For the determination of total nitrogen in biological nitrogen

fixation experiments many workers have used different micro analytical

methods. Mortenson in 1961, for example, used a simple method for

measuring nitrogen fixation by cell-free enzyme preparations of Clos-

tridium pasteurianum. The method comprised the measurement of the

ammonia produced during nitrogen fixation and could detect from 3 to 5

µg nitrogen fixed. Parker (1961) demonstrated a good method for the

measurement of total nitrogen in microgram amounts (in the range of

0.5 to 25 µg). He utilized a micro Kjeldahl type digestion to reduce all

the nitrogen to ammonia which was then collected in Conway's micro-

diffusion vessels. Mathies (1962) reported a simple, indirect sensitive

procedure for the determination of ammonia nitrogen at the microgram

and submicrogram level.

For the purpose of these experiments, different methods for the

determination of total nitrogen were attempted, for example, the Koch

and McMeekin (1924) Nessler method. In this case a precipitate usually

appeared during the time of color development, which interferred with

the actual spectrophotometric readings. To overcome this difficulty

different concentrations of the gum ghatti were tried but without any

significant improvement. For this reason the method was discarded.

The phenol reagent method (Folin and Ciocalteu, 1927), another spec-

trophotometric method, was tried but found to be unsuitable. It was

found that the phenol reagent (Folin reagent) gives a deep blue color

with sucrose solutions and ascorbic acid.

After this, attention was turned to the microdiffusion principle,

which, in conjunction with Kjeldahl digestion, was of value in consider-

ably diminishing the work in serial determinations and for all micro-

ranges increased the sensitivity of the determination. With quantities

as low as 1 µg nitrogen Tompkins and Kirk (1942) reported errors of

less than 0.3 per cent. On application of this method for the determination
of total nitrogen in the sugar syrup samples, it was found that there are
no sharp color changes in the boric acid-indicator mixture. Finally,
the micro-Kjeldahl method (Freedrich, 1933) was modified to suit the
purposes of the experimentation and utilized as follows:

1. Digest mixture:

The digest mixture was made of equal parts of concentrated H_2SO_4
and saturated $KHSO_4$ solution containing 0.2 per cent sodium selenate.

2. Boric acid:

Ten grams of the reagent grade boric acid were introduced into a
one liter flask, 200 ml ethyl alcohol (95%) were added and then about
700 ml deionized distilled water. The boric acid was brought into
solution and ten ml of the mixed indicator (bromo-cresol green and
methyl red) added. On mixing, the whole was brought to the desired
end point color of faint reddish by the addition of a little alkali (NaOH),
and the mixture made up to 1 liter.

3. Mixed indicator:

The mixed indicator contained bromocresol green 0.033% and methyl
red, 0.066% in ethyl alcohol (95%). This mixed indicator was found to
be more advantageous for the titration than the methylene blue-methyl
red indicator and keeps indefinitely.

4. Potassium hydroxide:

For the alkalinization of the digest or aliquot parts thereof, 40 per cent potassium hydroxide appeared to be the most suitable and was, therefore, utilized.

5. Standard acid:

Standard N/100 hydrochloric acid was prepared as follows: Hydrochloric acid approximately 0.1 $\underline{N}$ was prepared and standardized against standard 0.1 $\underline{N}$ sodium carbonate (anhydrous) using methyl red as an indicator. The standard N/100 acid was then prepared by dilution.

6. Procedure:

Upon digestion of high concentrations of sucrose there was a great deal of charring. To overcome this difficulty the sugar syrup was diluted to 10^{-2}, and three milliliters were digested by 10 ml of the digestion mixture. After digestion, the Kjeldahl flask was cooled down under tap water, diluted with about 20 ml deionized distilled water and the solution then transferred to a 50 ml volumetric flask. The Kjeldahl digestion flask was washed several times with deionized distilled water, and the washings added to the contents of the 50 ml volumetric flask.

A water blank sample of 10 ml volume was treated with 9 ml of 40 per cent potassium hydroxide and distilled into 10 ml of the mixed indicator-boric acid mixture diluted (1:1). The distillation lasted

usually for 5 1/2 minutes. This was used as a comparison for the titration of the sugar syrup samples.

A volume of eight milliliters out of the 50 ml volumetric flask containing the digested sugar sample to be analyzed were placed in a Kjeldahl digestion flask and after the addition of 10 ml of 40 per cent KOH was distilled into 10 ml of mixed indicator-boric acid mixture. Following the distillation the indicator color change was titrated back to the color of the blank indicator using N/100 hydrochlorid acid.

B. Nitrogen-Fixation Investigation.

Preliminary experiments of this investigation were to determine any increase of total nitrogen in Mycophil broth and plain 30 per cent sucrose solution. Both were seeded with one ml of sugar syrup containing high yeast count. The experiment was set up as follows:

Two samples, 50 ml each of Mycophil broth were transferred into two-125 ml Erlenmeyer flasks; into another two flasks similar volumes of plain 30 per cent sucrose solution were placed. Each flask was seeded with one milliliter of sugar syrup of high yeast count. Flask number one and three were left to stand at room temperature, and flasks, two and four were incubated at 37° C. The nitrogen determination was carried out using the previously mentioned modified micro-Kjeldahl method (Friedrich, 1933) for each flask, at zero time, two and five days.

C. Study with Washed Yeast Cells.

In this case, a 14 day old Mycophil yeast culture was centrifuged at 1300 rpm for 15 minutes, washed by centrifugation three times with 30 per cent sucrose solution. The yeast cells were kept refrigerated in 30 per cent sugar and used in experiments as described in the previous paragraph.

The effect of yeast inoculae on culture media containing different nitrogen sources was studied as follows:

Fifty milliliters 30 per cent sucrose solution containing few crystals of ammonium sulfate were inoculated with 1 ml yeast suspension in water, and the amount of total nitrogen was determined at zero time and every hour thereafter for a nine hour incubation period at room temperature. This experiment was repeated using glutamic acid, aspartic acid, B-alanine and threonine as nitrogen sources instead of ammonium sulfate.

IV. EFFECT OF CATIONS AND ANIONS, HYDROGEN ION CONCENTRATION, TEMPERATURE, AND SUGAR CONCENTRATION ON YEAST GROWTH

A. Effect of Cations and Anions on Growth and Nitrogen Fixation.

It has been established that the cations and anions play a significant role in nitrogen fixation and growth of microorganisms. Therefore, the

role of trace elements on nitrogen fixation and growth of yeast cells

under investigation was made the subject of these studies.

1. Growth media:

The growth medium was Mycophil broth prepared as described above.

The pH of the medium was adjusted to 4. 4 using 0. 1 $\underline{N}$ hydrochloric acid.

2. Reagents:

The salts of the different cations and anions used in the investigation

were prepared as 0. 1 molar solutions of ferrous sulfate, K_2HPO_4,

sodium molybdate, boric acid, sodium citrate and calcium chloride.

3. Methods:

The inoculum for cations and anions studies was prepared as follows:

The growth medium was inoculated with 5 ml of a yeast suspension in

water and placed on a shaker at room temperature for five days. The

yeast cells were centrifuged at 2500 rpm for 15 minutes, washed three

times with 2 per cent sterile sucrose solution, and then suspended in

30 per cent sucrose solution.

This inoculum was adjusted to a specific optical density as follows:

One milliliter of the yeast suspension was diluted to 10 ml with water

and then measured at 700 mµ against 10 ml water as a blank using

Spectronic 20 spectrophotometer and red filter. The optical density

recorded was 1. 95.

In this investigation, the methods and procedures reported by Takha (1964) were used. A series of 125 ml Erlenmeyer flasks were used for the experiment; each flask contained 25 ml of 30 per cent sucrose solution and a specific concentration of the cation or anion to be studied for its stimulating effect on nitrogen fixation and growth of the osmophilic yeasts. Each flask was seeded with 2 ml of the yeast suspension prepared, and placed on a shaker for ten days at room temperature. The cations and anions used in the investigation were iron, phosphorus, molybdenum, boron and calcium.

In these experiments two control flasks were prepared, one (I) as a control for the nitrogen fixation studies, and the second (II) as a control for the effect of cations on growth.

Control I was a flask containing 25 ml of 30 per cent sucrose solution and 2 ml of the yeast suspension. This control was prepared to measure the biomass at zero time. Immediately after seeding 10 ml of the sugar solution was centrifuged at 3000 rpm in a weighed small centrifuge tube for 10 minutes. The centrifugate was washed with deionized distilled water by centrifugation at the same speed and for the same time as above. The tube was then placed in an oven of 80° C for 24 hours to dry to constant weight; thus, the difference in weight is the biomass weight at zero time incubation.

Control II was a flask prepared in the same manner as Control I but was placed on the shaker together with the test flasks, which contained

the different concentration of cations or anions. These were shaken for the ten day incubation period.

After ten days on the shaker, 10 ml of each flask were treated as described for Control I. The amount of nitrogen in the biomasses was then determined using the modified micro-Kjeldahl method (Friedrich, 1933). At the same time one ml of each flask was utilized to determine the total nitrogen of the tested cultures.

B. Effect of Hydrogen Ion Concentration.

It is well known that the growth of microorganisms and particularly yeasts is influenced to a great extent by changes in hydrogen-ion concentration. Therefore, in the present work, the effect of changes in hydrogen-ion concentration of culture media on the growth of the yeast cells under investigation was studied. The procedure described by Ōnishi (1957b) was used. The growth medium of this series of experiments was Mycophil broth containing 30 per cent sucrose. Buffer solutions of pH 3, 4, 5, 6 and 7 were prepared using 0.2 $\underline{M}$ - Na_2HPO_4 and 0.1 $\underline{M}$ - citric acid (pH 3, 4.11 ml Na_2HPO_4 and 15.89 ml citric acid; pH 4, 7.71 ml Na_2HPO_4 and 12.29 ml citric acid; pH 5, 10.30 ml Na_2HPO_4 and 9.70 ml citric acid; pH 6, 12.63 ml Na_2HPO_4 and 7.37 ml citric acid; pH 7, 16.47 and Na_2HPO_4 and 3.53 ml citric acid). The experiment was set up as follows: Seven and 8 tenths ml of the Mycophil broth medium containing 2 per cent and 30 per cent sucrose respectively were pipetted into a series of standardized test tubes, then 2 ml of the buffer solutions

and 0.2 ml of yeast suspension in water were added. The optical density

for each tube (at 700 mu against water and using red filter) was recorded

at zero time and after 24 hours using Spectron-ic 20 spectrophotometer.

C. Effect of Culture Temperature.

It is quite clear that the temperature of incubation plays a signifi-

cant role in the growth rate of microorganisms. Numerous investigations

of the effect of culture temperature on microorganisms growth have been

made. Van Halteren (1950) showed that yeast is protected against the

effect of heat by a concentrated solution of sugars. At the same time

Ōnishi (1959b) found that Saccharomyces rouxii was able to multiply at

40° C in the presence of sugars at concentrations higher than 30 per cent.

Therefore, in the present work the effect of culture temperature on

growth of yeast cells under investigation was a subject of study.

In this investigation Mycophil broth containing 30 per cent sucrose

was used as the growth medium; the medium was buffered with a solution

at pH 4 (7.71 ml 0.2 $\underline{M}$ - Na_2HPO_4 and 12.29 ml 0.1 $\underline{M}$ - citric acid)

and osmophilic yeast cells suspended in water were used as the inoculum.

The experiment was set up as follows: Seven and ninty-eighth one

hundredths ml Mycophil broth, 2.0 ml buffer solution (pH 4) and 0.02

ml of the yeast suspension were pipetted into 3 standardized test tubes.

One was kept at refrigerator temperature, the second at room temperature

and the third at 56° C for 20 hours. The optical density of each tube was

measured at zero time and after 20 hours of incubation using Spectronic

20 spectrophotometer with the red filter (at 700 mu using water as a

blank).

D. Effect of Sugar Concentration.

The yeast cells under investigation grow in the sugar samples con-

taining as high as 65.5 per cent sucrose. The question arises now if

these yeast cells are true osmophilies. To answer that question the

effect of sucrose concentration on growth of these yeast cells was

studied.

Three different sucrose concentrations (2 %, 30% and 60%) were

investigated for their effect on growth of the yeasts. The experiment

was set up as follows: Mycophil broth media containing the different

concentrations of sucrose were prepared and used as growth media. A

volume of 7.8 ml of these Mycophil media containing the various concen-

trations of sucrose were pipetted into three standardized test tubes. Two

ml buffer pH 4 (7.71 ml 0.2 $\underline{M}$ - Na_2HPO_4 and 12.29 ml 0.1 $\underline{M}$ - citric

acid) and 0.2 ml of the yeast suspension prepared as described earlier

were added. The optical density was then recorded for these preparations

using the Spectronic 20. The measurements were at 700 mu using red

filter and water as a blank.

V. UTILIZATION OF AMMONIUM SULFATE AND DIFFERENT

AMINO ACIDS AS NITROGEN SOURCES IN THE

PRESENCE AND ABSENCE OF SUCROSE

It is well established that most yeast strains can use ammonium salts for aerobic growth in the presence of a suitable carbon source. Yeasts growing under conditions found in nature most probably derive at least part of their nitrogen requirements from amino acids occurring free or produced by the hydrolysis of the proteins found in the natural growth media. The ability of the yeast cells under investigation to use ammonium sulfate and some amino acids for growth was studied.

The growth media including salts and growth factors and the culturing method applied in these studies were similar to those mentioned earlier under growth media and culturing method.

A. Growth Experiments.

The growth experiments in this investigation were conducted according to the methods used by Meyer and Benziman (1959). The yeast growth was determined turbidimetrically using the Spectronic 20 spectrophotometer at 700 mu and red filter.

Washed yeast cells were suspended in sterilized deionized distilled water and the amount of yeast suspension, which on dilution to 10 ml gives an optical density reading 0.175, was determined. This amount of yeast suspension was then pipetted into the number of test tubes necessary for

the respective experiment, the necessary amounts of salts, growth factors, amino acids, and sucrose added (from concentrated stock solutions) and the whole made up to 10 ml. This was the basic set up for the experimental procedure. Due to several variatives in procedure the exact amount of salts, growth factors, amino acids and sucrose (2% and 30%) are presented with the results.

VI. BIOSYNTHESIS AND COMPOSITION OF AMINO ACID POOL IN OSMOPHILIC YEAST

The growth of yeast cells in sucrose solutions and the induction of enzyme synthesis carried out in the absence of an external nitrogen source attracted our attention to the point that in such inductions the nitrogen employed by the cell in fabricating the new enzyme molecule must derive from some preexisting nitrogenous compounds in the cell. One was immediately faced with the obvious necessity of identifying the components thus employed. Ability to form enzyme in the absence of an external supply of nitrogen is far more widespread among the yeasts than among the bacteria. Thus, it was of some interest to investigate our yeast cells under investigation for the presence and composition of amino acid pool and the effect of growth media on that pool.

A. Growth Media.

In this investigation a complete medium was used. It was composed of Mycophil broth containing 30 per cent sucrose, salts and growth factors.

Other growth media were used in the study to compare their effects on the composition of the amino acid pool of the yeast cells isolated from sugar samples. One medium was Mycophil broth with growth factors, etc., but contained no sucrose, and the other was composed only of 30 per cent sucrose plus the salts and growth factors.

B. Method of Cultivation.

A volume of two-hundred and fifty milliliters of the growth media above were seeded with 3 ml of washed yeast cells suspended in water. The cultures were allowed to stand on a shaker for one week at room temperature. The yeast cells were then centrifuged at 3000 rpm for 5 minutes, washed with deionized distilled water three times and finally suspended in water and kept in the refrigerator until extraction and analysis could be accomplished.

C. Extraction of Pool Samples.

In designing methods for obtaining pool samples the research was primarily guided by the studies of Gale (1947). Two procedures gave the most consistent results, and were at the same time the simplest.

1. The boiling method:

In this method a yeast suspension (10 per cent by volume of cells) was placed in a boiling water bath for ten minutes and then cooled. The material was then centrifuged at 3000 rpm for 5 minutes and the supernate retained for analysis.

2. Alcohol method:

The method reported by Kretovich and Krauze (1961) was followed.
The yeast cells were fixed in 10 ml of 96 per cent ethanol and allowed to
extract over 24 hours at room temperature. The alcohol yeast mixture
was then centrifuged at 3000 rpm for 5 minutes. The centrifugate was
again extracted in the same volume of 80 per cent ethanol for 24 hours.
The combined extracts were exposed to a jet of cold air to evaporate the
alcohol and the residue was then dried over solid NaOH in a vacuum
disicator. This was dissolved in 3 ml 10 per cent aqueous isopropanol
and was then ready for analysis.

D. Method of Analysis.

The method used for the analysis of the free amino acids in yeast
extracts was ascending unidirectional paper chromatography. Methods
from the manual of paper chromatography and paper electrophoresis
(Block and Durrum, 1955) were used.

1. Solvents:

Although a great many solvents have been employed for the separation
of amino acids and peptides, two proved to be the most useful in this
investigation.

(a) Phenol solvent: One hundred milliliters of metal-free
water were dissolved in 400 ml of Mallinckrodt Gilt label redistilled
phenol by gentle warming. The addition of 0.04 per cent 8-quinolinol

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(8-hydroxyquinoline) (Block, 1950) to the phenol retards its decomposition.
The solvent was stored in a dark bottle in the refrigerator where the cold
caused separation into 2 layers. When this solvent was to be used, the
bottle was vigorously shaken and the desired quantity of the emulsion
removed and gently warmed to affect solution.

A beaker containing 0.3 per cent ammonia was placed in the chroma-
togram chamber to allow the more basic amino acids to travel further in
the solvent. The paper was exposed to 1:4 ammonia solution for four
minutes before placed in the chromatogram chamber.

(b) Butanol-acetic acid solvent: Sixty ml of glacial acetic acid
were added to 500 ml of a freshly shaken mixture of equal volumes of
water and n-butanol. After the layers separated in a separatory funnel,
the upper layer was used as the moving phase solvent. An aliquot of the
lower layer (25-100 ml) was placed in the chromatogram chamber (Woiwod,
1949) in a small beaker.

2. Color reagent:

A 0.25 per cent ninhydrin in n-butanol was prepared.

3. Standard amino acids:

Standard solutions (1 mg/ml) of the amino acids were prepared for
reference materials in the paper chromatography experiments.

4. Paper:

The paper used in the investigation was Whatman No. 1, size 41 x
11.5 cm.

5. Unknown:

Samples of extract were weighed in small capillary tubes by difference
(weight was between 13 to 15 mg).

6. Time of run:

Paper chromatograms were allowed to stand overnight in the chroma-
tography chamber.

EXPERIMENTAL RESULTS

It was elected to study the properties of osmophilic yeasts, their ability to fix atmospheric nitrogen, their growth, the effect of some cations and anions on growth and nitrogen fixation, the yeast ability to utilize ammonium sulfate and different amino acids as nitrogen sources and the composition of the free amino acids biosynthesized by these yeast cells in different culture media.

I. CLASSIFICATION OF THE YEAST CELLS

UNDER INVESTIGATION

The present work was devoted to study those aspects mentioned above for yeast cells isolated from sugar syrup samples. Classification of the main yeast type found growing in sucrose syrups was attempted. The methods employed and the classification system referred to under materials and methods were essentially the same as those outlined in the monographs by Wickerham (1951) and by Lodder and Kreger-Van Rij (1962).

The results obtained from the taxonomic experiments are shown in Table 1. According to the characteristics shown in the table, this yeast resembles Saccharomyces sp. in regard to the vigorous fermentation of glucose and other sugars, and in the inability to assimilate nitrate,

TABLE 1

Morphological, cultural and physiological characteristics
of yeast cells under classification

Vegetative cells after 10 days' incubation on cornmeal agar, at 26° C:

Cells were round or very short oval, single or in pairs.   The cells usually measure (3-7) X (3-8) µ.

Growth on cornmeal agar after 2 weeks at 26° C: The streak culture was white cream colored, dull glistening, raised and almost smooth.

Ascospore formation: Not found.

Growth at moderate osmotic pressure: Very good growth.

Fermentation of sugars
 ┌─ Fermented: glucose, galactose (weak), maltose and saccharose.
 └─ Not fermented: lactose.

Assimilation of sugars
 ┌─ Assimilated: glucose and galactose.
 ├─ Partially assimilated*: maltose, saccharose
 └─ Not assimilated: lactose.

Assimilation of nitrate: Absent.

Ethanol as sole source of carbon: No growth.

Production of starch: Negative.

Ability to grow at 37° C: Very slight growth.

Growth in absence of vitamins (vitamin deficiency test): Positive.

Ability to liquify gelatin: Absent.

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* give 1+ readings

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but differs in its inability to assimilate most of the sugars fermented. These yeast cells closely resembled Saccharomyces italicus and Saccharomyces steineri since they fermented glucose, galactose, maltose and saccharose, but they differed from Saccharomyces italicus in that the latter could assimilate nitrate. The yeast cells differed from Saccharomyces steineri in the ability of the latter species to assimilate maltose and saccharose, and in the formation of primitive pseudomycelia consisting of tree like formations.

Owing to the ability of the yeast being classified to assimilate glucose and galactose, they resemble Saccharomyces acidifaciens, but the latter ferments only glucose.

From these characteristics shown in Table 1, the yeast strain studied was considered as a new species of the genus Saccharomyces.

II. NITROGEN-FIXATION STUDIES

In the present work, in order to study the ability of the yeast under investigation to fix atmospheric nitrogen, several experiments were devised and carried out.

In this case an experiment to compare the total nitrogen of Mycophil broth and 30 per cent sucrose cultures after 2 and 5 days was devised. Some of the cultures were incubated at room temperature and the others at 37° C. The total nitrogen was determined as described in the section

on the methods at zero time, 2 and 5 days. The data from these studies are presented in Table 2.

It is clear that there was a remarkable decrease in the nitrogen content, either of the Mycophil broth culture or of the 30 per cent sucrose. The decrease in nitrogen in the case of Mycophil broth cultures was more significant than in the case of sucrose cultures.

Another experiment was carried out using 50 ml of 30 per cent sucrose solution which was inoculated with one milliliter of Mycophil broth culture of yeasts. The quantity of total nitrogen in this case was determined at zero time, 1, 2, 3, 4, 22 and 24 hours as shown in Table 3.

From these data presented in Table 3, when compared with the data of Table 2, it is to be noted that the decrease in the nitrogen content significantly occurs during the first 2 hours of incubation.

A. Study with Washed Yeast Cells.

A 14 day old yeast culture was centrifuged at 1400 rpm for 15 minutes and washed three times by centrifugation with 30 per cent sucrose solution. The supernate was discarded and the yeast cells were transferred to an Erlenmeyer flask containing 50 ml of 30 per cent sucrose solution. The culture was incubated at room temperature and the total nitrogen determined as usual at zero time and after different incubation times. The results obtained are shown in Table 4.

TABLE 2

Nitrogen fixation studies of osmophilic yeasts in Mycophil
broth and in 30% sucrose at R. T. and 37° C

Time in Days	MgN/ml of the culture			
	Mycophil broth		30% sucrose	
	I R. T.	II 37° C	III R. T.	IV 37° C
0	1.030	1.032	0.342	0.342
2	0.350	0.235	0.171	0.145
5	0.377	0.209	0.194	0.119

TABLE 3

Nitrogen fixation studies of osmophilic yeasts grown
in 30% sucrose for 24 hours

Time (hrs.)	ml N/1000 HCl	mgN/ml
0	0.350	1.68
1	0.200	0.96
2	0.160	0.77
3	0.150	0.73
4	0.150	0.73
22	0.146	0.70
24	0.140	0.67

TABLE 4

Nitrogen-fixation studies with washed yeast cells

Time (days)	ml N/1000 HCl	mgN/ml
0	0.175	0.510
1	0.160	0.456
2	0.190	0.541
3	0.21	0.598
5	0.23	0.655

These data show that there was a slight increase in total nitrogen after the second day of incubation. This indicates that atmospheric nitrogen was fixed as there was no other source of nitrogen. However, before concluding that these yeasts have the ability to fix atmospheric nitrogen, more experiments were conducted and the results are found in the next section. These experiments dealt with the effect of cations and antions on growth and possible nitrogen-fixation.

In the above experiments it was observed that there was a decrease of total nitrogen, especially if the growth media contained a nitrogen source. The following experiment was set up to investigate the effect of yeast inocula on culture media containing different nitrogen sources. Ammonium sulfate and certain amino acids were utilized. The procedure is found in the section on methods. The results obtained are shown in Table 5. They demonstrated that there was a significant decrease in total nitrogen, which was in agreement with the observations found in the experiments which results are shown in Table 2.

In this series of experiments, it was found that there was an apparent decrease in the total nitrogen. Certainly there was no increase to indicate nitrogen fixation.

TABLE 5

Growth of osmophilic yeast in 30% sucrose solution
containing different nitrogen sources

Time (hrs.)	Ammonium sulfate	mgN/ml			
		L-glutamic	L-aspartic	B-alanine	DL-threonine
0	9.63	1.45	0.68	2.37	2.08
1	9.46	1.11	0.48	2.02	1.57
2	9.12	1.08	0.51	1.77	1.65
3	9.06	1.14	--	1.79	1.69
4	8.64	1.11	0.48	1.82	1.74
5	8.27	1.08	0.48	1.80	1.77
6	7.89	0.91	0.46	2.17	1.54
7	8.65	0.88	0.51	2.05	1.54
8	8.61	0.88	0.51	1.95	1.66
9	8.55	0.94	0.53	1.85	1.79

III. <u>EFFECT OF CATIONS AND ANIONS, SUGAR CONCENTRATION,</u>

<u>HYDROGEN-ION CONCENTRATION AND TEMPERATURE</u>

<u>ON THE GROWTH OF OSMOPHILIC YEAST</u>

A. <u>Effect</u> <u>of</u> <u>Cations</u> <u>and</u> <u>Anions</u> <u>on</u> <u>Growth</u> <u>and</u> <u>Nitrogen-Fixation.</u>

In the present work the effect of changes in concentration of different cations and anions on the growth of osmophilic yeast was studied. At the same time the effect of these cations and anions on the ability of the yeast cells to fix atmospheric nitrogen was observed.

1. <u>Effect</u> <u>of</u> <u>iron:</u>

Different concentrations of ferrous sulfate were added to the yeast cultures and the amount of biomass and its nitrogen content and the total nitrogen of the culture after ten days of shaking at room temperature were determined as described under materials and methods. The results obtained in this case are shown in Table 6 and presented graphically in Figure 1. These data showed that there was no significant increase in total nitrogen compared to Control I. At the same time it was found that there was an increase in the biomass of those cultures treated with ferrous sulfate, compared to that culture which contained no ferrous sulfate, (control II).

2. <u>Effect</u> <u>of</u> <u>citrate:</u>

In this case two flasks were prepared as follows: One contained 0.3 ml 0.1 $\underline{M}$ Fe $SO_4 \cdot 7H_2O$ solution (308.7 mg/L), and the other contained the same amount of ferrous sulfate plus 0.1416 gm sodium citrate (5410 mg/L). The data obtained are shown in Table 7, where it was found that

TABLE 6

Effect of concentration of ferrous sulfate on growth
and nitrogen fixation of osmophilic yeasts

No.	mg $FeSO_4 7H_2O$ per liter	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I*	- -	800	25.25	49
II**	- -	1200	26.74	43.40
1	102.9	1570	33.88	51.80
2	205.8	1210	31.36	49.70
3	308.7	1320	29.82	50.16
4	411.6	1320	28.42	51.10
5	514.5	1090	25.34	46.20

I* - Control for N-fixation studies.
II** - Control for the effect of cations and anions studies.

TABLE 7

Effect of sodium citrate concentration on growth
and nitrogen fixation of osmophilic yeasts

No.	mg/L salts	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I	- -	800	25.25	49
II	- -	1200	26.74	43.40
1	308.7 ferrous sulfate	1350	29.68	47.60
2	ferrous sulfate +5410 sodium citrate	1190	25.62	46.90

FIGURE 1. Effect of initial concentration of ferrous sulfate on growth and nitrogen fixation of osmophilic yeasts.

  I. Control for N-fixation studies

  II. Control for trace elements effect studies

☐ Biomass

▨ Total N

▧ N in Biomass
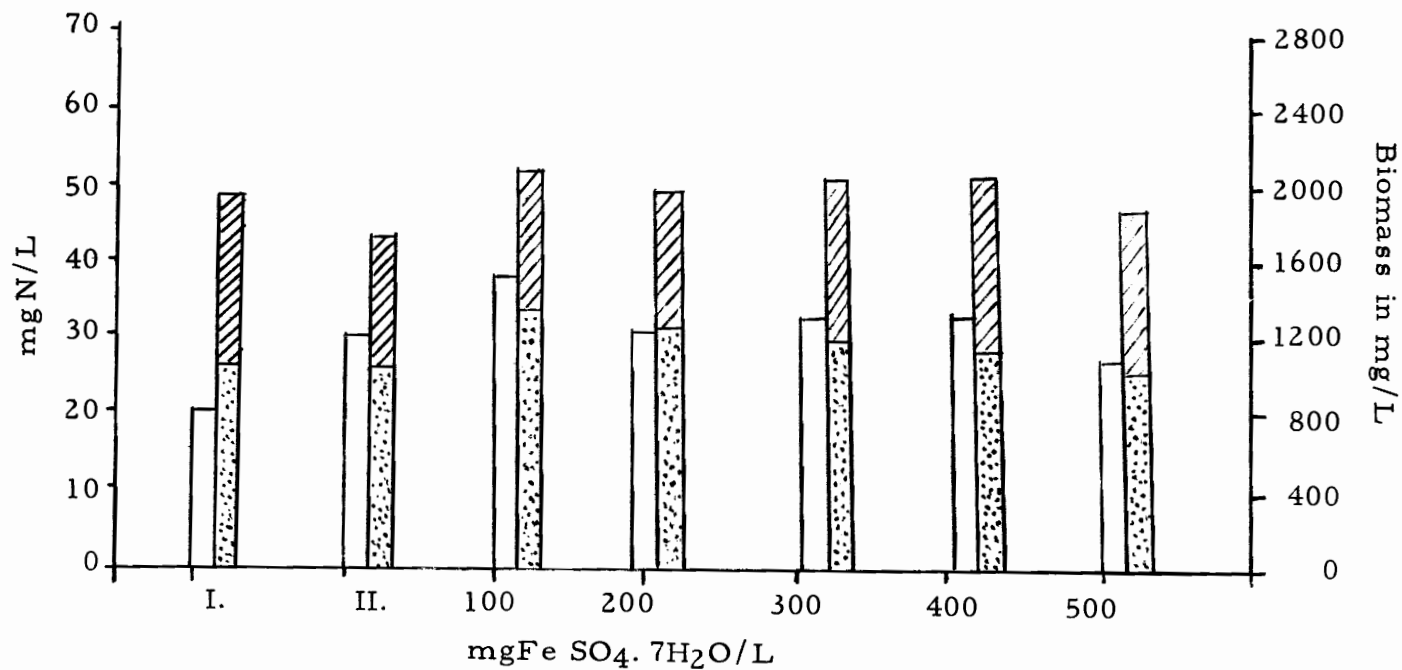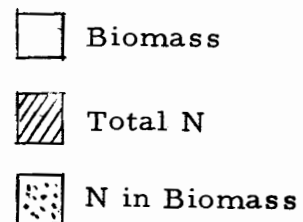
there was a decrease in the total nitrogen compared to control I. It was also clear that there was no growth enhancement in the culture that contained iron and citrate compared to the culture that contained ferrous sulfate and no citrate.

3. Effect of phosphorus:

The effect of phosphorus concentration was studied as described in the case of ferrous sulfate and citrate. The total nitrogen, nitrogen in the biomass and the amount of biomass were determined and the results are shown in Table 8 and Figure 2.

The addition of K_2HPO_4 increased the biomass as compared to media containing no phosphate, while the nitrogen control of the biomass and the total culture remained the same. It is clear that phosphorus stimulated the growth of the yeast cells more efficiently than iron. Concerning nitrogen fixation, it was found that there was a slight increase in the total nitrogen as a result of the addition of phosphate.

4. Effect of molybdenum:

The same series of experiments were carried out to study the effect of molybdenum on the growth and possible nitrogen fixation of osmophilic yeasts. The biomass of the yeast cells and its nitrogen content and the total nitrogen content were determined as usual and compared with the controls (I, II). The results obtained are shown in Table 9 and presented graphically in Figure 3.

TABLE 8

Effect of K_2HPO_4 concentration on growth and
nitrogen fixation of osmophilic yeasts

No.	mg K_2HPO_4/L	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I	--	800	25.25	49
II	--	1200	26.74	43.40
1	64.5	1460	32.48	51.10
2	129.0	1590	33.46	51.80
3	193.5	1790	34.30	53.20
4	258.0	2060	34.72	54.60
5	322.5	2500	35.14	56.90

TABLE 9

Effect of $Na_2MoO_4 \cdot 2H_2O$ concentration on growth and
nitrogen fixation of osmophilic yeasts

No.	mg Na $MoO_4 \cdot 2H_2O$ per liter	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I	--	800	25.25	49
II	-	1200	26.74	43.40
1	89.6	1250	23.10	46.90
2	179.2	1610	33.32	49.00
3	268.8	1640	33.46	48.30
4	358.4	1530	27.72	47.60
5	448.0	1960	34.44	51.10

FIGURE 2: Effect of initial concentration of K_2HPO_4 on growth and nitrogen fixation of osmophilic yeasts.

    I.   Control for N-fixation studies

    II. Control for trace elements effect studies

☐ Biomass

▨ Total N

▦ N in biomass

FIGURE 3. Effect of initial concentration of $Na_2MoO_4 \cdot 2H_2O$ on growth and nitrogen fixation of osmophilic yeasts.

I. Control for N-fixation studies

II. Control for trace elements effect studies

☐ Biomass

▨ Total N

⊡ N in Biomass
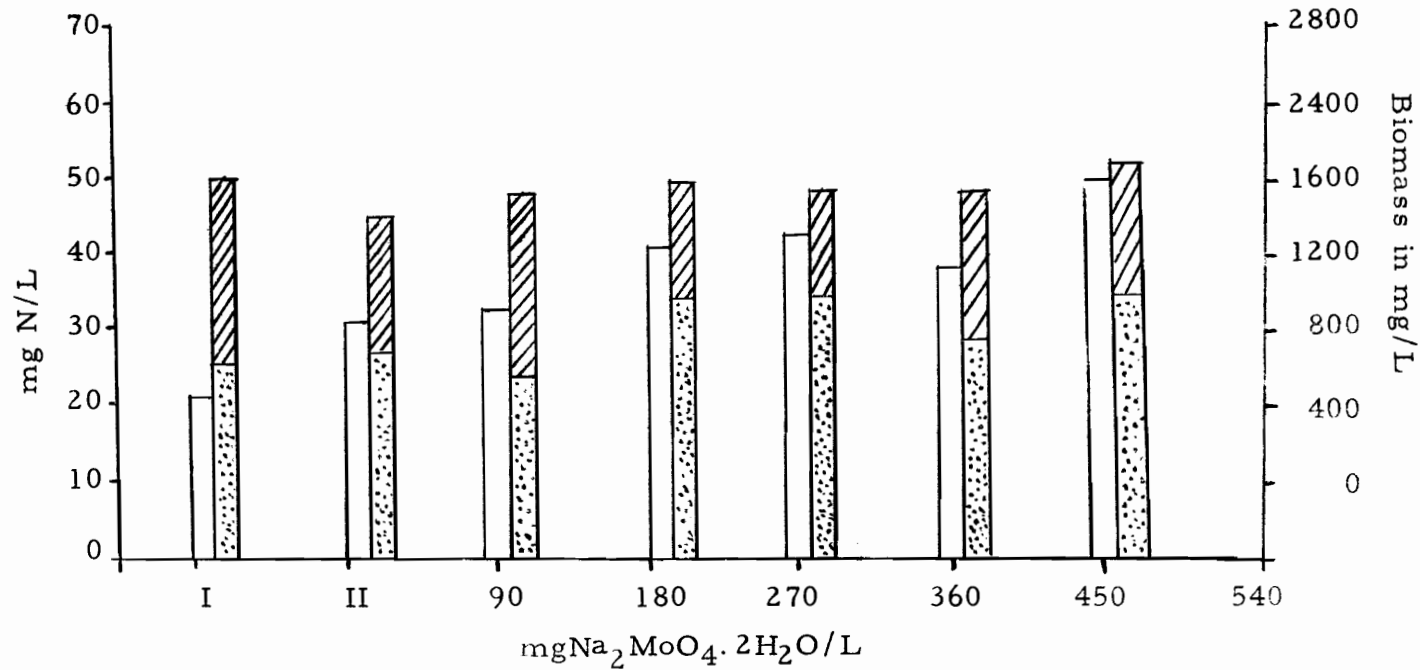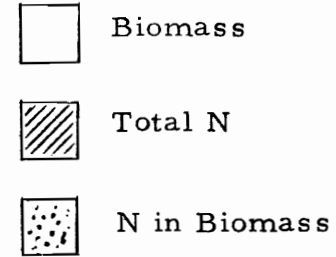
It was found that molybdenum enhanced the growth of yeast cells when comparing their biomass to that of control II, but it did not stimulate any significant increase in total nitrogen as compared to control I.

5. Effect of boron:

The effect of boron concentration on growth and nitrogen fixation of osmophilic yeasts was carried out using different concentrations of boric acid in a manner similar to the above experiments. Table 10 and Figure 4 present the data obtained in this investigation. It was clear from these data that there was no increase in total nitrogen compared to control I. Concerning the effect of boron on growth, there was little enhancement compared to the effect of phosphorus and molybdenum.

6. Effect of calcium:

Calcium chloride was used and the overall experimental set up was similar to the above experiments. The data obtained are shown in Table 11 and presented graphically in Figure 5. These data showed that there was no significant increase in the total nitrogen which indicated that these yeast cells were unable to fix atmospheric nitrogen. On the other hand, calcium enhanced the growth of the yeast cells to a degree similar to that of iron.

It was found that under the above experimental conditions, the yeast cells required from 240-360 mg per liter K_2HPO_4, 100-300 mg per liter ferrous sulfate, 180-270 mg per liter $Na_2MoO_4 \cdot 2H_2O$, 23-69 mg per

TABLE 10

Effect of boric acid concentration on the growth and
nitrogen fixation of osmophilic yeasts

No.	mg H_3BO_3/L	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I	--	800	25.25	49
II	--	1200	26.74	43.40
1	22.9	1600	32.20	47.60
2	45.8	1230	25.20	44.80
3	68.7	1570	33.60	46.20
4	91.6	1220	25.20	43.40
5	114.5	1370	29.40	44.80

TABLE 11

Effect of calcium chloride concentration on growth and
nitrogen fixation of osmophilic yeasts

No.	mg $CaCl_2$/L	mg/liter		
		Biomass	N-in biomass	Total nitrogen
I	--	800	25.25	49
II	--	1200	26.74	43.40
1	41.1	1090	24.50	44.80
2	82.2	1140	24.64	46.90
3	123.3	1350	26.32	45.50
4	164.4	1520	33.32	46.90
5	205.5	1260	30.24	44.10

FIGURE 4. Effect of initial concentration of boric acid on growth and nitrogen fixation of osmophilic yeasts.

I. Control for N-fixation studies

II. Control for trace elements effect studies

Biomass

Total N

N in Biomass
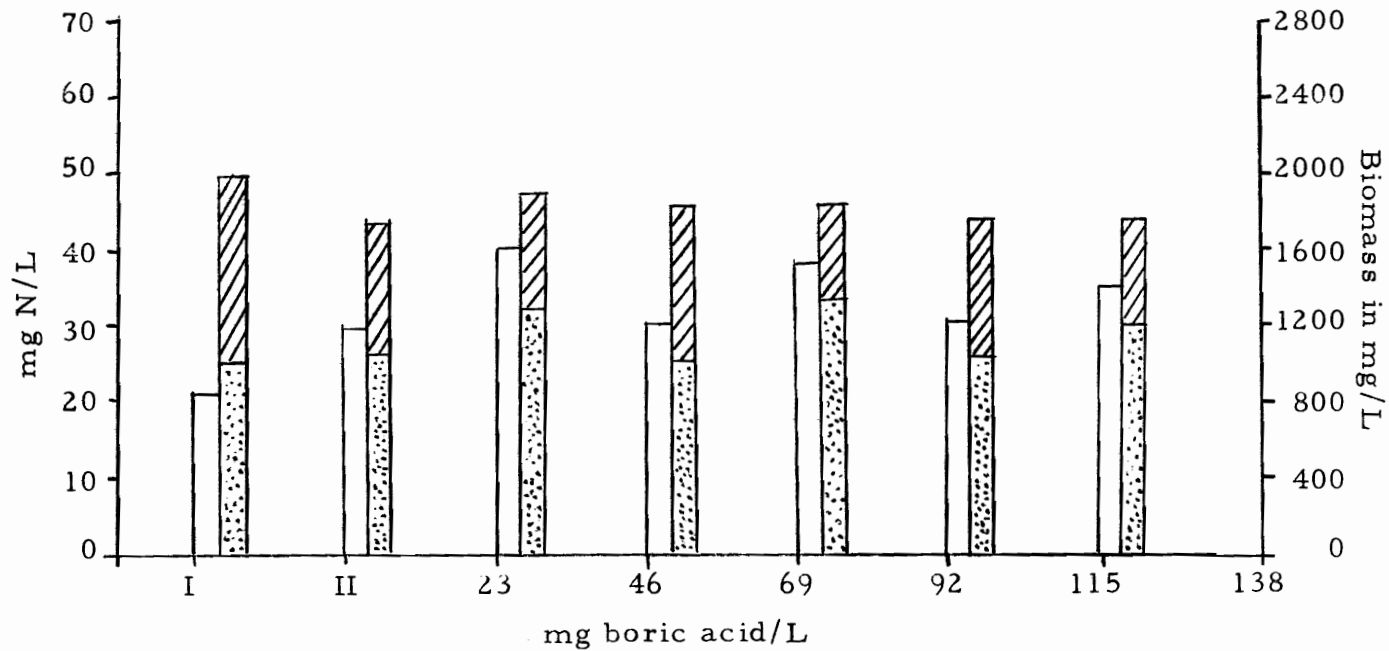
FIGURE 5. Effect of initial concentration of $CaCl_2$ on growth and nitrogen fixation of osmophilic yeasts.

I. Control for N-fixation studies
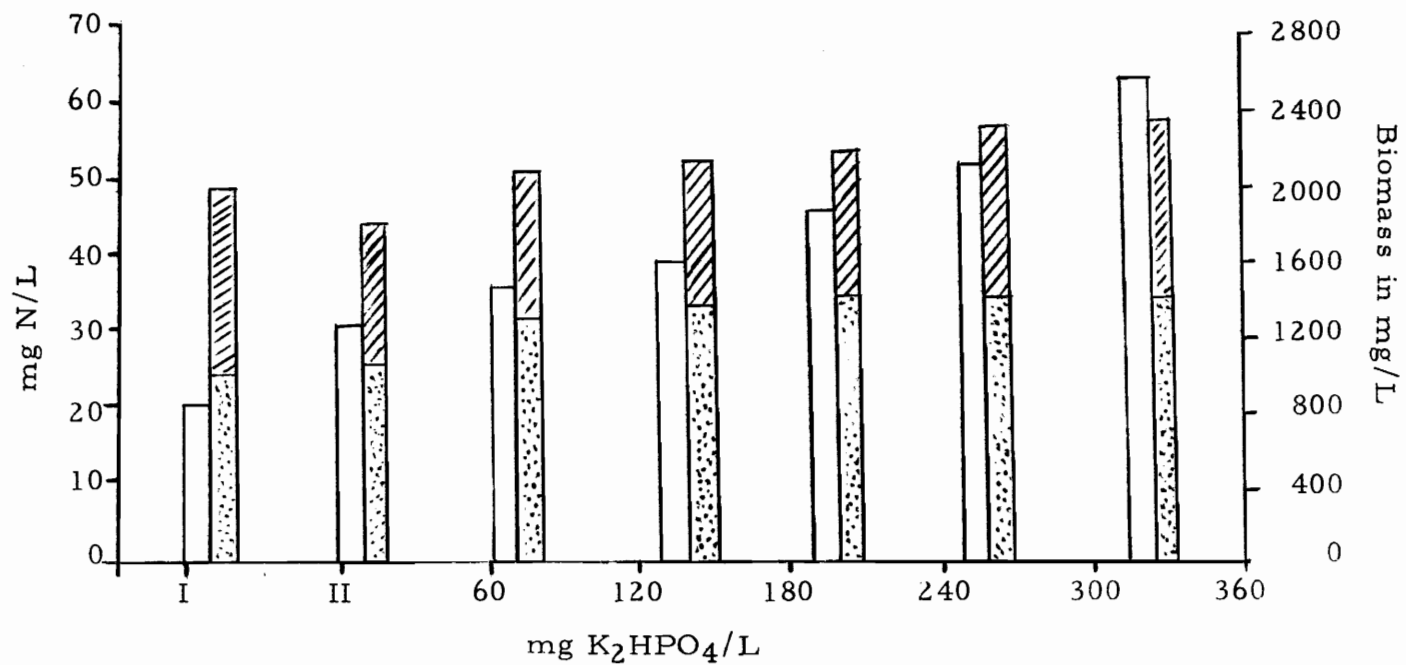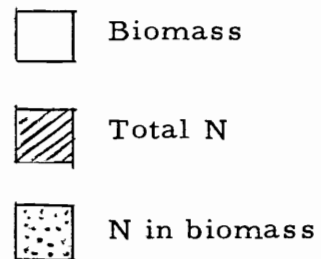
II. Control for trace elements effect studies

Biomass

Total N

N in Biomass
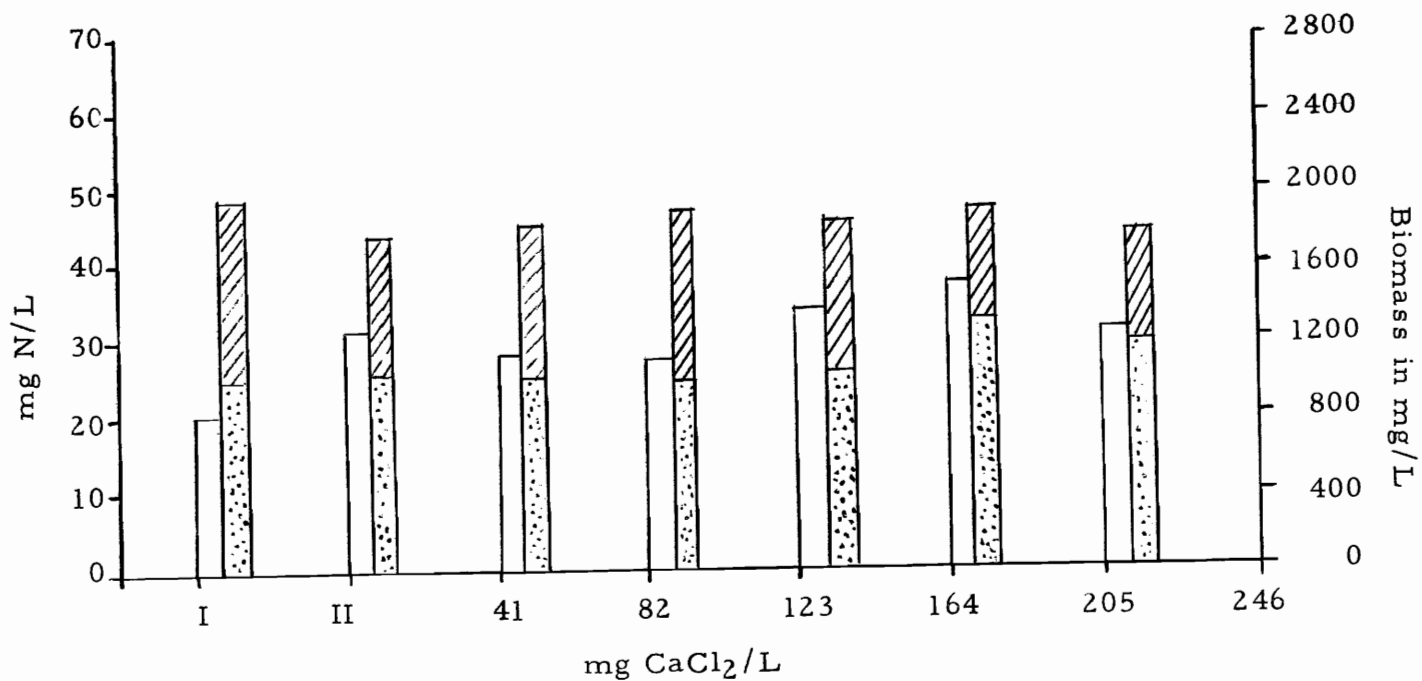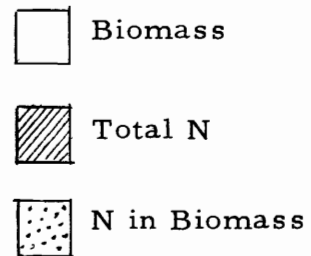
liter boric acid, and 123-164 mg per liter calcium chloride to reach maximum growth under the conditions of these experiments.

In summary, these data showed that there was no significant increase in total nitrogen of yeast cultures in the presence of the cations and anions tested, but at the same time there was an increase in the biomass which indicated that there must be another source of nitrogen other than the atmosphere; and thus the ability of the osmophilic yeasts to fix atmospheric nitrogen was ruled out.

As there was no increase in total nitrogen, therefore, the cations and anions tested were unable to stimulate the yeast cells to fix atmospheric nitrogen. On the other hand, the only established effect of the cations and anions was the enhancement of yeast growth as indicated by biomasses increase.

The above work indicated that the sugar samples may contain adequate nitrogen to support the growth of osmophilic yeasts.

In order to establish this possibility different sugar samples received from the Amalgamated Sugar Company were analyzed for the total nitrogen using the micro-Kjeldah method. The data obtained are presented in Table 12. From the table it is clear that the sugar samples contain 1.5 to 2.5 mg N per gram of sucrose.

B. Effect of Sucrose Concentration on Growth of Yeast Cells.

The effect of different sucrose concentrations on growth of yeast cells isolated from the sugar syrup samples was studied. The experiment was

TABLE 12

Determination of nitrogen in some sugar samples

Sample No.	ml N/1000 HCl	mg N/gm sugar
642	0.25	2.50
647	0.16	1.60
687	0.24	2.40
688	0.18	1.80
682	0.17	1.70
689	0.15	1.50
TC 166c	0.12	1.20
Blank sugar*	0.08	0.80

* Reagent grade Saccharose from Mallinckrodt

set up as described under materials and methods. The growth of yeast cells was determined by measuring the optical density at 700 mµ using red filter. Samples were removed at zero time, 20 and 40 hours. Another experiment was carried out using different experimental mixtures and different amounts of the inoculum. The experiment was set up as follows: Three Mycophil broth media containing 2%, 30% and 60% of sucrose respectively were prepared. Three different tubes each containing 8.9 ml Mycophil broth of different sucrose concentrations were set up. To each tube 1 ml of phosphate-citric acid buffer at pH4 and 0.1 ml of yeast suspension were added. The optical density was recorded at zero time and after 24 and 53 hours of incubation at room temperature.

The results of these experiments are shown in Table 13 and graphically presented in Figure 6. These data show that the yeast cells grew well in low sugar concentrations during the first 24 hours of incubation. After that period, they grew more efficiently at high sugar concentrations.

In summary, the data obtained indicate that these yeast cells are osmophilic since they grew better at high sugar concentrations than at low ones.

C. Effect of Hydrogen-ion Concentration on Growth of Osmophilic Yeasts.

The effect of hydrogen ion concentration on the growth of osmophilic yeasts in media containing 2 per cent and 30 per cent sucrose was investigated according to the procedure reported by Ōnishi (1957b). The results obtained in this investigation are shown in Table 14 and presented

TABLE 13

Effect of sucrose concentration on growth
of the yeast cells

Expt. No.	Time in hrs.	Optical density		
		Mycophil broth containing		
		2% sucrose	30% sucrose	60% sucrose
I	0	0.250	0.180	0.105
	20	0.385	0.355	0.120
	40	0.670	>2.00	0.150
II	0	0.065	0.035	0.010
	24	0.080	0.055	0.012
	53	0.375	0.460	0.165

TABLE 14

Effect of hydrogen-ion concentration on
the growth of osmophilic yeasts

pH	Optical density (after 24 hrs.)				
	3	4	5	6	7
Mycophil broth containing 2% sucrose	0.355	0.395	0.480	0.460	0.425
Mycophil broth containing 30% sucrose	0.275	0.295	0.285	0.275	0.220
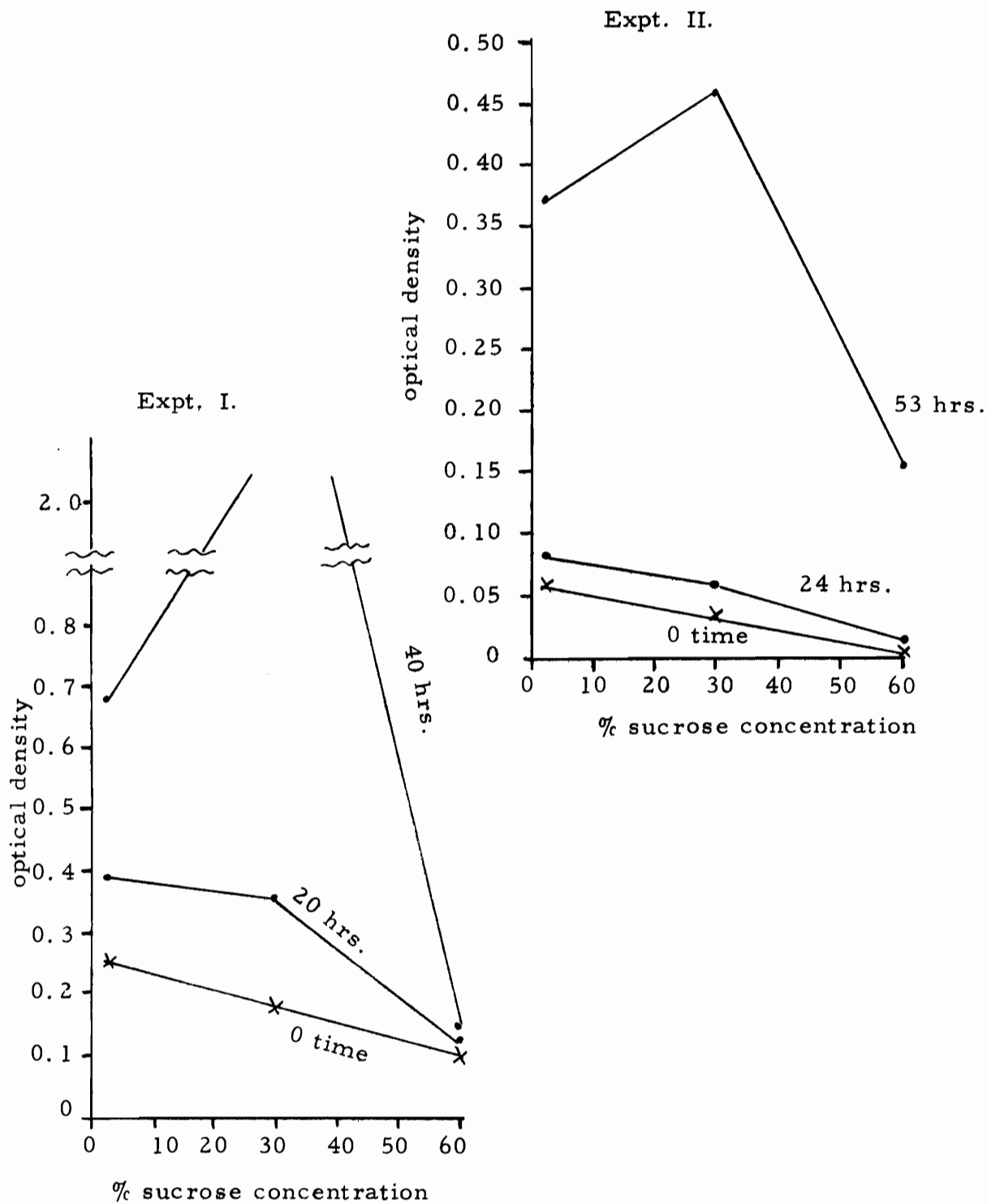
FIGURE 6.   Effect of sucrose concentration on growth of yeast cells.

graphically in Figure 7. These data indicate that the osmophilic yeasts tested were able to grow in a wide pH range of 3 to 7 in both media containing 2 per cent and 30 per cent sucrose.

D. Effect of Culture Temperature.

The influence of temperature on growth of osmophilic yeasts was investigated according to the methods used by Ōnishi (1959b). The experiment was set up as described under materials and methods. The optical density for each tube was recorded at zero time, 20 and 40 hours. The data obtained in this investigation are shown in Table 15. These data indicate that there was no growth at all at refrigerator temperature and 56° C, while there was excellent growth at room temperature.

IV. UTILIZATION OF AMMONIUM SULFATE AND AMINO ACIDS AS NITROGEN SOURCES IN THE PRESENCE AND ABSENCE OF SUCROSE

It is well known that, if the supply of other nutrients is adequate, ammonium nitrogen such as ammonium sulfate can be utilized by yeasts as the sole source of nitrogen in a conventional medium. Yeasts growing under conditions found in nature most probably derive at least part of their nitrogen requirements from amino acids occurring free or produced by the hydrolysis of the proteins found in the natural growth media, thus, the ability of the yeast cells isolated from the sugar samples to use ammonium sulfate and amino acids for growth was made the subject of the present investigation.
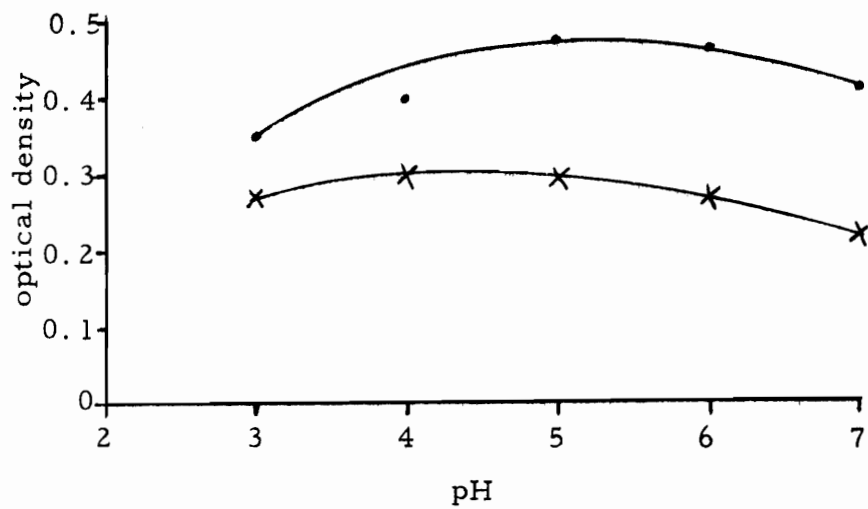
FIGURE 7. Effect of hydrogen-ion concentration on growth of yeast cells.

TABLE 15

Effect of culture temperature

Time in hrs.	Optical density Incubation temperature		
	Refrig. temp.	R. T. (26° C)	56° C
0	0.045	0.038	0.040
20	0.040	0.065	0.045
40	0.035	>2.000	0.038

First of all, the utilization of ammonium sulfate in the presence and absence of sucrose was studied according to the method described in materials and methods. The method involved the determination of yeast growth turbidimetrically. Different ammonium sulfate concentrations together with growth factors and salts were utilized as media. The growth increment in each case was then calculated by subtracting the zero time optical density reading from the ninth or last reading, then dividing the difference by the zero time reading.

The same experiment was repeated using different amino acids instead of the ammonium sulfate. The results obtained in each case are shown in Tables 16, 17, 18, 19 and 20. From these data one can compare the maximum growth increment of the osmophilic yeasts and the corresponding nitrogen concentration in the presence of the different nitrogen sources. This comparison is illustrated in Table 21. It is clear that the yeast cells utilized ammonium sulfate and proline as nitrogen sources more efficiently than the other sources tested. It is also obvious that the growth in the presence of sugar was better than in its absence. At the same time, yeast cells grown in the presence of sugar utilized more nitrogen.

Other experiments were carried out to study the utilization of ammonium sulfate, glutamic acid, aspartic acid, threonine, proline, alanine and

TABLE 16

Utilization of ammonium sulfate in the presence
and absence of sucrose

No.	ml 0.01 M amm. sulfate	ml growth F* and salts	ml yeast suspens.	ml H$_2$O	Growth increment in 9 hrs. X 10	
					In absence of sucrose	In presence of 5 ml 2% sucrose
1	0.1	0.5	0.3	9.1	16.0	16.2
2	0.2	0.5	0.3	9.0	14.8	15.0
3	0.4	0.5	0.3	8.8	17.2	18.0
4	0.6	0.5	0.3	8.6	17.2	19.0
5	0.8	0.5	0.3	8.4	15.1	17.2
6	1.6	0.5	0.3	7.6	14.8	14.9
7	2.0	0.5	0.3	7.2	17.5	17.8
Blank	--	0.5	--	9.5	--	--

* Growth factors

TABLE 17

Utilization of L-glutamic acid in the
presence and absence of sucrose

No.	ml 0.01 M L-glutamic acid	ml growth F* and salts	ml yeast suspens.	ml H$_2$O	Growth increment in 9 hrs. X 10	
					In absence of sucrose	In presence of 5 ml 2% sucrose
1	0.1	0.5	0.3	9.1	3.2	no increment
2	0.2	0.5	0.3	9.0	no increment	4.40
3	0.4	0.5	0.3	8.8	" "	3.33
4	0.6	0.5	0.3	8.6	" "	no increment
5	0.8	0.5	0.3	8.4	" "	0.97
6	1.6	0.5	0.3	7.6	" "	2.88
7	2.0	0.5	0.3	7.9	" "	2.88
Blank	--	0.5	--	9.5	--	--

* Growth factors

TABLE 18

Utilization of DL-threonine in the
presence & absence of sucrose

No.	ml 0.01 M DL-threonine	ml growth F* and salts	ml yeast suspens.	ml H$_2$O	Growth increment in 9 hrs. X 10	
					In absence of sucrose	In presence of 5 ml 2% sucrose
1	0.1	0.5	0.3	9.1	2.12	3.97
2	0.2	0.5	0.3	9.0	1.91	3.82
3	0.4	0.5	0.3	8.8	1.70	3.95
4	0.6	0.5	0.3	8.6	2.22	4.40
5	0.8	0.5	0.3	8.4	1.53	4.00
6	1.6	0.5	0.3	7.6	1.84	4.00
7	2.0	0.5	0.3	7.2	2.32	5.01
Blank	--	0.5	--	9.5	--	--

* Growth factors

TABLE 19

Utilization of B-alanine in the presence
and absence of sucrose

No.	ml 0.01 M B-alanine	ml growth F* and salts	ml yeast suspens.	ml H$_2$O	Growth increment in 9 hrs. X 10	
					In absence of sucrose	In presence of 5 ml 2% sucrose
1	0.1	0.5	0.3	9.1	5.80	6.9
2	0.2	0.5	0.3	9.0	5.10	8.8
3	0.4	0.5	0.3	8.8	6.28	6.6
4	0.6	0.5	0.3	8.6	6.00	8.0
5	0.8	0.5	0.3	8.4	6.10	7.3
6	1.6	0.5	0.3	7.6	6.20	6.9
7	2.0	0.5	0.3	7.2	6.60	7.6
Blank	--	0.5	--	9.5	--	--

* Growth factors

TABLE 20

Utilization of L-proline in the presence
and absence of sucrose

No.	ml 0.01 M L-proline	ml growth F* and salts	ml yeast suspens.	ml H₂O	Growth increment in 9 hrs. X 10	
					In absence of sucrose	In presence of 5 ml 2% sucrose
1	0.1	0.5	0.3	9.1	14.7	15.3
2	0.2	0.5	0.3	9.0	12.6	16.2
3	0.4	0.5	0.3	8.8	10.0	16.8
4	0.6	0.5	0.3	8.6	10.8	16.6
5	0.8	0.5	0.3	8.4	10.8	15.1
6	1.6	0.5	0.3	7.6	9.5	16.9
7	2.0	0.5	0.3	7.2	9.5	16.4
Blank	--	0.5	--	9.5	--	--

* Growth factors

TABLE 21

Maximum growth increment of osmophilic yeasts and
corresponding nitrogen concentration in the
presence and absence of sucrose

Nitrogen source	µg N/10 ml		Growth increment in 9 hrs. X 10	
	In absence of sucrose	In presence of 5 ml 2% sucrose	In absence of sucrose	In presence of 5 ml 2% sucrose
ammonium sulfate	112.06	140.05	17.20	18.00
L-glutamic A	14.00	42.00	3.20	4.40
DL-threonine	14.00	84.00	2.12	4.40
B-alanine	28.00	42.00	6.10	8.80
L-proline	14.00	42.00	14.70	16.20

methionine in the presence of 30 per cent sucrose. The experiments were set up as shown in Table 22 for every nitrogen source tested.

Similar mixtures were prepared in the same fashion except that they did not contain sucrose; instead, 5 ml of water were added to make the total volume 10 ml. The yeast cells were added at zero time and the optical density was recorded. The experimental mixtures were shaken at room temperature for 9 hours, during which time the optical density was recorded at 1, 3, 5, 7 and 9 hours. The data obtained in this investigation are shown in Table 23 and presented graphically in Figures 8 and 9. These results are in good agreement with those results obtained from the experiments that were carried out earlier; i.e., the results indicate that the yeast cells tested, utilized ammonium sulfate, L-proline, B-alanine and DL-threonine very efficiently. It is clear also that the growth of the yeast cells in the presence of sucrose was better than in its absence. It was also found that optical density readings of the cultures containing 30 per cent sucrose were lower than those containing no sugar. It was possible that this phenomenon was due to optical properties of the sucrose varying with changes in sugar concentrations. This possibility was ruled out by measuring the optical density of different sucrose concentrations (2%, 5%, 10% and 30%) at 700 mu using water as a blank. No difference was observed.

TABLE 22

Experimental set up for the investigation of utilization of
nitrogen sources in the presence of 30% sucrose

ml 0.01 $\underline{M}$ nitrogen source soln.	Growth F* and salts	ml 30% sucrose	ml yeast suspens. (in H_2O)	ml water
0.6	0.5	5	0.3	3.6
--	0.5	5	--	4.5 (Blank)

* Growth factors

TABLE 23

Utilization of different nitrogen sources by yeast cells in the
presence and absence of 30% sucrose

hrs.	Optical density													
	amm. SO_4		L-glutamic A		L-aspartic A		DL-threonine		L-proline		B-alanine		DL-methionine	
	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose
0	.135	.115	.135	.115	.137	.110	.141	.115	.135	.112	.139	.115	.135	.115
1	.115	.095	.105	.091	.115	.095	.112	.100	.103	.092	.112	.082	.108	.095
3	.265	.225	.210	.200	.251	.215	.270	.218	.278	.205	.265	.225	.275	.200
5	.280	.235	.210	.201	.262	.215	.285	.225	.295	.215	.280	.235	.291	.212
7	.280	.245	.210	.201	.262	.210	.285	.235	.295	.215	.282	.245	.291	.215
9	.275	.245	.215	.191	.250	.205	.280	.225	.288	.210	.275	.240	.285	.215

I* in absence of sucrose
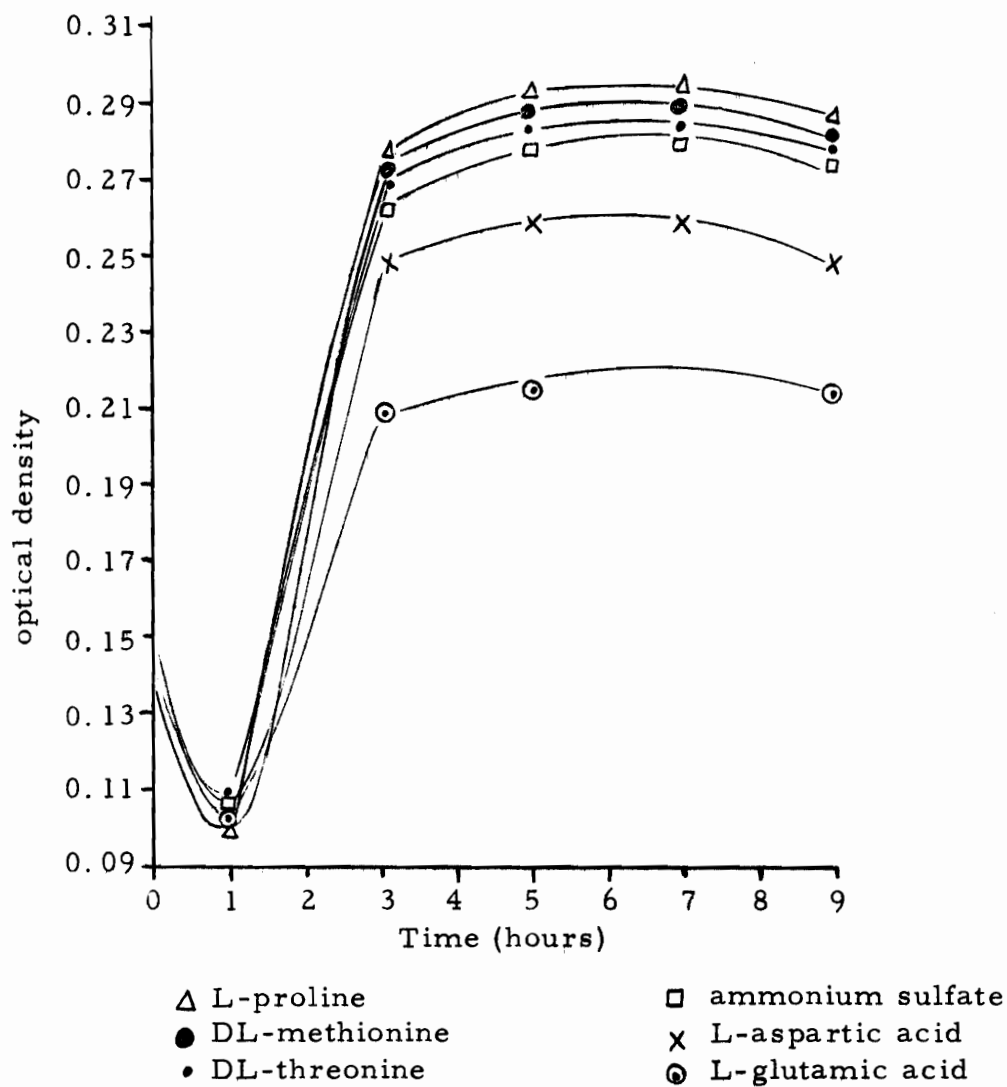
FIGURE 8. Growth curves of yeast cells grown in media
containing different nitrogen sources.
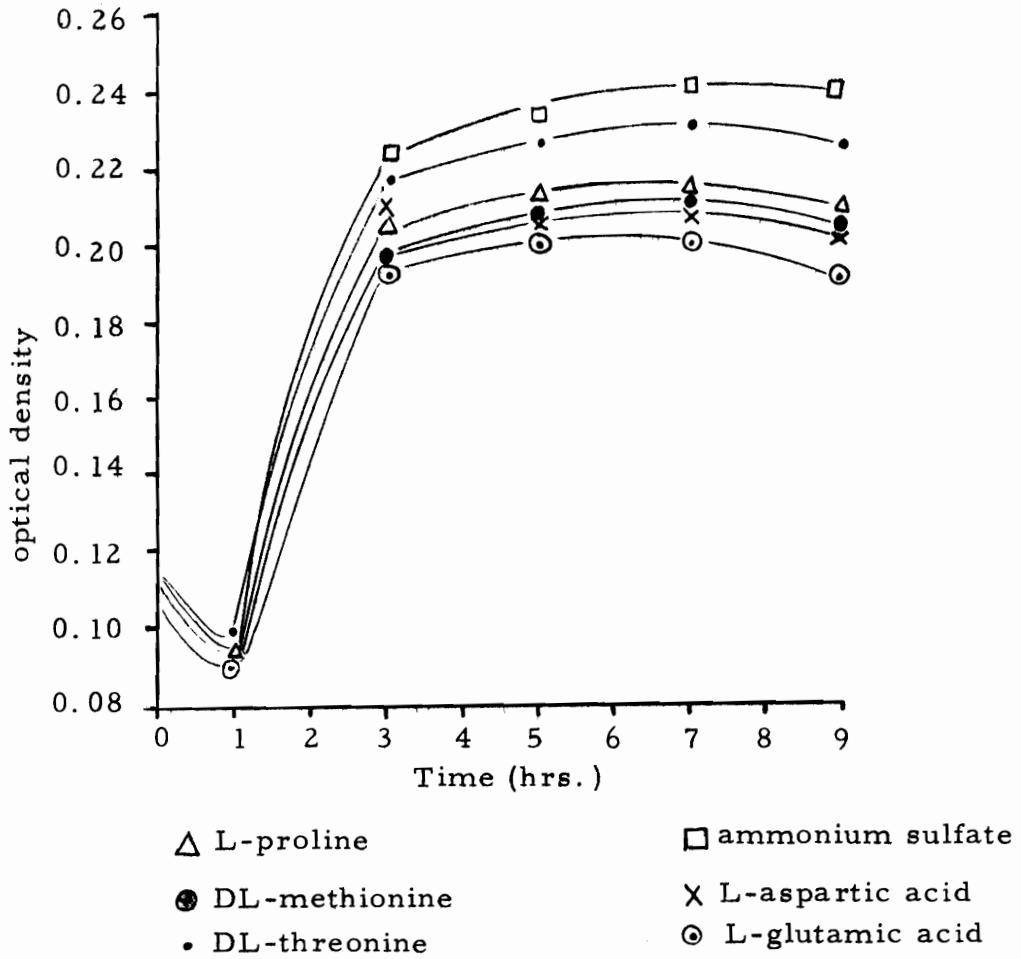
FIGURE 9. Growth curves of yeast cells grown in media containing different nitrogen sources and 30% sucrose.

It was suggested that possibly high sucrose concentrations changed the optical properties of the yeast cells; thus the sudden change from a water suspension of yeast used in the above experiments as inoculum to the 30 per cent sucrose medium might cause a difference in optical density of the cell. This possibility was investigated by carrying out experiments in which the yeast cells used were cultured in media containing sucrose concentrations similar to those used in the investigation media. The yeast cells were then harvested by centrifugation and kept in the corresponding sucrose concentration. Otherwise the experimental procedure was similar to that in the last experiments.

Mycophil broth containing 30 per cent sucrose was used as the culture medium. The yeast cells grown in the latter medium were washed three times with 30 per cent sucrose solution by centrifugation and then were suspended in 30 per cent sucrose solution. The experiment was set up as shown in Table 24.

Other experimental mixtures were prepared in the same fashion using deionized distilled water in place of the 30 per cent sucrose solution. The results obtained in this case are shown in Table 25 and presented graphically in Figures 10 to 13. From these results, it is clear that the optical density readings of the tubes containing 30 per cent sucrose were lower than those containing the same amount of yeast cells and no sucrose. Therefore, the results indicate that the lower optical density readings

TABLE 24

Experimental set up for utilization of nitrogen sources by
yeast cells cultured in Mycophil broth containing 30% sucrose

ml 0.01 $\underline{M}$ nitrogen source	ml growth F* and salts	ml 30% sucrose	ml yeast suspension (in 30% sucrose)
0.6	0.5	8.8	0.1
--	0.5	9.5	-- (Blank)

* Growth factors

TABLE 25

Utilization of nitrogen sources by yeast cells cultured in
media containing 30% sucrose

hrs.	optical density													
	amm. SO$_4$		L-glutamic A		L-aspartic A		DL-threonine		L-proline		B-alanine		DL-methionine	
	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose
0	.120	.060	.120	.060	.120	.060	.120	.060	.120	.060	.120	.060	.120	.060
1	.112	.058	.120	.060	.115	.057	.117	.057	.115	.058	.115	.059	.111	.055
3	.115	.070	.123	.070	.120	.065	.122	.062	.119	.062	.120	.062	.120	.060
5	.140	.075	.125	.075	.130	.072	.132	.070	.131	.068	.136	.064	.141	.063
7	.145	.075	.125	.075	.130	.072	.135	.070	.135	.070	.140	.064	.142	.063
9	.165	.085	.135	.080	.142	.082	.152	.073	.155	.080	.155	.068	.151	.070

I* in absence of sucrose

FIGURE 10. Growth curves of yeast cells grown in media containing ammonium sulfate (A) and L-aspartic acid (B) in the presence and absence of 30% sucrose.

FIGURE 11. Growth curves of yeast cells grown in media containing L-glutamic acid (A) and DL-threonine (B) in the presence and absence of 30% sucrose.
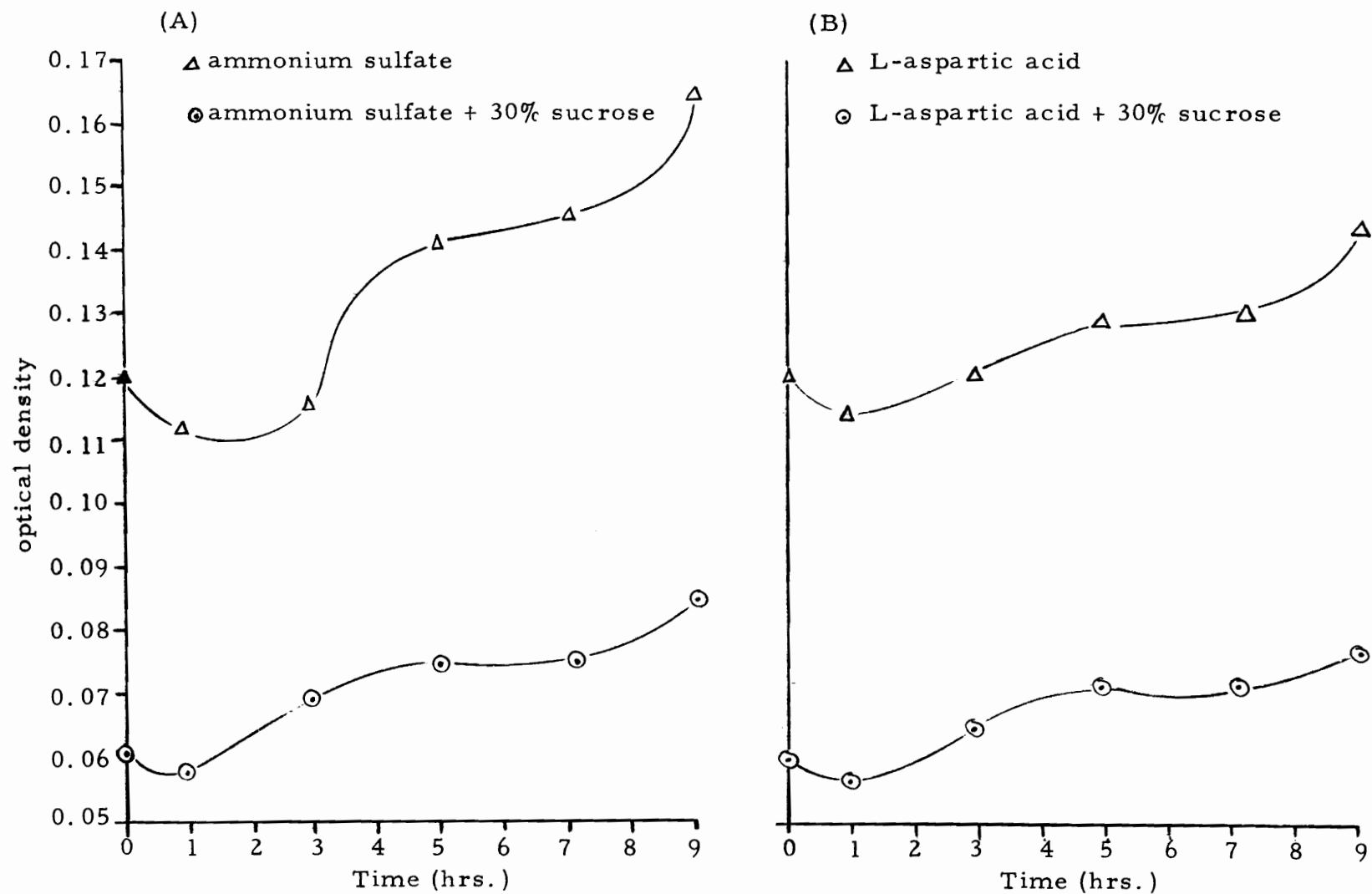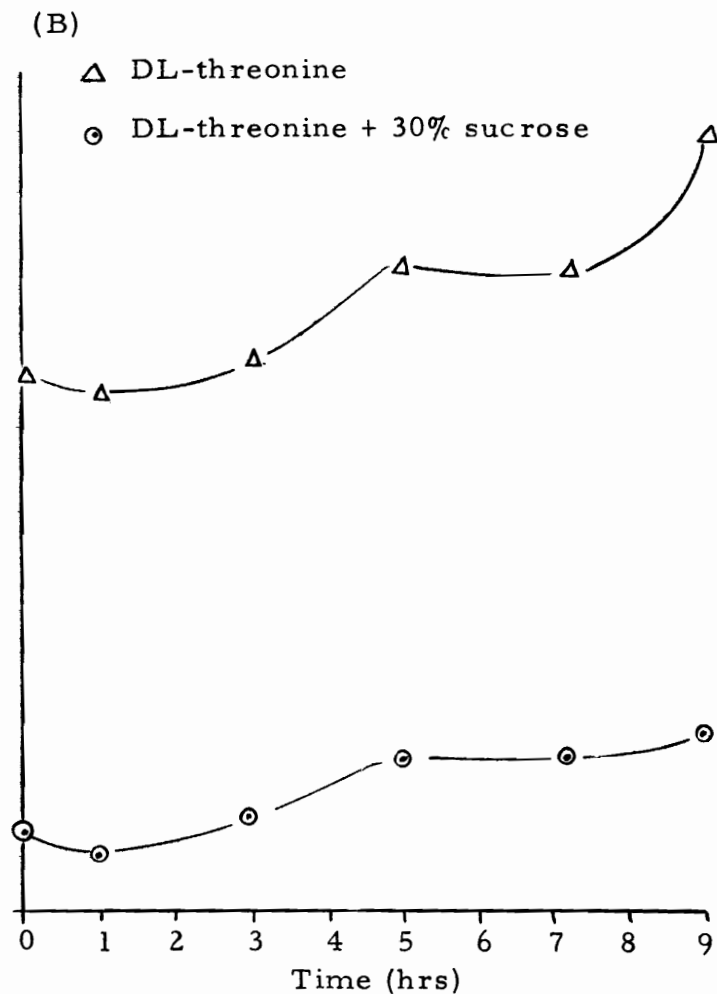
FIGURE 12. Growth curves of yeast cells grown in media containing L-proline (A) and B-alanine (B) in the presence and absence of 30% sucrose.

FIGURE 13.   Growth curve of yeast cells grown in media
containing DL-methionine in the presence
and absence of 30% sucrose.

could be attributed to a change in the optical density of the yeast cells caused by difference in sugar concentration. These results are also in accord with those obtained in the previous experiments, where it was found that yeast utilized ammonium sulfate and proline more efficiently than the other nitrogen sources investigated.

The last experiment was repeated using 2 per cent sucrose solution instead of the 30 per cent. The experiment was set up as shown in Table 26 for each nitrogen source. Similarly, another set of tubes was prepared in the same manner using deionized distilled water instead of the 2 per cent sucrose. The optical density was then recorded at 700 mμ for each tube at zero time and after 1, 3, 5, 7 and 9 hours. The results obtained in this experiment are shown in Table 27 and presented graphically in Figures 14 to 17.

From these data, it is clear that during the 9 hours of incubation, the yeast cells grew better in the presence of 2 per cent sucrose than in its absence. At the same time, ammonium sulfate and L-proline were utilized more efficiently than the other nitrogen sources tested, a finding that agrees also with the other experiments of this series.

Comparing the growth increments of the yeast cells in the last two experiments, it was found, as shown in Table 28, that the yeast cells grew better in the presence of sugar, and that the growth during that period of incubation (9 hours) was higher in the presence of 2 per cent

TABLE 26

Experimental set up for utilization of nitrogen sources by yeast cells
cultured in Mycophil broth containing 2% sucrose

ml of 0.01 $\underline{M}$ nitrogen source	ml growth F* and salts	ml 2% sucrose	ml yeast suspension (in 2% sucrose)
0.6	0.5	8.6	0.3
--	0.5	9.5	-- (Blank)

* Growth factors

TABLE 27

Utilization of nitrogen sources of yeast cells cultured
in media containing 2% sucrose

Hrs.	Optical density													
	amm. SO$_4$		L-glutamic A		L-aspartic A		DL-threonine		L-proline		B-alanine		DL-methionine	
	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose	I*	sucrose
0	.070	.070	.070	.070	.070	.070	.070	.070	.070	.070	.070	.070	.070	.070
1	.125	.132	.103	.122	.130	.135	.125	.135	.120	.143	.120	.135	.123	.145
3	.125	.135	.103	.122	.130	.135	.138	.138	.120	.143	.120	.135	.123	.145
5	.150	.161	.096	.120	.137	.145	.158	.168	.150	.168	.140	.160	.153	.167
7	.205	.210	.119	.135	.20	.20	.205	.215	.210	.218	.195	.210	.185	.205
9	.290	.285	.163	.175	.28	.29	.265	.282	.287	.305	.265	.275	.240	.255

I* in absence of sucrose
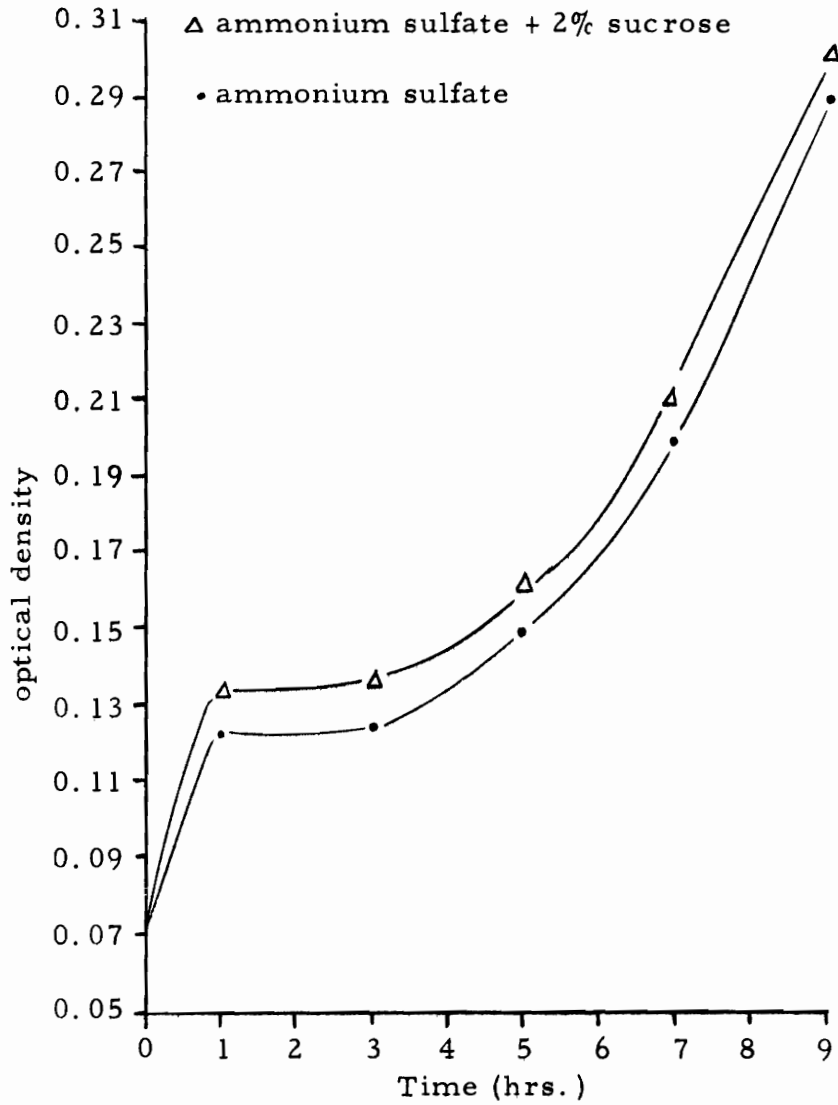
FIGURE 14. Growth curve of yeast cells grown in media
containing ammonium sulfate in the presence
and absence of 2% sucrose.
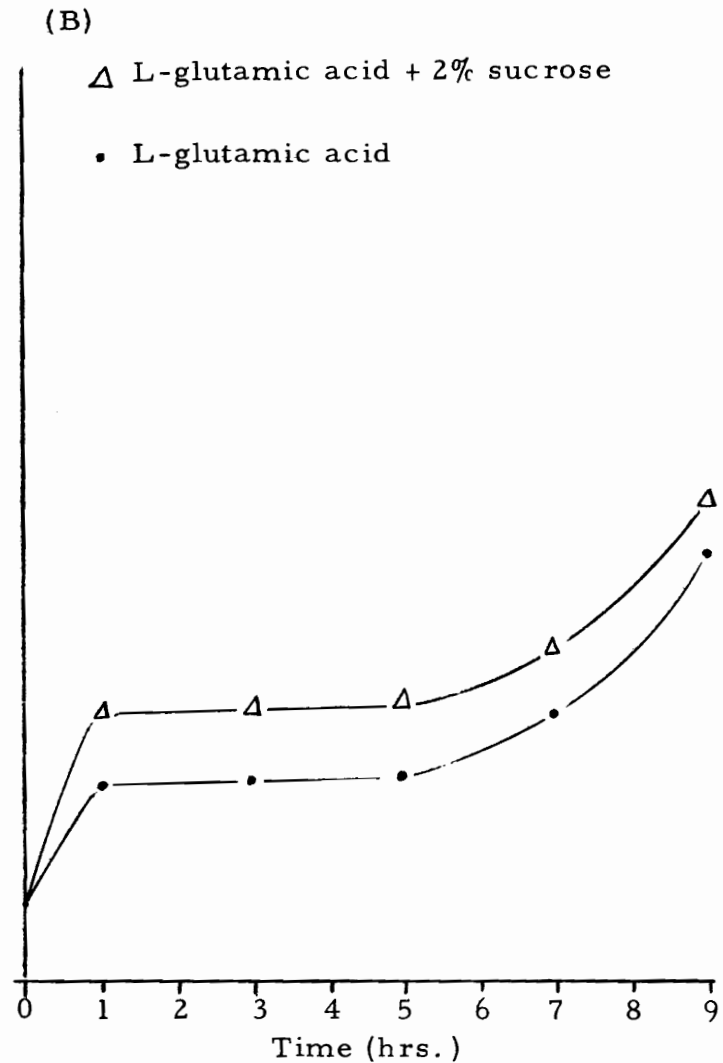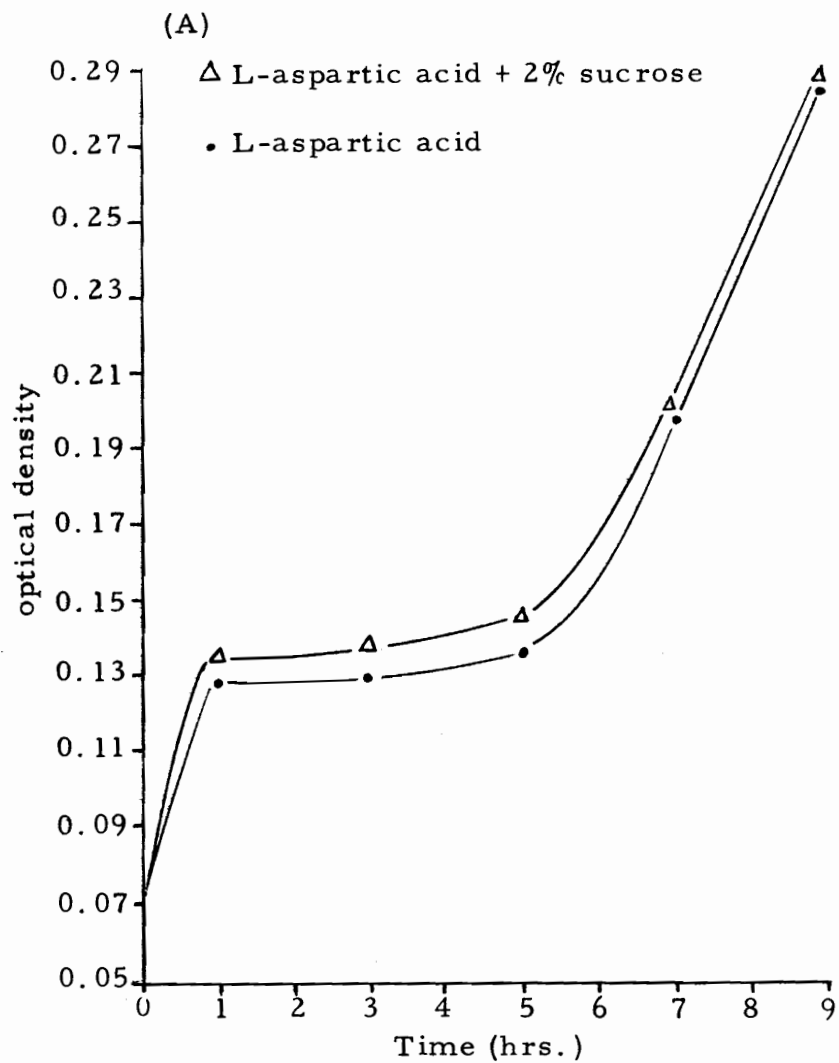
FIGURE 15. Growth curves of yeast cells grown in media containing L-aspartic acid (A) and L-glutamic acid (B) in the presence and absence of 2% sucrose.
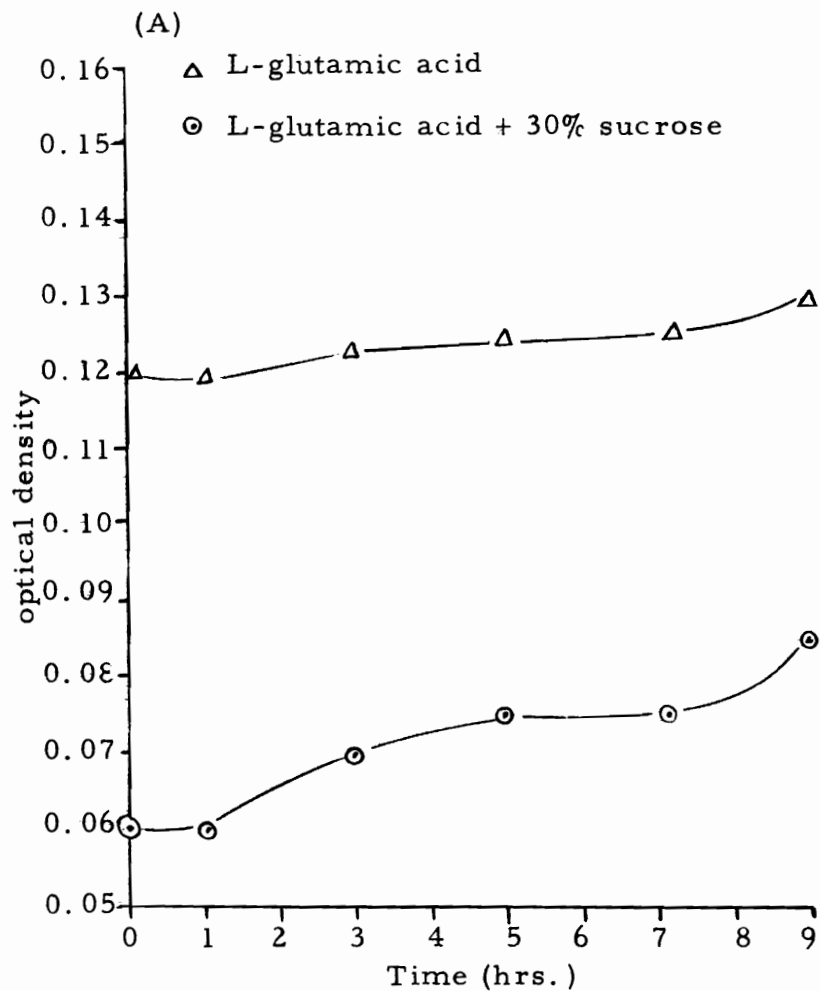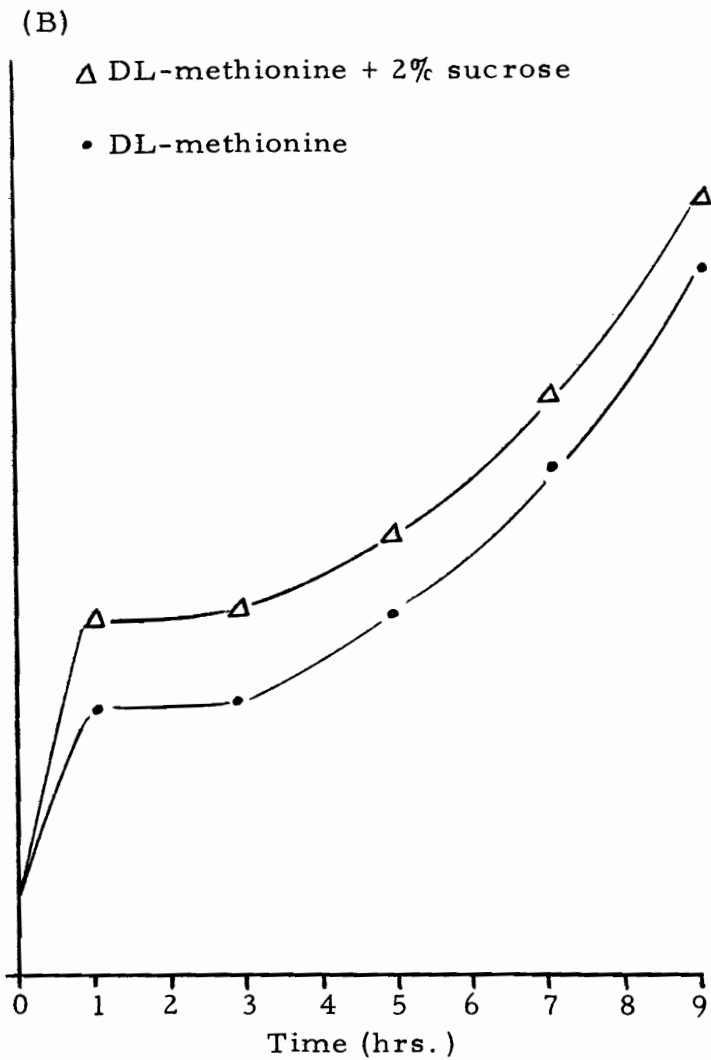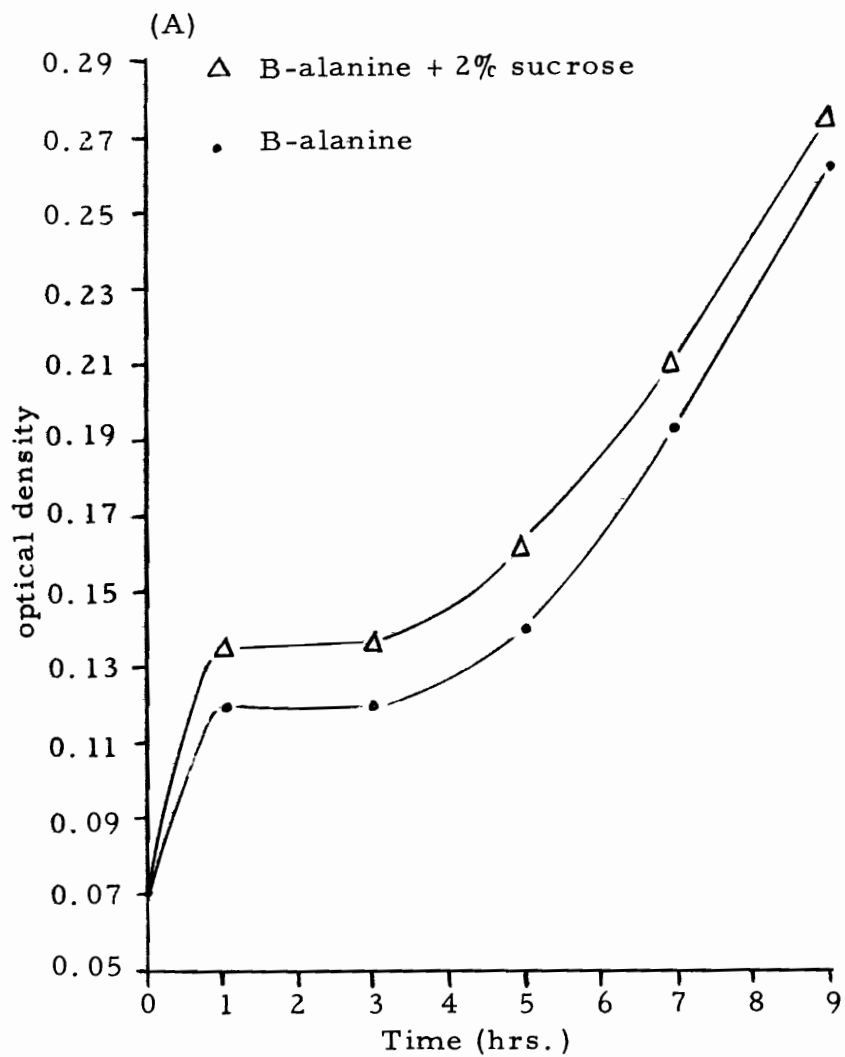
FIGURE 16.   Growth curves of yeast cells grown in media containing B-alanine (A) and
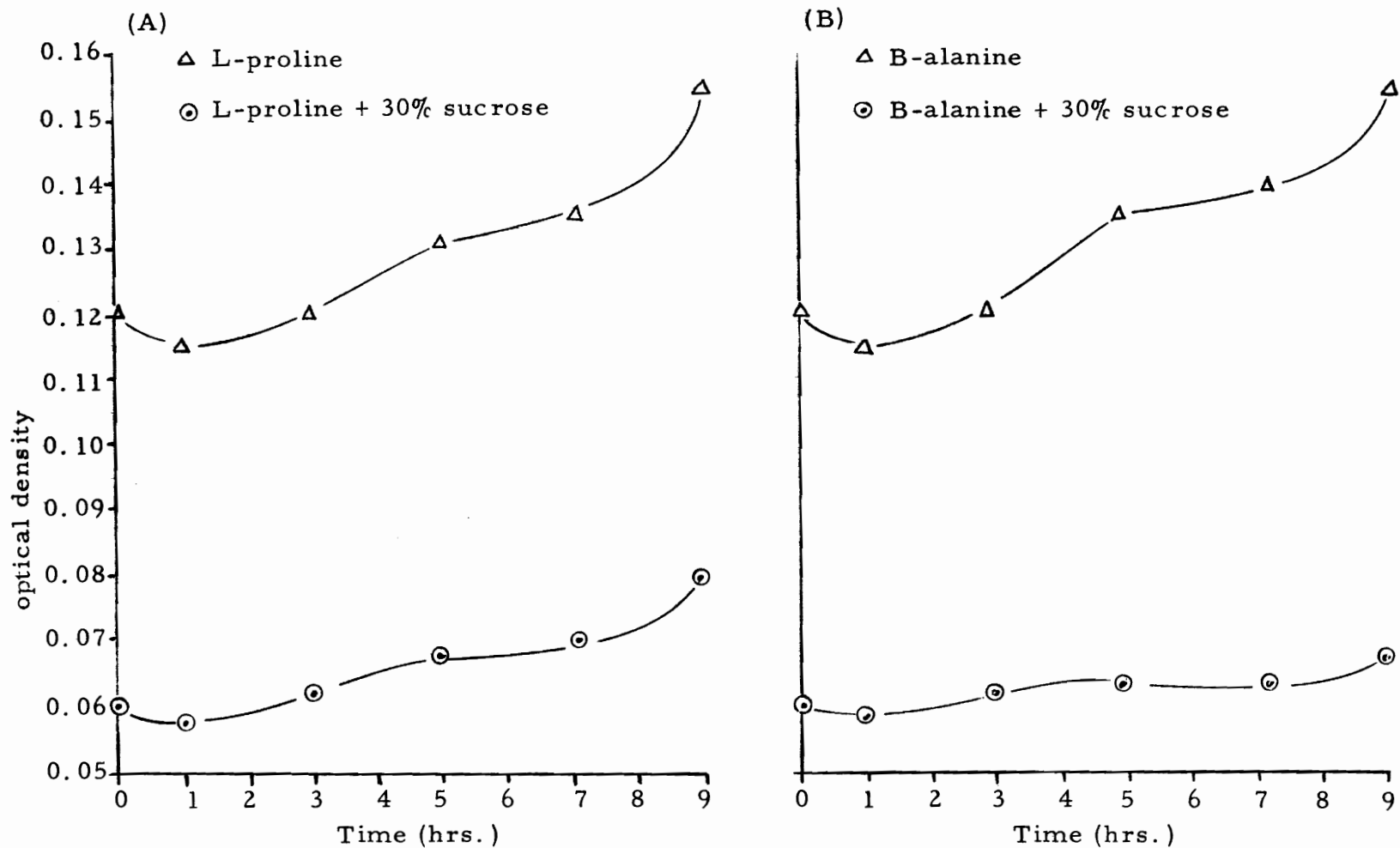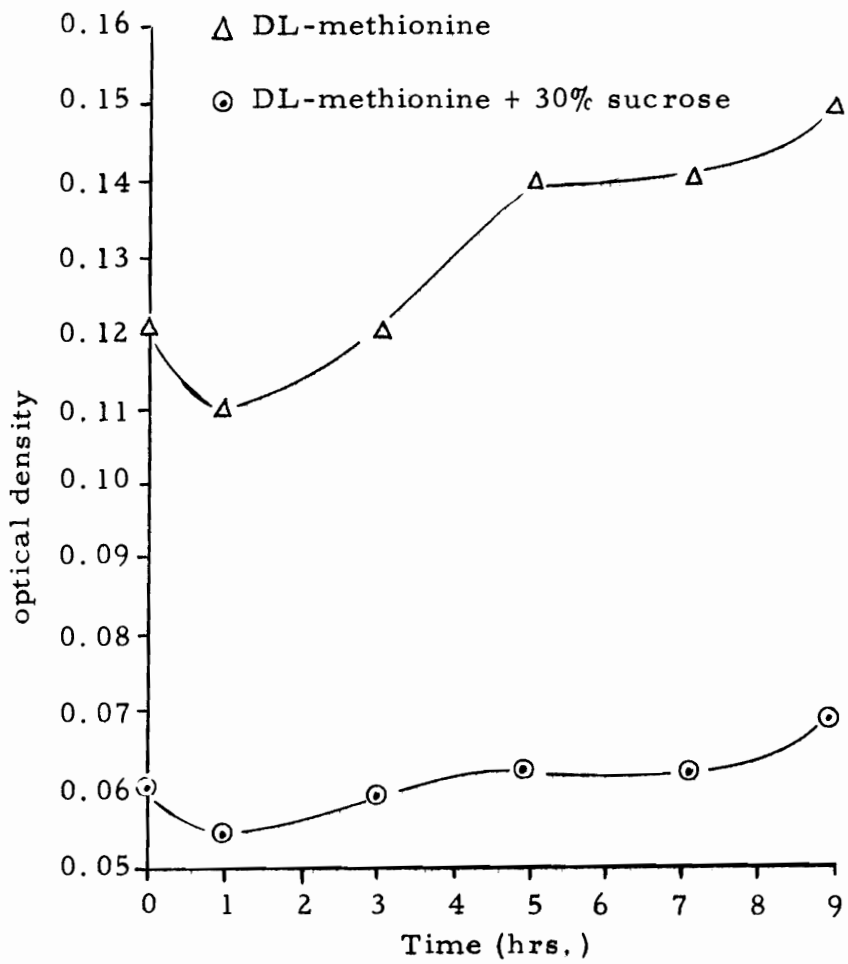DL-methionine (B) in the presence and absence of 2% sucrose.
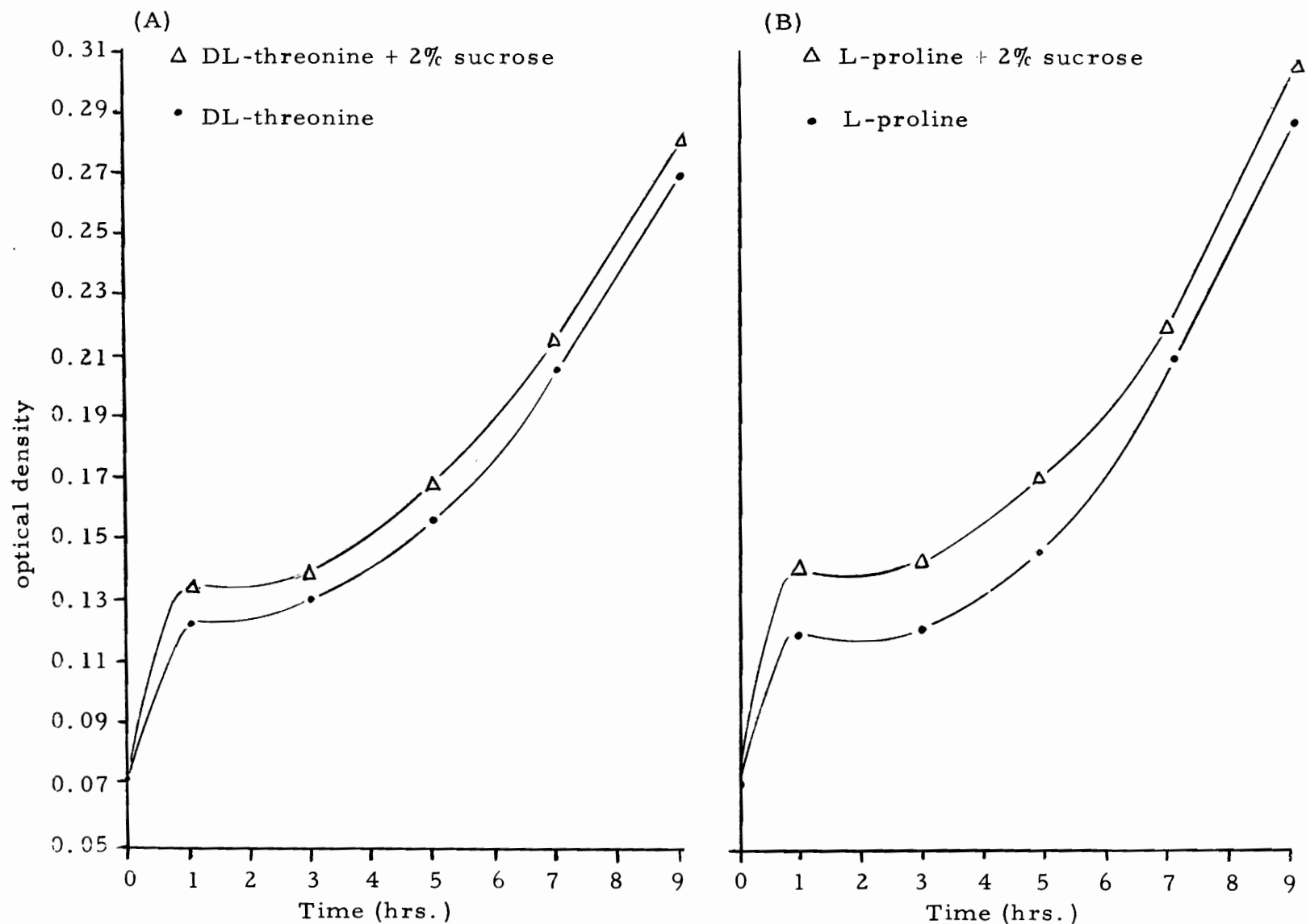
FIGURE 17. Growth curves of yeast cells grown in media containing DL-threonine (A) and L-proline (B) in the presence and absence of 2% sucrose.

TABLE 28

Growth increment of yeast cells cultured in media containing 2%
and 30% sucrose in the presence of different nitrogen sources

Nitrogen source	Growth increment* in 9 hrs. X 10			
	2% sucrose		30% sucrose	
	In absence of sucrose	In presence of sucrose	In absence of sucrose	In presence of sucrose
ammonium sulfate	31.4	30.8	3.7	4.1
L-glutamic acid	13.2	15.0	1.2	3.3
L-aspartic acid	30.0	31.4	1.8	3.3
DL-threonine	27.8	30.2	2.6	2.2
L-proline	31.0	33.0	2.9	3.3
B-alanine	27.8	29.2	2.9	1.3
DL-methionine	24.2	26.4	2.5	1.5

* The growth increment is defined as: $(B-A)/A$, where B is the
optical density at the end of the experiment and A, the reading at
its beginning.

sucrose than in 30 per cent. Concerning the utilization of nitrogen sources, the data showed that ammonium sulfate was utilized more efficiently than the amino acid sources and that L-proline, B-alanine, DL-threonine and L-aspartic acid sustained the growth of the yeast cells very well.

V. COMPOSITION OF FREE AMINO-ACIDS BIOSYNTHESIZED IN YEAST CELLS GROWN IN DIFFERENT CULTURE MEDIA

The role of free amino acids in enzyme synthesis of microorganisms was established after it was found that the induction of enzyme synthesis was dramatically carried out in the absence of an external nitrogen source. In such cases the nitrogen utilized by the cell in synthesizing the new enzyme molecule must derive from some preexisting nitrogenous compounds in the cell. The ability to form enzyme in the absence of an external supply of nitrogen is far more widespread among the yeasts than among the bacteria. It would appear that the ability of yeasts to get along without an external source of nitrogen in enzyme synthesis is due to the presence of free amino acids acting as an internal supply.

The method employed to identify the composition of free amino acids biosynthesized by the yeast cells was unidirectional paper chromatography as described under materials and methods.

In the first series of experiments, butanol-acetic acid-water was used as the solvent to compare the efficiency of both boiling water and

alcohol extraction methods employed for obtaining the amino acid pool

extracts of yeast cells grown in Mycophil broth containing 30 per cent

sucrose. In this case equal amounts (by weight) of both extracts were

used on the chromatogram paper. At the same time, some standard

amino acid spots were also used for identifying some of the free amino

acids separated on the chromatogram.

The photograph of the chromatogram obtained in this experiment is

shown in Figure 18. From this chromatogram it is clear that the alcohol

extract method was more efficient than the boiling water method in

obtaining free amino acid extracts. Therefore, the alcohol extracts

were used in all analysis experiments to be mentioned below. It is also

clear that the butanol-acetic acid-water solvent efficiently separated the

free amino acids individually present in the extracts. The standard

amino acids used for identification of amino acids in the unknown extracts

were tyrosine, cysteine and B-alanine. As is shown on the chromatogram

(Figure 18) the only amino acid identified in the unknown mixture was

tyrosine.

The latter experiment was repeated with the alcohol extract as the

unknown and using another series of standard amino acids. These were

L-glutamic acid, L-aspartic acid, L-valine, L-leucine, L-isoleucine,

L-phenylalanine, L-lysine and DL-histidine. The photographs of the

chromatograms obtained in this case are shown in Figures 19 and 20.

TABLE 29

Table of Abbreviations

H_2O-Ext. = water extract
Alc-Ext. = alcohol extract
L-tyr. = L-tyrosine
L-cyst. = L-cysteine
L-ala. = L-alanine
L-glu. = L-glutamic acid
L-asp. = L-aspartic acid
L-val. = L-valine
UN = unknown (alcohol extract)
L-isoleu. = L-isoleucine
L-leu. = L-leucine
L-phe. = L-phenylalanine
L-lys. = L-lysine
DL-his. = DL-histidine
L-ser. = L-serine
L-thr. = L-threonine
L-arg. = L-arginine
L-gly. = L-glycine
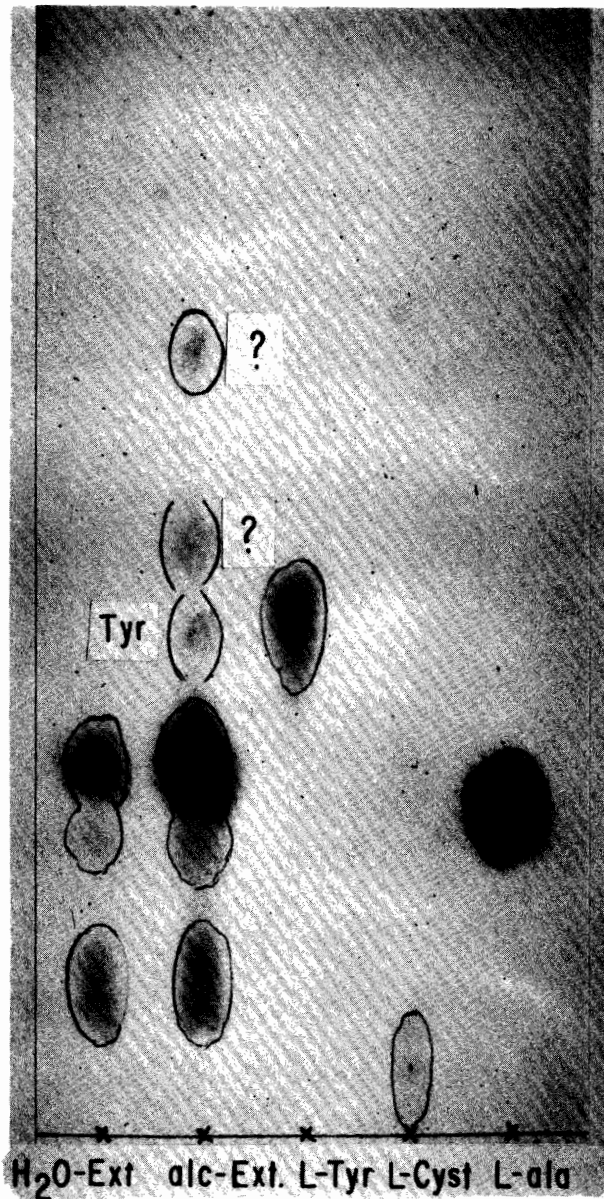
FIGURE 18.   Chromatogram of water and alcohol extracts of free amino
acids in yeast cells using Butanol-acetic acid-water
solvent.   The growth media used in this case were Mycophil
broth containing 30% sucrose.   The identified amino acid
was tyrosine.

FIGURE 19.   Chromatogram of free amino acids in yeast extract (un.)
showing identification of glutamic acid, aspartic acid and
valine.   The growth media were Mycophil broth containing
30% sucrose.   The solvent used was Butanol-acetic acid-
water mixture.
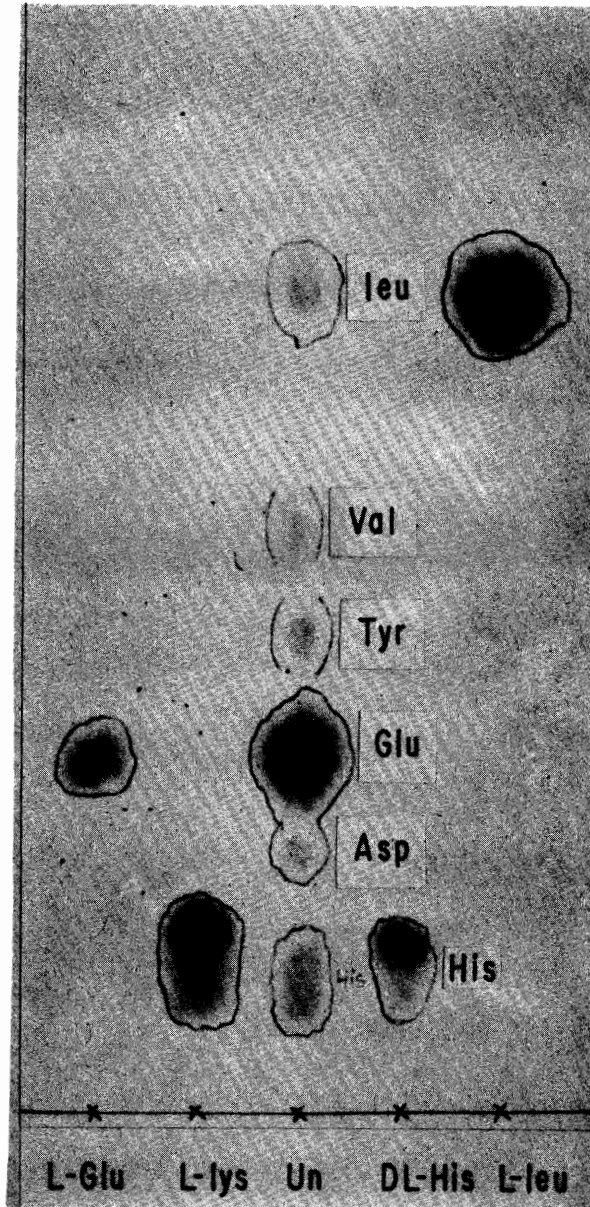
FIGURE 20. Chromatogram of free amino acids in yeast extract (un.)
showing identification of histidine and leucine. The growth
media used were Mycophil broth containing 30% sucrose.
The solvent was Butanol-acetic acid-water mixture.

Figure 19 identifies glutamic acid, aspartic acid and valine, while Figure 20 indicates the presence of histidine and leucine in the unknown mixture.

Yeast cells grown in Mycophil broth media containing 30 per cent sucrose were found to have glutamic acid, aspartic acid, histidine, tyrosine, valine and leucine in the free amino acid pool.

A further series of experiments was carried out using the standard phenol solvent prepared as described in the section on materials and methods to determine most of the other free amino acids that might be present in the amino acid pool. Usually, in these experiments, the phenol solvent was not used for more than two runs, then replaced by a fresh sample. The standard amino acids used for identification of the separated free amino acids were L-glutamic acid, L-aspartic acid, L-serine, L-threonine, L-arginine, L-lysine, L-glycine and DL-histidine.

The photographs of the chromatograms are shown in Figure 21 and 22. The amino acids identified in Figure 21 are glutamic acid and aspartic acid; while those identified in Figure 22 are glycine, lysine and histidine.

In summary, the composition of amino acid pool of these osmophilic yeast grown in Mycophil broth containing 30 per cent sucrose and analyzed using standard butanol-acetic acid-water and phenol as solvents in a unidirectional paper chromatography, were L-glutamic acid, L-aspartic
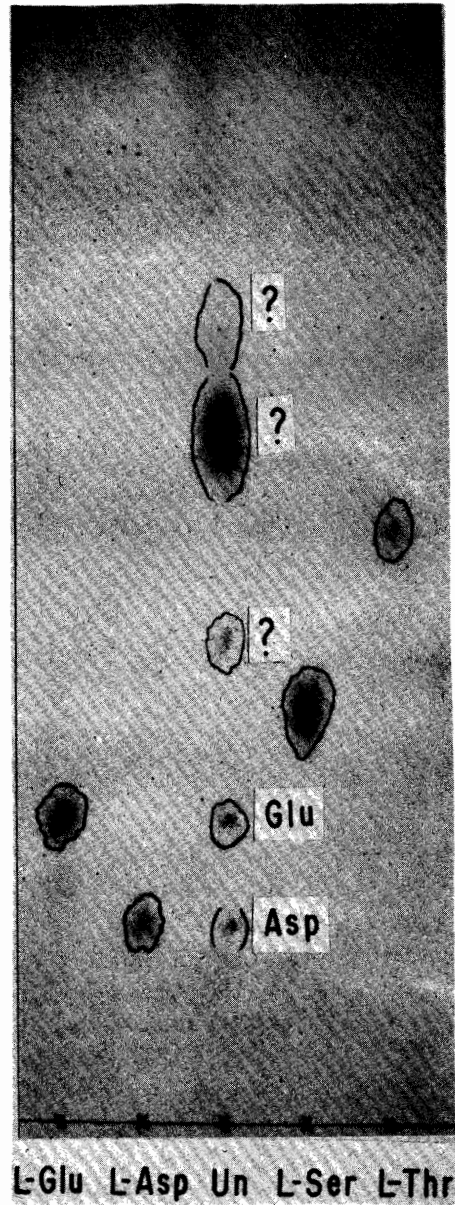
FIGURE 21. Chromatogram of free amino acids in yeast extract (un.)
showing identification of aspartic acid and glutamic acid.
The growth media were Mycophil broth containing 30%
sucrose. The solvent used was phenol.
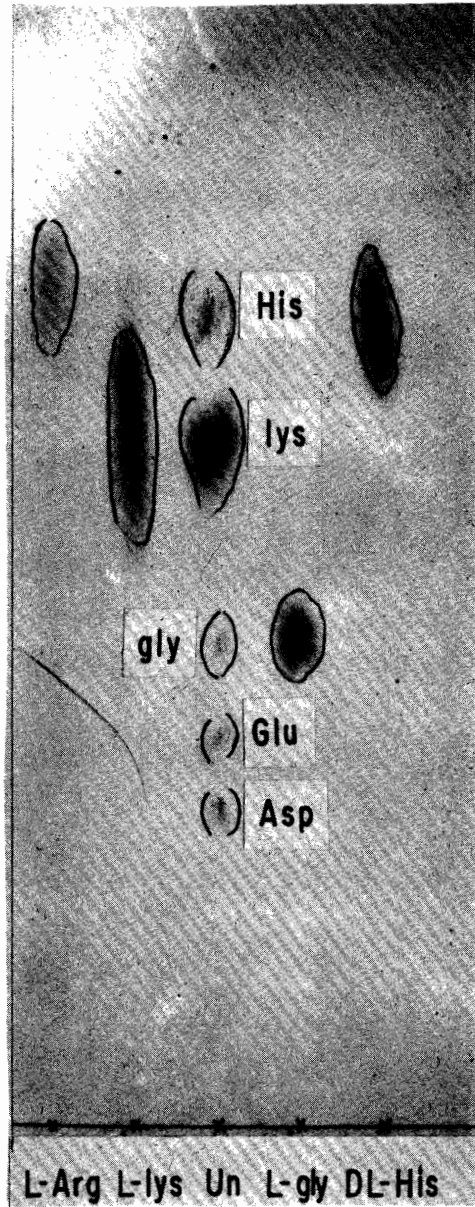
FIGURE 22. Chromatogram of free amino acids in yeast extract (un.)
showing identification of glycine, lysine and histidine.
The growth media were Mycophil broth containing 30%
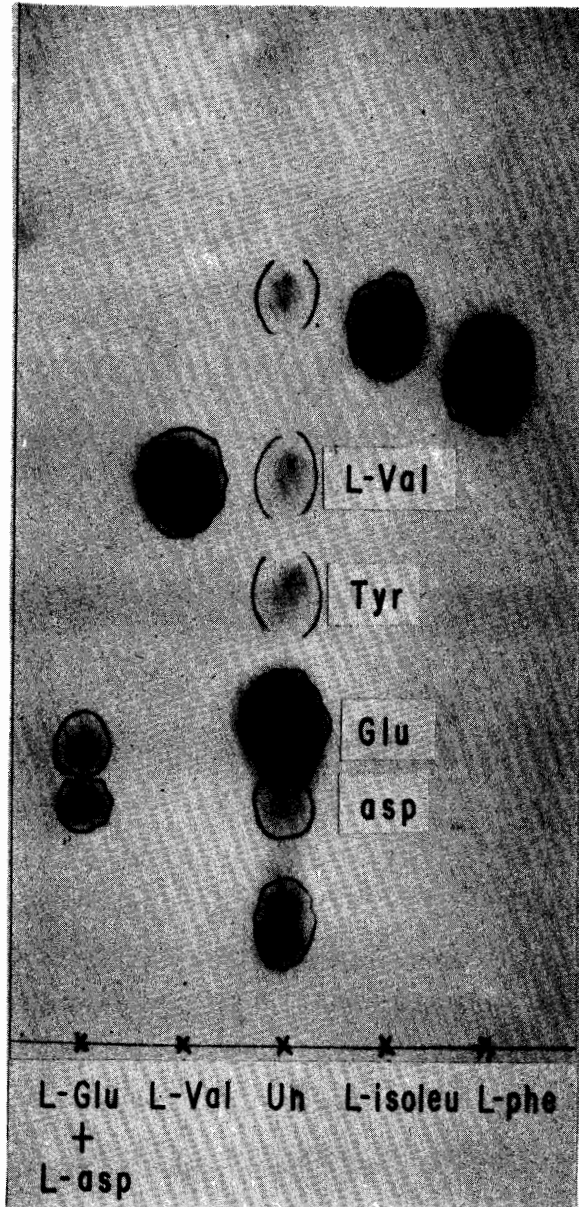sucrose. The solvent used was phenol.

acid, DL-histidine, L-tyrosine, L-valine, L-leucine, L-glycine and
L-lysine.

A.   Effect of Growth Media on Pool Composition.

After establishing the presence of the amino acids described above
in the amino acid pool of yeast cells studied, we were also interested
in the effect of growth media on the composition of the pool.

In this case, two different growth media were used to be compared
with the medium of the last experiment.   One of the two media used was
Mycophil broth without sucrose, and the second was 30 per cent sucrose
with no added nitrogen source but containing added growth factor.   The
methods of cultivation, the procedures used for obtaining the free amino
pool extracts and the methods of analysis were similar to those used
above in the last experiments and described in the section on materials
and methods.

The comparison in this investigation was conducted using equal
amounts by weight (13-15 mg) of the free amino acid alcohol extracts
obtained from yeast cells grown in the three different media.

The photographs of the chromatograms are shown in Figures 23 and
24.   Figure 23 presents the chromatogram obtained using butanol-acetic
acid-water solvent, while Figure 24 presents that obtained with the
standard phenol solvent.
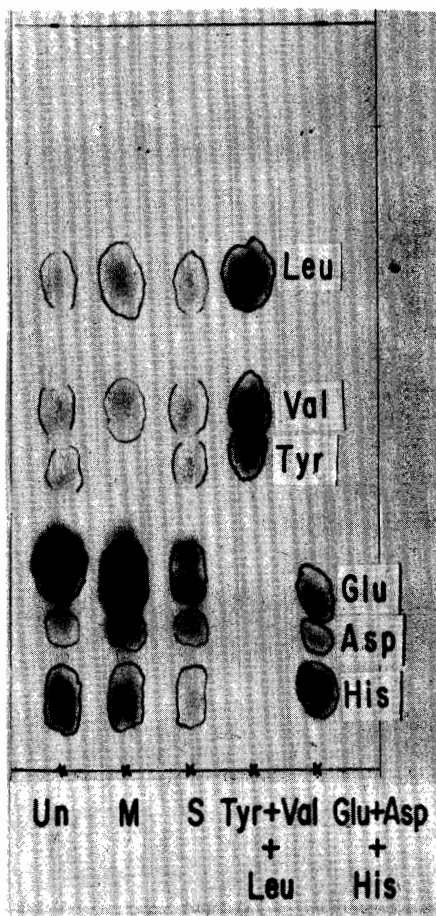
FIGURE 23. Chromatogram showing composition of amino acids in amino acid pools of yeast cells grown in Mycophil broth containing 30% sucrose (un.); in Mycophil broth without sucrose (M); and in 30% sucrose (S). The solvent used was butanol-acetic acid-water mixture.
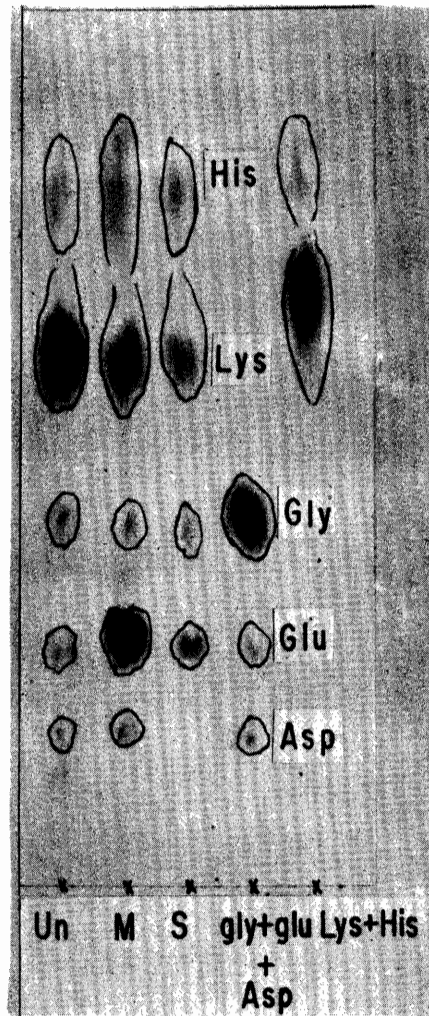
FIGURE 24. Chromatogram showing composition of amino acids in amino acid pools of yeast cells grown in Mycophil broth containing 30% sucrose (un); in Mycophil broth without sucrose (M); and in 30% sucrose (S). The solvent used was phenol.

From these chromatograms, it was distinctly evident that most of the free amino acids identified as members of amino acid pool of yeast cells grown in Mycophil broth containing 30 per cent sucrose were found to be present in the pools of yeast cells grown in both Mycophil broth without sucrose and in a 30 per cent sucrose solution.

The only amino acid which could not be identified as a member of the free amino acids of yeast cells grown in Mycophil broth without sucrose was tyrosine.

Since equivalent amounts of yeast extracts (13-15 mg) were used by comparing the intensity of the color spot of the chromatogram, the amount of each amino acid in the pool could be designated as 3+, 2+ and 1+ and a rough estimate of the comparative amount of the amino acids in the unknown mixtures could be made. The comparison is shown in Table 30. The data indicated that glutamic acid, aspartic acid, histidine and lysine were not significantly affected by differences in culture media. It was also found that the level of lysine decreased in the presence of Mycophil broth containing no sucrose.

TABLE 30

Composition of free amino acid pools of yeast cells grown in
different media and determined by paper chromatography

Amino acids	Mycophil broth containing 30% sucrose (UN)*	Mycophil broth containing no sucrose (M)**	30% sucrose (S)***
L-glutamic acid	+++	+++	++
L-aspartic acid	+	++	+
L-glycine	+	trace	trace
L-lysine	+++	++	+
DL-histidine	++	++	+
L-tyrosine	+	-	trace
L-valine	+	+	trace
L-leucine	+	+	trace

* (UN) Represent free amino acids extract from yeast cells grown in
Mycophil broth medium containing 30% sucrose.

** (M) Represent free amino acids extract from yeast cells grown in
Mycophil broth medium containing no sucrose.

*** (S) Represent free amino acids extract from yeast cells grown in
30% sucrose medium.

DISCUSSION

Osmophilic yeasts play an important role in the spoilage of dried fruits, concentrated fruit juice, honey, maple syrup, curing brines, salted meat and sugar. In this laboratory a new sugar-tolerant yeast strain was isolated from sugar syrup samples. There was no information available concerning its taxonomic properties, growth, ability to fix atmospheric nitrogen, effect of trace elements, effect of sucrose concentration on growth, effect of variation in hydrogen-ion concentration and culture temperature on growth. It was considered to be of interest to study the utilization of ammonium sulfate and certain amino acids and the composition of the free amino acid pool under different culture conditions of this "osmophilic" yeast. Therefore, the present studies were devoted to investigate these points.

The first studies in this work were carried out to determine the taxonomic properties of the yeast strain. The methods and the classification system employed were essentially the same as those outlined in the monographs by Wickerham (1951) and by Lodder and Kreger-Van Rij (1952).

It was found in the classification studies reported herein that the yeast cells under investigation resembled Saccharomyces species in the manner in which they fermented glucose and other sugars, and in their

inability to assimilate nitrate, but differed in their inability to assimilate most of the sugars fermented. The yeast cells closely resembled Saccharomyces italicus and S. steineri since they fermented glucose, galactose, maltose and saccharose. They differed from S. italicus in that the latter could assimilate nitrate; and from S. steineri in the ability of S. steineri to assimilate maltose and saccharose, and in the formation of primitive pseudomycelium consisting of tree-like formations, which the unknown species did not do.

Owing to the ability of this unknown yeast to assimilate glucose and galactose, it resembles Saccharomyces acidifaciens, but the latter species ferments only glucose. Therefore, this unknown yeast was regarded as a new species of the genus Saccharomyces.

Experiments concerned with the ability of this osmophilic yeast to fix atmospheric nitrogen were set up. These measured and compared the total nitrogen of both Mycophil broth cultures and 30 per cent sucrose cultures after 2 and 5 days' incubation. Some cultures were incubated at room temperature and others at 37° C. This experiment showed a decrease in the nitrogen content, either of Mycophil broth or of the 30 per cent sucrose cultures. It was also found that the decrease in nitrogen in the case of cultures incubated at 37° C was somewhat greater than in those incubated at room temperature.

Another experiment was carried out using 50 ml of 30 per cent sucrose solution inoculated with one milliliter of Mycophil broth yeast culture. The amount of total nitrogen in this case was determined at zero time, 1, 2, 3, 4, 22 and 24 hours. This experiment showed that the decrease in total nitrogen observed in the above experiment occurred significantly in the first two hours of incubation.

Other studies were carried out using washed yeast cells. The data obtained in this case showed that there was a slight increase in total nitrogen after the second day of incubation. This result differed from the other experiments of this series and indicated a source of nitrogen external to the culture itself. At this writing, this source is unknown.

In an attempt to find an explanation for the decrease in total nitrogen of the yeast culture described above, several experiments were set up using different nitrogen sources to further investigate the metabolism of this yeast on the total nitrogen of these cultures. The nitrogen sources used were ammonium sulfate and certain amino acids, i. e., glutamic acid, aspartic acid, B-alanine, and DL-threonine. These studies showed that there was a decrease in total nitrogen similar to that observed in the earlier experiments, and that the decrease was significant in the first two hours of incubation. These observations are similar to the findings obtained by Bakhadur (1956) on Dakhar yeast. The author used sucrose and ammonium sulfate as carbon and nitrogen sources respectively. He

found that increasing the carbon to nitrogen ratio in the culture media above 1.42 would result in nitrogen loss by the yeast cells. It was also found interesting by Kretovich and Krauze (1961) that after the addition of ammonium phosphate to baker's yeast cultures, the content of free amino acids and amides decreased. The latter authors did not give any explanation for the above observation; they merely stated that "this fact deserves attention and further experimental work."

It has been found by Krylova (1962) and Singh (1962) that the presence of trace elements had an effect on growth and nitrogen fixation of micro-organisms. Therefore, the effect of trace elements on growth and nitrogen fixation was studied to establish their role on growth and their efficiency in stimulating yeast cells to fix atmospheric nitrogen. The trace elements used were iron as ferrous sulfate, phosphorus as K_2HPO_4, molybdenum as $Na_2MoO_4.2H_2O$, boron as boric acid, calcium as calcium chloride and the citrate ion as sodium citrate.

The studies indicated that there was no increase in total nitrogen. Therefore, the cations and anions tested were unable to stimulate the yeast cells to fix the atmospheric nitrogen. On the other hand, the only established effect of trace elements was the enhancement of yeast growth as indicated by the increase in biomass.

The studies indicated also that phosphorus stimulated the growth of yeast cells more efficiently than the other anions and cations tested.

Therefore, it could be concluded that phosphorus is essential for the growth of yeast as it plays an important part in carbohydrate metabolism and it is concerned with the adenosine phosphate-adenosine diphosphate reaction and other metabolic reactions.

It was also found that iron, calcium, boron and molybdenum enhanced growth of the yeast cells. The role of iron in enhancing growth of yeast cells may be attributed to the fact that its presence is essential for full catalase and cytochrome activity. The enhancement of calcium ion to yeast growth is in accord with the findings of many workers as Fulmer et al., (1921). They found that certain amounts of calcium definitely increased the amount of yeast growth. The finding that boron and molybdenum stimulated yeast growth was in agreement with the data obtained by Singh (1962) and Krylova (1962). Even though no nitrogen was fixed by the osmophilic yeast under study here, they found that the development and fixation of molecular nitrogen by microorganisms were stimulated by boron and molybdenum.

As the above studies showed that there was no significant increase in total nitrogen of yeast cultures, the ability of yeast to fix atmospheric nitrogen was ruled out. Attention, therefore, was turned to the sugar samples to learn what would act as the nitrogen source for these yeast cells. That these samples may contain nitrogen was established after analyzing different sugar samples received from Amalgamated Sugar

Company for total nitrogen. These analyses showed that the sugar samples analyzed contained from 1. 5 to 2. 5 mg nitrogen per gram, which would be adequate to support the growth of yeast.

Therefore, the above studies suggested the hypothesis that, as the sugar is manufactured, it may be subjected to contamination by nitrogen containing substances. These factors deserve attention and further experimental work.

Concerning the decrease in total nitrogen observed in the earlier studies, one could suggest that the decrease may be attributed to ammonia liberation as it was found by Bernard (1963) that ammonia was liberated in the process of fixation of atmospheric nitrogen by Azotobacter spp. Another possibility may be suggested for this decrease in total nitrogen. The nitrogen may be incorporated into the yeast nucleic acids during the lag phase; in this case it could not be determined by the micro-Kjeldahl method used in these studies. In the present work, there was no chance to investigate these two possibilities and we suggest they deserve further considerations.

Studies on the effect of sucrose concentration on the growth of yeast cells indicated that the yeast grew better in low sugar concentrations in the first 24 hours of incubation; and after that period, they grew more efficiently at high sugar concentrations. No concentration limits were established, but they will grow at 65. 5 per cent regularly in sugar

company tanks; hence, it may be concluded that the yeast under investigation is an osmophilic yeast strain.

Other studies were carried out to investigate the effect of hydrogen-ion concentration on growth of the yeast cells. The studies indicated that the osmophilic yeasts tested were able to grow in a wide pH range of 3 to 7 in Mycophil broth containing 2 or 30 per cent sucrose. These findings were in accord with those obtained by English (1954). He found that sugar-tolerant Saccharomyces rouxii were able to grow in a wide pH range of 1.8 to 8.0 in a high glucose concentration of 46 per cent. In support of the latter findings, a similar phenomenon in a medium of high sugar concentration was also observed with a salt-tolerant soy yeast, Saccharomyces rouxii (Ōnishi, 1961b).

Studies of the effect of temperature on yeast culture growth indicated that the yeast cells were unable to grow either at refrigerator temperature or at 56° C, and they grew poorly at 37° C. On the other hand there was a very good growth of yeast cells incubated at room temperature (26° C). These observations were similar to the data obtained by Ōnishi (1959b). He found that many osmophilic yeasts including Saccharomyces rouxii, were not able to multiply at 40° C in the presence of sugars at concentrations lower than 30 per cent.

In conclusion, the above studies devised to study the effect of sucrose concentration, hydrogen-ion concentration, and culture

temperature on growth of the yeast cells isolated from sugar syrup samples of high yeast count, indicated that these yeast cells were osmophilic yeasts and, specifically, were a sugar-tolerant strain.

Another series of studies was conducted to investigate the utilization efficiency of ammonium sulfate and different amino acids as nitrogen sources by this osmophilic yeast. The first experiments in this series of studies were carried out to study the growth of the yeast cells in the presence of different concentrations of ammonium sulfate and various amino acids as inorganic and organic nitrogen sources respectively. The growth of the yeast cells was determined turbidimetrically. In each case, the growth increment was calculated by subtracting the original optical density reading from the reading taken after nine hours, then dividing by the original reading. The experiments were carried out in the presence and absence of 2 per cent sucrose. These studies indicated that ammonium sulfate supported the growth of yeast (expressed in growth increment) better than the other nitrogen sources tested. It was also found that the growth of the yeast cells was better in the presence of sucrose than in its absence. At the same time, yeast cells grown in the presence of sugar utilized more nitrogen. Therefore, the growth of the yeast cells in the presence of sugar was directly proportional to the amount of nitrogen available.

Similar studies were carried out to study the utilization of nitrogen sources in the presence of 30 per cent sucrose as compared with 2 per cent sucrose. These studies indicated that the results obtained were in good agreement with those obtained in the above studies with 2 per cent sucrose, i.e., the yeast cells grew very well in the presence of ammonium sulfate. The studies showed also that the yeast cells utilized L-proline, B-alanine and threonine more efficiently than the other amino acids in the presence of 30 per cent sucrose. It was also found that the optical density readings of cultures containing the 30 per cent sucrose were lower than those containing no sugar. It was suggested that this observation might be due to certain optical properties of sucrose concentrations. This possibility was ruled out by measuring the optical density of different sucrose concentrations (2%, 5%, 10% and 30%) at 700 mu. It was found that there was no change in optical density readings of the above sugar concentrations where yeast were not involved.

The other possibility suggested for the above observation was the sudden change in sucrose concentration between the water suspension of yeast used in the above studies as inoculating culture and the 30 per cent sucrose media. Therefore, these studies were repeated using inoculum cultured in media containing sucrose concentrations similar to those used in the investigation media, i.e., the growth media which contained 30 per cent sucrose were inoculated with yeast cells cultured

in media containing 30 per cent sucrose, and those carried out in the presence of 2 per cent sucrose were inoculated with yeast cells cultured in media containing 2 per cent sucrose.

These studies indicated that the optical density readings of cultures containing 30 per cent sucrose were also lower than those containing 2 per cent sucrose; therefore, this observation may be attributed to general optical properties of the yeast cells when in sucrose solutions. The changes in optical properties of the cells are different at different concentrations of sucrose.

The studies showed that during the 9 hours of incubation the growth of the yeast cells was more efficient in 2 per cent than in 30 per cent sucrose. The opposite situation might be observed if the incubation period was longer than 24 hours. The above findings were in accord with Thorne's (1946) data. He demonstrated that in the presence of glucose, a number of amino acids could sustain growth and that their efficiency was, on the average, about 60 per cent of that of ammonium salts. Similar observations were also made by Ōnishi (1957b) who found that in conventional (low sugar concentration) media Saccharomyces rouxii could fully utilize ammonium sulfate. On the other hand, Schultz and Pomer (1948) reported other studies undertaken with a considerable number of Torula and Saccharomyces strains and found that some amino acids were used as both nitrogen and carbon donors, whereas others

were not capable of utilization in the absence of an additional carbon source. Similar observations were made by Meyer and Benziman (1959). They found that Saccharomyces cerevisiae Hansen could use all of the amino acids for growth when glucose was present.

Therefore, in conclusion concerning the above studies, the yeast cells under investigation utilized ammonium sulfate more efficiently than the other nitrogen sources tested. At the same time some of the amino acids investigated such as proline, alanine, threonine and aspartic acid sustained growth more efficiently than the other amino acids.

The importance of free amino acids in enzyme synthesis of micro-organisms was established, after it was found that the induction of enzyme synthesis was dramatically carried out in the absence of an external nitrogen source. In such cases the nitrogen utilized by the cell in synthesizing the new enzyme molecule must derive from some preexisting nitrogenous compounds in the cell. The ability to form enzyme in the absence of an external supply of nitrogen is far more widespread among the yeasts than among the bacteria. Therefore, the present studies were conducted to determine the composition of amino acid pools biosynthesized by yeast cells under investigation. The yeast cells were cultured in Mycophil broth containing 30 per cent sucrose. In this studies, two methods for obtaining the free amino acids extracts were tried and compared on unidirectional paper chromatography using a butanol-acetic

acid-water solvent. The two extraction methods were boiling water method and ethyl alcohol method. The comparative studies showed that the alcohol method was more efficient in obtaining the free amino acid extracts than the boiling water. The individual free amino acids in the extracts were identified using standard amino acids on the chromatograms for comparison. A phenol-water mixture was also used as a solvent in some of the studies.

The amino acids identified as members of the amino acid pool of yeast cells grown in Mycophil broth containing 30 per cent sucrose were L-glutamic acid, L-aspartic acid, DL-histidine, L-tyrosine, L-valine, L-leucine, L-glycine and L-lysine. These findings were in accord with the results obtained by Taylor (1947), who employed the amino acid decarboxylase procedure to survey a variety of yeasts and bacteria for the presence of free amino acids in their internal environment. Analyses were made for arginine, glutamic acid, histidine, lysine and tyrosine. Of the three yeast types examined, all possessed detectable quantities of these five amino acids. The only amino acid that could not be identified as a member of the free amino acids biosynthesized by yeast cells under investigation was arginine, while the other four amino acids reported by Taylor (1947) were identified. The observation of the latter author on the existence of free amino acid pools in yeast were readily confirmed with strain K of Saccharomyces cerevisiae. The

results obtained in these studies agreed also with those obtained by
Halvorson and Spiegelman (1953) who examined the free amino acid
pool content of exponential phase yeast cells, grown in complete
medium. The examinations revealed that of 16 amino acids sought
by bioassay methods, all could be found in detectable amounts. The
major components were glutamic acid, aspartic acid and serine. The
latter amino acid could not be identified among the free amino acids
biosynthesized by the yeast cells of the present investigation. This
almost complete absence of serine might be explained by the primarily
aerobic mechanisms involved in the utilization of that compound.

Comparing the amino acids identified in the pool of the osmophilic
yeast studied with the survey made by Halvorson and Spiegelman (1953)
for different yeast species as well as of different representatives of
Saccharomyces cerevisiae as found in Table 31, it was clear that the
osmophilic yeast did not contain alanine, serine, glutamine, arginine
and hydroxyproline.

After establishing the presence of the amino acids described above
in the amino acid pool of the yeast under investigation, it was considered
of interest to investigate the effect of growth media on the amino acid
pool composition. In addition to the above medium, two other media
were used, i.e., Mycophil broth containing no sucrose and 30 per cent
sucrose containing no further nitrogen source.

TABLE 31

Amino acid pool composition of various yeast strains
as determined by paper chromatography

Amino acids

1. alanine	5. threonine	9. lysine
2. serine	6. glutamic acid	10. arginine + valine
3. glycine	7. aspartic acid	11. tyrosine
4. leucine	8. glutamine + alanine	12. hydroxyproline

Strain	1	2	3	4	5	6	7	8	9	10	11	12
P. strain A	+++	++	++	+++	+	++	++	++	+	++	+	-
R. 427a	++	++	+	++	+	+++	++	++	tr.*	++	+	+
R. 427A	+++	++	+	+++	+	+++	++	++	+	++	-	-
S. cerevisiae sex A	++	++	++	+++	+	++	++	+++	+	++	+	-
S. cerevisiae (strain K)	+++	++	++	+++	+	+++	++	+++	+	++	+	-
S. carlsbergensis (y-379)	+++	++	++	++	++	+++	++	+++	+	-	-	-
S. carlsbergensis (y-1005)	+++	+	++	++	+	+++	+	+++	+	+	-	-
S. fragilis (VN)	+++	++	++	+++	+	+++	+	++	+	-	-	-
S. chevalieri	+++	++	++	+++	++	+++	++	++	+	+	+	-
S. ludwigii	+++	++	tr.	++	+	+++	++	+++	+	+	-	-
S. italicus (y-1434)	++	+	+	++	+	++	++	++	tr.	-	+	-

* tr. = trace

These data were compiled from Halvorson and Spiegelman article (1953).

These studies indicated that most of the free amino acids identified as members of the amino acid pool of yeast cells grown in Mycophil broth containing 30 per cent sucrose were found to be present in the pools of yeast cells grown in both Mycophil broth without sucrose and in 30 per cent sucrose without a further nitrogen source.

The only amino acid which could not be identified as a member of the free amino acids of yeast cells grown in Mycophil broth without sucrose was tyrosine. In this case it could be accounted for the absence of the latter amino acid in terms of its biosynthetic pathway. It is well established in a large number of organisms, including yeasts, that p-hydroxyphenylpyruvic acid is a precursor of tyrosine. The p-hydroxy-phenylpyruvic acid is formed from sugar via sedoheptulose diphosphate and a 7-carbon deoxy-keto-heptose which undergoes cyclization. The product is gradually dehydrogenated to the aromatic state and takes on a pyruvic side chain to form the immediate precursor, p-hydroxyphenyl-pyruvic acid. Therefore, the absence of tyrosine as a member of the free amino acid pool of yeast cells grown in Mycophil broth containing no sucrose could be attributed to the lack of sugar.

Since equal amounts by weight (13-15 mg) of yeast extracts were used on the paper chromatograms, a rough comparison was made in these studies for the free amino acids separated as 3+, 2+ and 1+ according to the intensity of the colored spot. It was found that L-glutamic acid,

L-aspartic acid, DL-histidine and L-lysine were not significantly affected by differences in culture media. That observation was in good agreement with the data obtained by Nagai quoted from Spiegelman and Halvorson (1955) article. He found that growth of yeast in media of high sugar concentration leads to the disappearance of some amino acids in the amino acid pool. Only glutamic acid, histidine and small amounts of aspartic acid were found in such cells. The author also found that pool levels were readily lowered by exposing cells to a nitrogen-free medium containing sucrose. This finding was also confirmed by our studies, as it was noted that the level of free amino acids identified as members of the amino acid pool of the yeast cells under investigation was lowered by exposing the cells to the nitrogen-free medium (30 per cent sucrose), but none of these free amino acids disappeared entirely.

The studies showed also that the level of lysine decreased in the presence of Mycophil broth containing no sugar. This observation might be explained in terms of lysine biosynthesis. It is well known that the biosynthesis of lysine in yeasts and fungi is carried out through the alpha-aminoadipic acid pathway. This pathway involves the condensation of alpha-ketoglutaric acid with acetyl CoA to give homocitric acid which undergoes rearrangement to give homoisocitric acid. The latter acid is dehydrogenated to give oxaloglutaric acid, which is decarboxylated giving rise to alpha-ketoadipic acid. The alpha-ketoadipic acid is

transaminated to give rise to alpha-aminoadipic acid which will give rise to lysine through other series of reactions. Therefore, it is clear from that pathway that lysine biosynthesis depends upon the presence of alpha-ketoglutarate which is a product of the tricarboxylic acid cycle. Thus, the availability of alpha-ketoglutaric acid depends to a great extent upon the presence of sugar; and as the yeast cells investigated were grown in media containing no sugar, the biosynthesis of lysine in these cells would be mediated through the deamination of glutamic acid to furnish the alpha-ketoglutarate to a certain degree.

These studies indicated also that the level of free amino acids was lowered by exposing the yeast cells to the nitrogen-free medium (30% sucrose) even though none of these amino acids disappeared entirely. This fact explains the ability of the yeast cells under investigation to get along without an external source of nitrogen in enzyme synthesis, i. e., they have an internal nitrogen supply which can be used, to a limited extent, in enzyme synthesis.

SUMMARY

1.   An osmophilic yeast strain was isolated from sugar syrup samples of high yeast count.   The taxonomic characteristics of this yeast strain were determined.   These characteristics showed that it is a new species of the genus Saccharomyces.

2.   A series of experiments was conducted to investigate the ability of this yeast strain to fix atmospheric nitrogen.   The studies showed that in yeast cultures there was a decrease in total nitrogen, particularly in the first two hours of incubation.   In this series of experiments, only one experimental yeast culture showed an increase in total nitrogen. Therefore, the ability of the yeast cells to fix atmospheric nitrogen was ruled out.

3.   The role of cations and anions in enhancing growth and possibly stimulating nitrogen-fixation by yeast cells was studied.   The cations and anions tested were iron as ferrous sulfate, citrate as sodium citrate, phosphorus as K_2HPO_4, molybdenum as $Na_2MoO_4.2H_2O$, boron as boric acid and calcium as calcium chloride.   These trace elements did not stimulate the fixation of atmospheric nitrogen.   The only established effect of these cations and anions was the enhancement of yeast growth as indicated by an increase in the biomass.   Phosphorus enhanced the

growth more efficiently than the other anions and cations tested. On the other hand citrate did not enhance the yeast growth to a significant degree.

4. The effect of sucrose concentration on yeast growth was investigated. It was found that the yeast cells grew well in lower sugar concentrations in the first 24 hours of incubation. After that period, they grew more efficiently at higher sugar concentrations. These findings indicated that this is an osmophilic strain.

5. Studies of the effect of temperature on yeast culture growth indicated that the yeast cells were unable to grow at refrigerator temperature or at 56° C and they grew poorly at 37° C. On the other hand there was very good growth of yeast cells when incubated at room temperature (26° C).

6. The effect of hydrogen-ion concentration on yeast growth was studied. It was shown that the yeast cells were able to grow over a wide pH range of 3 to 7 in Mycophil broth containing either 2 per cent or 30 per cent sucrose.

7. The efficiency of the utilization of various amino acids and ammonium sulfate as nitrogen sources by these osmophilic yeasts was studied. The studies indicated that ammonium sulfate was utilized more efficiently than the other nitrogen sources tested. At the same time some of the amino acids investigated such as proline, alanine, threonine and aspartic acid sustained growth better than L-glutamic

acid and DL-methionine. It was also found that the growth in 2 per cent sucrose was better than in 30 per cent sucrose during the nine hours incubation period. No experiments of longer duration were carried out.

8. A survey of the free amino acids synthesized by the yeast cells cultured in Mycophil broth containing 30 per cent sucrose was made using unidirectional paper chromatography. The solvents applied were butanol-acetic acid-water mixture and phenol. The identified amino acids were L-glutamic acid, L-aspartic acid, DL-histidine, L-tyrosine, L-valine, L-leucine, L-glycine and L-lysine.

9. Investigation of the effect of growth media on the free amino acid pool composition mentioned above was carried out. Besides the Mycophil broth medium, two other media were used. One was Mycophil broth containing no sucrose and the other was 30 per cent sucrose containing no other nitrogen source. This investigation showed that most of the free amino acids identified as members of amino acid pool when cultured in Mycophil broth containing 30 per cent sucrose were found to be present in the pools of yeast cells cultured in the other two media. Tyrosine was the only amino acid which could not be identified as a member of the free amino acids of yeast cells cultured in Mycophil broth containing no sucrose.

10. A rough comparison was made between the free amino acids identified in the yeast cells cultured in the three media mentioned above.

It was found that the content of L-glutamic acid, L-aspartic acid and DL-histidine was not significantly affected by differences in culture media. The total level of the free amino acids identified as members of the amino acid pool grown in nitrogen-free medium, i. e., 30 per cent sucrose, was lowered. It was also found that the level of lysine was significantly decreased in the case of yeast cells grown in Mycophil broth containing no sugar.

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RESEARCH PROPOSALS

submitted

by

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RESEARCH PROPOSALS

1. It has been found by Ōnishi (1960) that different yeast species and strains show different individual characteristics in their ability to produce various polyalcohols. Therefore, it is suggested that the osmophilic yeast strains under investigation be studied in relation to their ability to produce polyalcohols and the factors affecting their production.

2. If the production of polyalcohols by the present yeast is established, it is suggested that an attempt be made to convert the polyalcohol fermentation to ethanol fermentation. This can be accomplished using the techniques described by Ōnishi and Saito (1961). They used a medium containing high concentrations of yeast extract. The acetaldehyde formed as a metabolic intermediate leading to ethanol formation was trapped by the addition of sulfite and was identified as 2, 4-dinitrophenylhydrazone.

3. It was observed by many workers such as Ōnishi (1959) that osmophilic yeasts produce glycerol on a large scale. Therefore, it is suggested to study the glycerol production by the sugar-tolerant yeast separated from the sugar syrup samples.

4. As it was observed that the osmophilic yeast investigated grew well in sugar refinery tanks containing 65.5 per cent sucrose, it is

suggested that the relation between the sugar-tolerance and invertase activity be studied. The methods to be followed in this investigation are those described by Sato and Tanaka in 1961.

5. The yeast cells separated from the sugar syrup samples received from Amalgamated Sugar Company represent the major yeast contaminants of the sugar. Therefore, it is suggested to study the control of these yeast cells causing spoilage in refined sugar and intermediate products. The studies can be directed according to the methods used by Von Schelhorn (1951).

6. It has been observed, during my thesis studies, that there was a decrease in total nitrogen of yeast cultures containing different nitrogen sources. The suggestion proposed for this observation was that part of the nitrogen was involved in nucleic acid biosynthesis. The nucleic acid nitrogen, therefore, could not be determined by the micro-Kjeldahl method used for nitrogen determinations in my studies. Thus, it is suggested to study the nucleic acid biosynthesis by yeast cells using labeled nitrogen sources.

7. Another suggestion proposed for the nitrogen decrease was that it might be due to ammonia liberation. Thus, it is proposed that this possibility using the methods described by Bernard (1963) be investigated

8. Some workers studied the ability of yeasts to fix atmospheric nitrogen. For example, Ingram (1955) found that there was no increase

in total nitrogen which was in accord with the results obtained in the present studies. This finding indicated that the yeast cells were unable to fix atmospheric nitrogen. After that, Ingram used N-15 studies, where he found that the N-15 could find its way to the yeast cells and since there was no increase in total nitrogen, he suggested the possibility that there was an exchange of yeast nitrogen with that of the atmosphere. Therefore, it is suggested to study this possibility using the yeast which was investigated for my thesis work.

9. It was found by Bakhadur (1955) that changes in the carbon-nitrogen ratio affected the ability of Dkhar yeast to fix atmospheric nitrogen. Therefore, it is suggested to study the effect of changes in this ratio on the possible ability of yeast cells to fix atmospheric nitrogen.

10. In my thesis studies, a rough comparison for the free amino acids contents biosynthesized by yeast under different culture conditions was made. It is suggested to make quantitative studies in this regard using the methods described by Kretovich and Krauze (1961).

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