SOME UNIQUE MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF A YEAST ISOLATED FROM PICKLE FERMENTATION

by

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B.S., Taiwan Provincial College of Agriculture, 1959

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

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1964

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INTRODUCTION

A single celled yeast-like organism was isolated by A. F. Borg and J. L. Etchells from fermenting "overnight dill" pickles. Since this organism did not seem typical of known yeasts, this investigation was undertaken to study the morphological and cultural characteristics of the isolate. From the sausage-shaped cells and the pattern of vegetative reproduction, i.e., budding on a broad base, classification in the genus Saccharomycodes seemed logical. However this organism differs in fermentation pattern with S. ludwigii which is the only known species of this genus. On further observations and tests, it was found to differ from S. ludwigii in additional aspects such as cell shape and size, sexual cycle, and nutritional requirements. This may lead to the establishment of a new species of Saccharomycodes, or a new genus in the family Nadsonieae. Preliminary characterization of this organism formed the basis of the experimental data reported in this thesis.

REVIEW OF LITERATURE

The yeast considered here resembles in many ways a Saccharomyces-form organism described by Ludwig in 1886 (from Lodder and Kreger-van Rij, 1952). The cells of his culture were ellipsoidal to bottle shaped or lemon shaped and produced ascospores readily. His culture fermented glucose and sucrose, but not maltose or lactose.

Ludwig's description was incomplete. Three years later Hansen isolated an apparently identical organism from the exudation of an oak and named it Saccharomyces ludwigii. In subsequent studies, Hansen in 1891 found that this yeast reproduced vegetatively by budding on a broad base. This manner

of budding was suggestive of but different from formation of crosswalls seen in fission yeasts. Spores of this organism germinated in a special way. They fused two by two in the asci and resulting zygotes formed promycelia. In 1904 Hansen established the new genus Saccharomycodes for the organism, and named it S. ludwigii. (from Lodder and Kreger-van Rij, 1952).

Similar yeasts varying in one or more characteristics have been described from time to time. Kroemer and Heinrich in 1922 described in detail S. ludwigii var. vini isolated from grape must. It differed from S. ludwigii in cell and spore sizes, ring formation, and the resistance towards sulfurous acid. The original of this description was not available. No relative sizes or other comparative information is given by Lodder and Kreger-van Rij in their review of the taxonomy of this variety.

Hjort (1954) referred to a yeast isolated from hops by Behrens in 1896. Klöcker named it S. behresianus but its status is not clear. The description was incomplete and the culture no longer exists.

Castelli (1942) gave the specific name bisporus to a culture which differed from S. ludwigii in that it never formed more than two spores in each ascus. In his hands the spores never conjugated before germination. His cultures fermented glucose, levulose, mannose, sucrose, and 1/3 raffinose.

The existing cultures of Saccharomycodes were reviewed critically by Hjort (1954). Using standard strains of S. ludwigii, and Castelli's (1942) S. bisporus, he compared spore formation and germination, single-spore cultures, fermentation, and giant colony formation. He found that vegetative cells of S. bisporus were diploid and homozygous; and the germination pattern was similar to that of S. ludwigii. He named the organism S. ludwigii Hansen var. bisporus (Castelli).

Thus at the present time the genus Saccharomycodes seems to have only a single species with two varieties, namely vini and bisporus

The characteristics of S. ludwigii were summarized by Stelling-Dekker (1931) as follows : "In malt extract : Cells are sausage-shaped or lemon-shaped, 3-5 x 8-24 µ. A ring and a sediment. Vegetative reproduction by budding at the poles on a broad base. Spores : round, smooth, four per ascus. They conjugate before germination. Fermentation : Glucose +, Galactose -, Saccharose +, Maltose +, Lactose -. Assimilation of potassium nitrate : absent. Ethanol as sole source or carbon : Hardly any growth."

Guilliermond (1928) confirmed Hansen's observation that spores of S. ludwigii conjugate in the ascus before germination. This peculiar phenomenon was studied in some detail by Winge and Laustsen in 1939. Using single spore cultures, they showed that this yeast exists as a balanced double-heterozygote during the vegetative phase, and is heterothallic in the haplophase.

MATERIALS AND METHODS

The organism used in the present study was isolated by A. F. Borg and J. L. Etchells in December 1955 from fermenting "overnight dill" pickles. Four strains of the organism, all from the same source and designated as S-778, S-782, S-784, S-786, were obtained from cultures lyophilized in 1956. One culture of S. ludwigii was kindly supplied by Dr. Herman Phaff, University of California, Davis, California. Two cultures of S. ludwigii numbered 34-1-1 and 34-1-2 were sent by Dr. Anna Koskovâ-Kratochilovâ of the Chemicke Ustavy Sav in Bratislava, Czeckoslovakia. Culture 34-1-1 came originally from the collection of Dr. Kossikov, Institut genetiki An USSR, Moskow, and 34-1-2 from the Centraalbureau voor Schimmelcultures, Delft, Holland.

The experimental methods and media used in the present studies were mainly based upon those recomended by L. J. Wickerham in his publication "Taxonomy of Yeasts" (1951) and J. Lodder and N. J. W. Kreger-van Rij in their book entitled "The Yeasts, a Taxonomic Study" (1952).

Observations of cell morphology were made on wet mounts prepared from 72-hour cultures grown on malt extract-yeast extract agar slants at 25°C. Patterns of growth on dalmau plates were observed on Difco Morphology Agar supplemented with 0.5 % peptone. Observations were made on exposed cells and on growth under a cover glass on agar plates as recommended by Wickerham. For these observations, cultures were incubated at 25°C for 7 days.

To obtain ascospores, small inocula were spread on entire surfaces of Henrici's Vegetable Juice Agar slants containing 1 % yeast extract and incubated at 25°C. Cultures were examined for spores at regular intervals up to 3 months after inoculation.

Colony morphology was observed on malt extract-yeast extract agar plates inoculated at three points and incubated at 25°C for 3 weeks.

Germination of spores was observed on slide cultures. A small amount of dilute suspension of a sporulated culture was spread on a drop of malt extract-yeast extract agar solidified on a sterile slide. Cover glasses were placed over the inoculated medium and the cultures were examined every two hours.

Physiological properties of the organism were studies by the methods recommended by Wickerham. In certain cases it was necessary to modify the basal media to obtain growth. Wickerham's Bacto Yeast Nitrogen Base and Bacto Yeast Carbon Base were filter-sterilized in 10 x solutions. A 0.5 ml portion of 10 x concentration was added to 4.5 ml of water or supplement. For nitrogen assimilation tests, various nitrogen sources were added to the

Carbon Base media to give a final concentration of 0.1 %. Similarly, for carbon assimilation tests, solutions of various carbon sources were added to give 0.5 % in Nitrogen Base media supplemented with 0.5 % peptone.

Ability to ferment sugars was tested in Durham tubes using 1 % yeast extract solution containing 2 % of the sugar.

To determine the ability of the cultures to grow at moderate osmotic pressure, 10 % sodium chloride (NaCl) and 5 % glucose were added together with 0.5 % peptone to Nitrogen Base media.

The ability of the culture to liquify gelatin, to produce starch, to produce esters and to grow at 37°C was tested on Nitrogen Base medium plus 0.5 % peptone containing the appropriate additive (10 % gelatin), and tested under appropriate conditions. Twenty-four-day old cultures were stained with 0.02 N iodine to observe possible starch formation. The presence of organic esters was tested organoleptically.

An effort was made to determine the nutritional requirements of the culture described here. Since cultures failed to grow on Wickerham's Nitrogen Base plus glucose or on Carbon Base media, these media were supplemented with a variety of nitrogen sources including peptone, yeast extract, casamino acids, peptone hydrolysate*, yeast hydrolysate, lactalbumin hydrolysate, and mixtures of amino acids.

* peptone hydrolysate was prepared by hydrolyzing 2 gm of peptone in 100 ml 2 N HCl for 6.5 hours in autoclave under 15 lbs pressure.

RESULTS

Morphological Properties

Cells of the culture, microscopic examination showed, were mostly sausage-shaped, and occurred singly or in pairs. Vegetative reproduction took place

by budding on a broad base at the poles. The distinctive budding pattern of this culture and of S. ludwigii can be seen in Plates I, II, and III. The cells commonly had large vacuoles and a few granules.

Cell shape on dalmau plates was the same as on malt extract-yeast extract agar slants. Cells were arranged singly, in pairs, and in short chains. No pseudomycelium was seen on either aerobic or anaerobic portions. The size of Vegetative cells grown on malt extract-yeast extract broth are given below :

Culture	Length		Width	
	range	mean	range	mean
S-778*	3.8-10.0 μ	5.7 μ	2.8-4.4 μ	3.5 μ
S-782	3.5-6.4 μ	4.7 μ	2.2-4.4 μ	3.17 μ
S. ludwigii**	10.0-30.0 μ		5-8 μ	
S. bisporus***	12.0-20.0 μ		7-9 μ	

* S-778 was used as a typical culture throughout the present studies unless otherwise noted.

** Measurement was from Lodder and Kreger-van Rij (1952).

*** Measurement was from Castelli (1942).

Giant colonies developed on malt extract-yeast extract agar plates within 6 weeks have average diameters 12.5 mm. They are smooth and slightly undulate at edges, raised, very thick at the center. See Plate IV.

Sporulation occurred readily provided the medium was a sufficiently rich one. Henrici's V-8 juice agar supported little growth and no spores were observed on this medium. Wickerham's malt extract-yeast extract yielded good vegetative growth, but poor in spore formation. V-8 juice medium supplemented with 1 % yeast extract gave good growth and abundant sporulation in 4-6 weeks. One of the four strains examined (S-782) has not produced

PLATE I

Vegetative cells of S-773 from a 4-day culture on malt extract-yeast extract agar slant at 25°C. Magnification 1100.

PLATE I

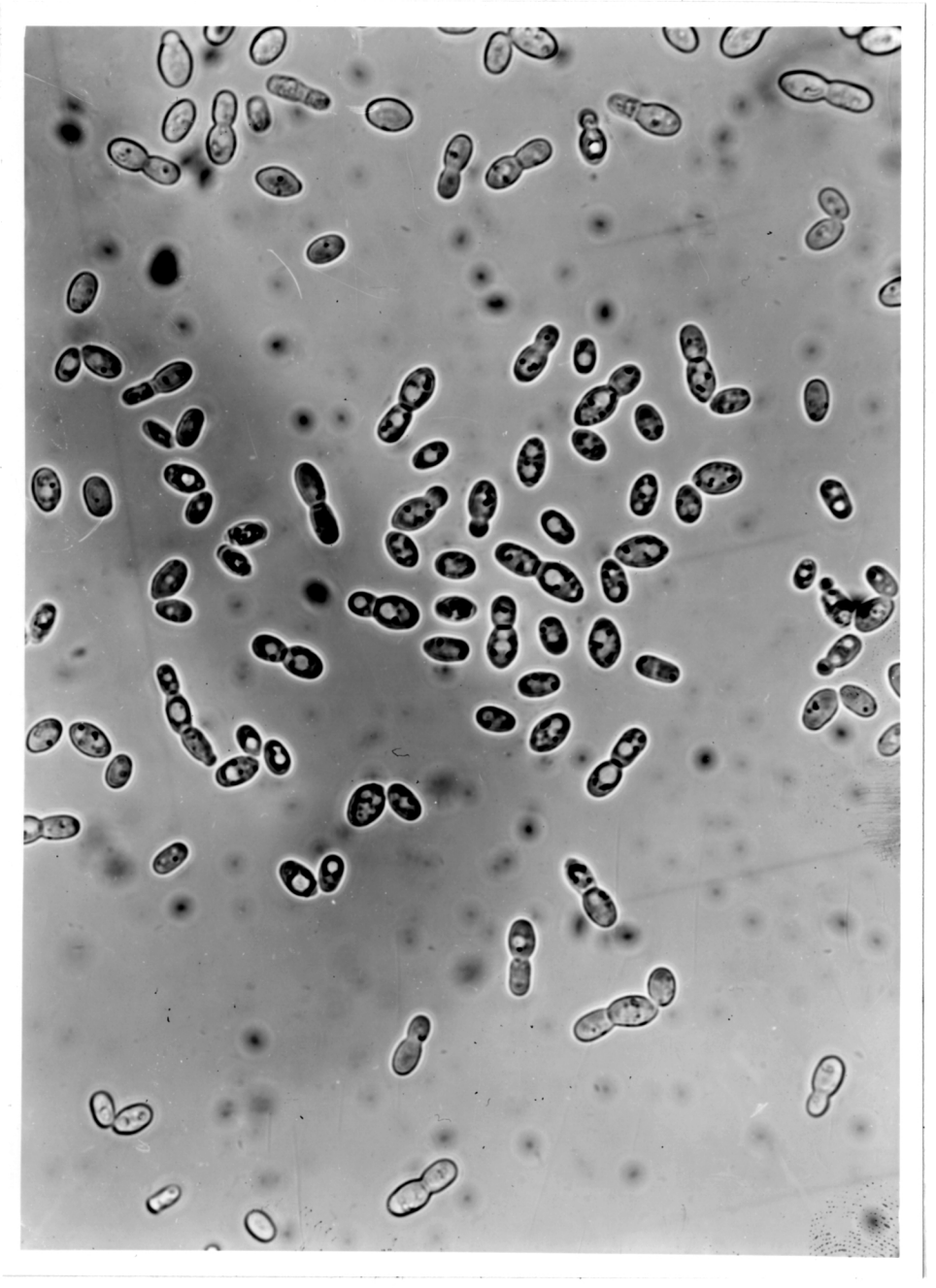


PLATE II

Vegetative cells and asci of *S. ludwigii* from a 1-week culture on malt extract-yeast extract agar slant at 25°C. Magnification 1100.

PLATE II

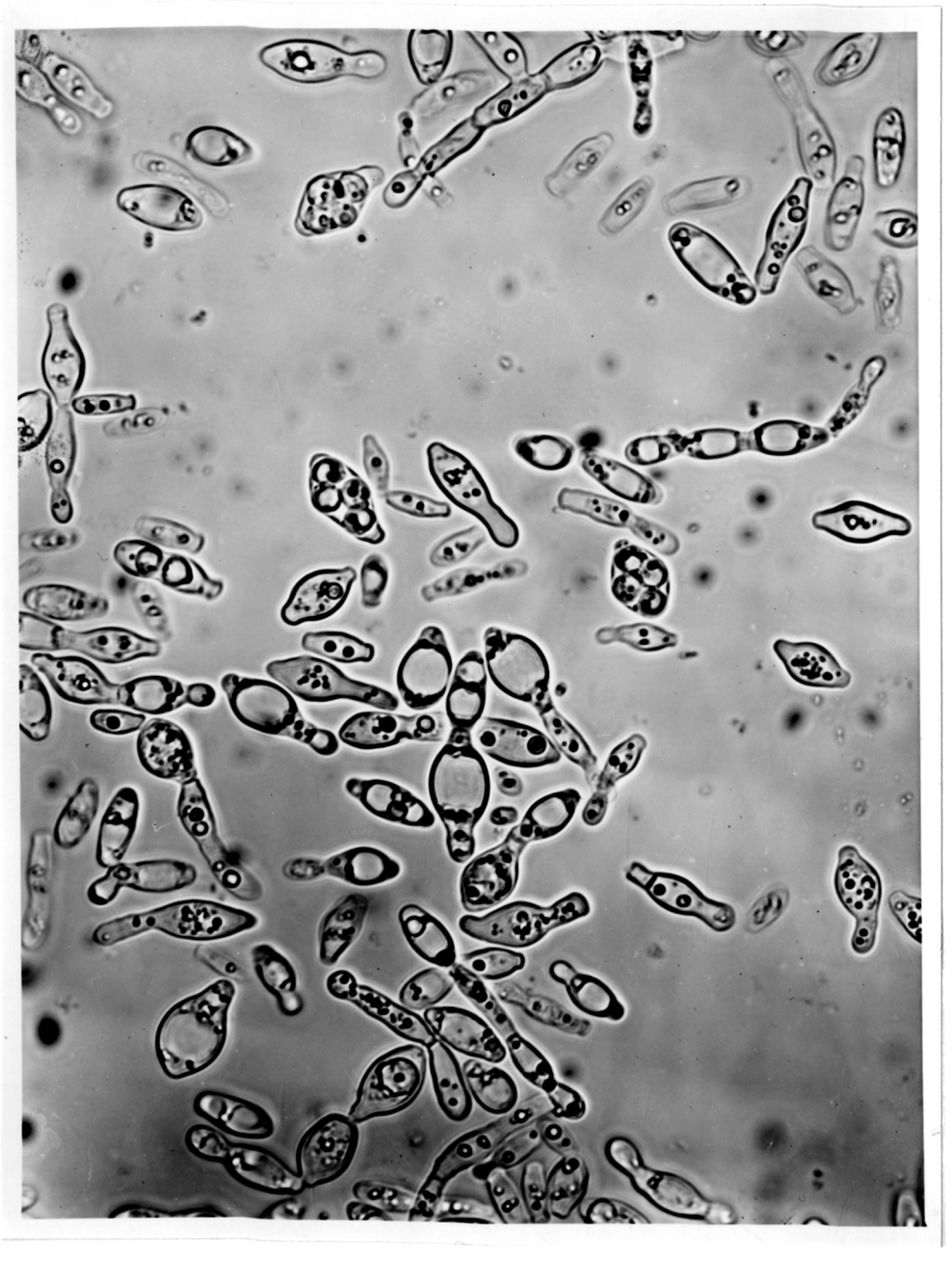


PLATE III

Vegetative cells and asci of *S. ludwigii* 34-1-2 from a 1-week culture on malt extract-yeast extract agar slant at 25°C. Magnification 1500.

PLATE III

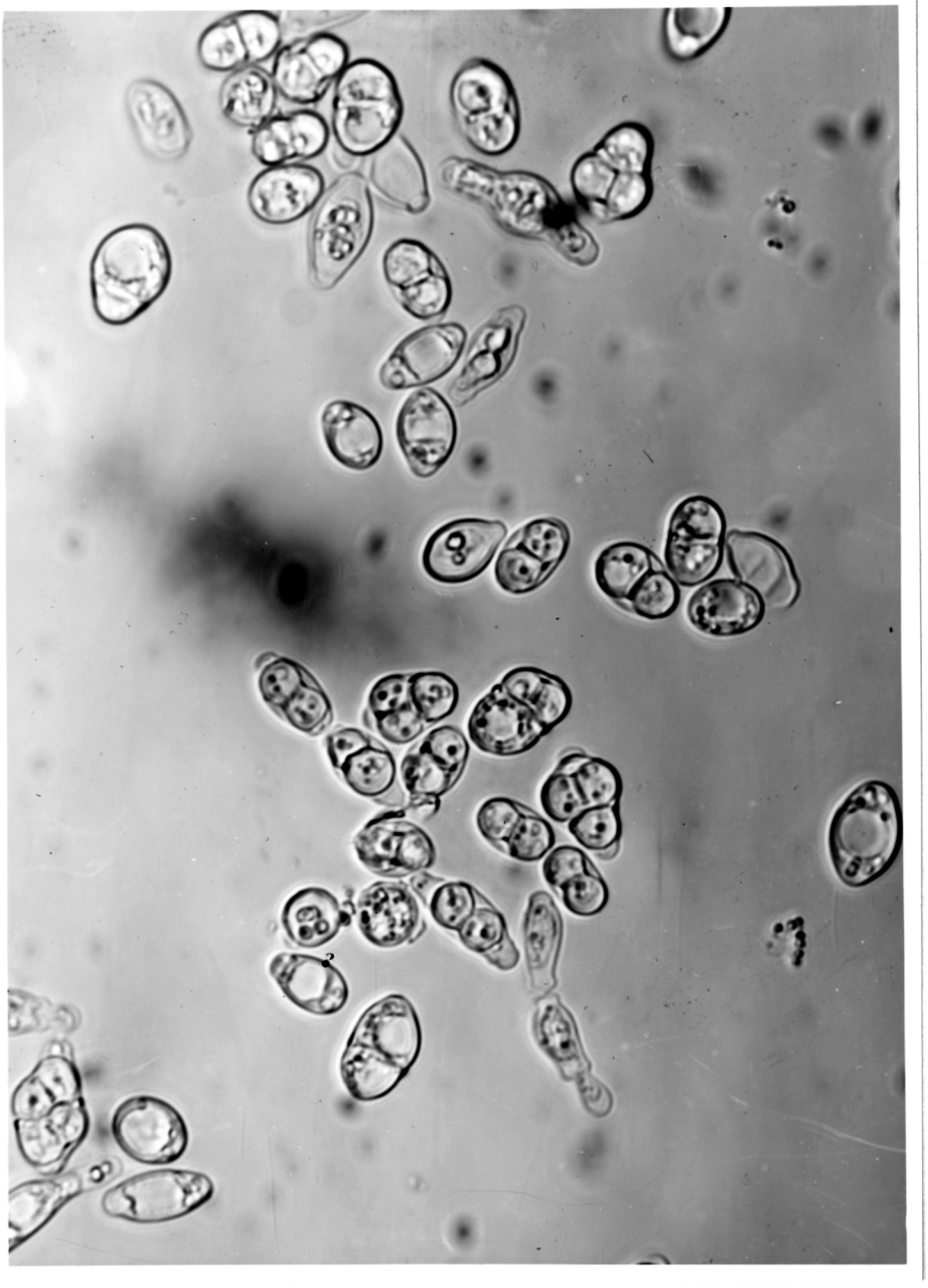
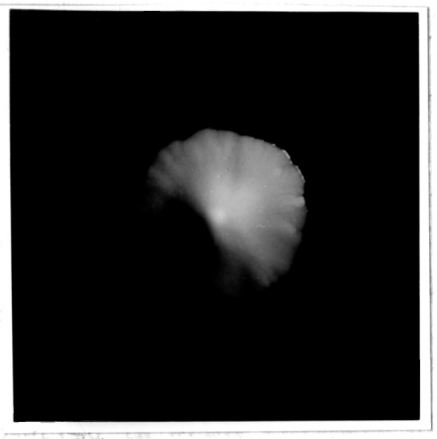


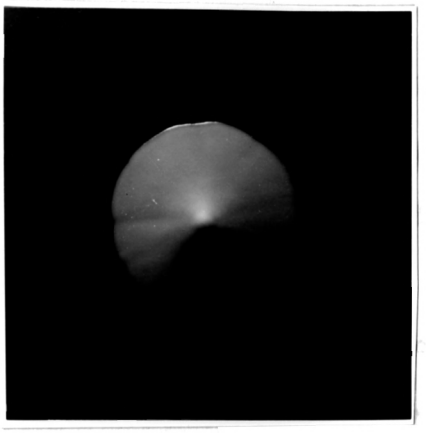
PLATE IV

Giant colonies observed on malt extract-yeast extract agar plates after 3 weeks at 25°C. a, S. ludwigii; b, S. ludwigii 34-1-1; c, S-778; d, S-782. Magnification 2.

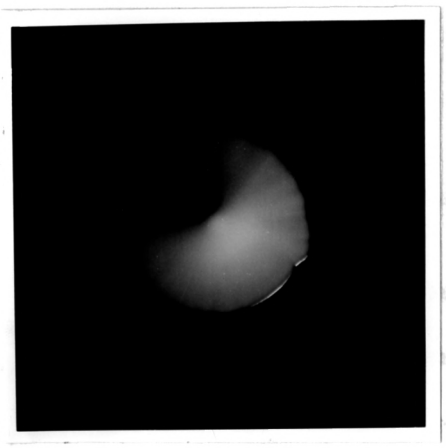
PLATE IV



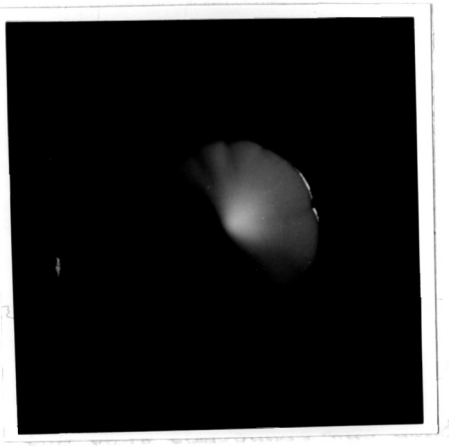
a



b



c



d

spores in any medium used.

Microscopically, sporulation began with the formation of a thickened wall immediately within the young ascus and the disappearance of internal visible structures. The spore wall then contracts away and leaves spaces at the poles(see Plate V).

The ascospores produced were round and smoth. Each ascus contained 1 or 2 spores, but never more. The spores measured 2.5-6.0 μ with a mean of 4.5 μ. For a comparison of spores in these cultures with those of S. ludwigii, see Plates II, III, and VI.

Spores germinated by the outgrowth of budlike processes. This could be observed in slide cultures after 14-15 hours of incubation. Plate VII is a series of photographs taken at intervals during the process of outgrowth. No conjugation of spores within the ascus was observed, though this point was not tested critically. Conjugation occurred only in subculture made by transferring sporulated cultures to a fresh medium. No conjugation was observed in any case where subcultures were made from cultures repeatedly transferred in vegetative state. Distinct conjugation tubes were formed as can be seen in Plate VIII.

Physiological Properties

The ability of the organisms to assimilate various nitrogen and carbon sources was judged by the presence or absence of growth of the organism in a liquid medium where one or the other of the test compounds was used as the sole nitrogen or carbon source.

All four strains assimilated peptone and yeast extract, but not ammonium sulfate, potassium nitrate, urea, or asparagine when these were provided as the sole source of nitrogen. All strains assimilated glucose, and galactose,

PLATE V

Sporulating cells of S-778 on V-8 juice agar slants after approximately one month at 25°C. Magnification 1500.

PLATE V

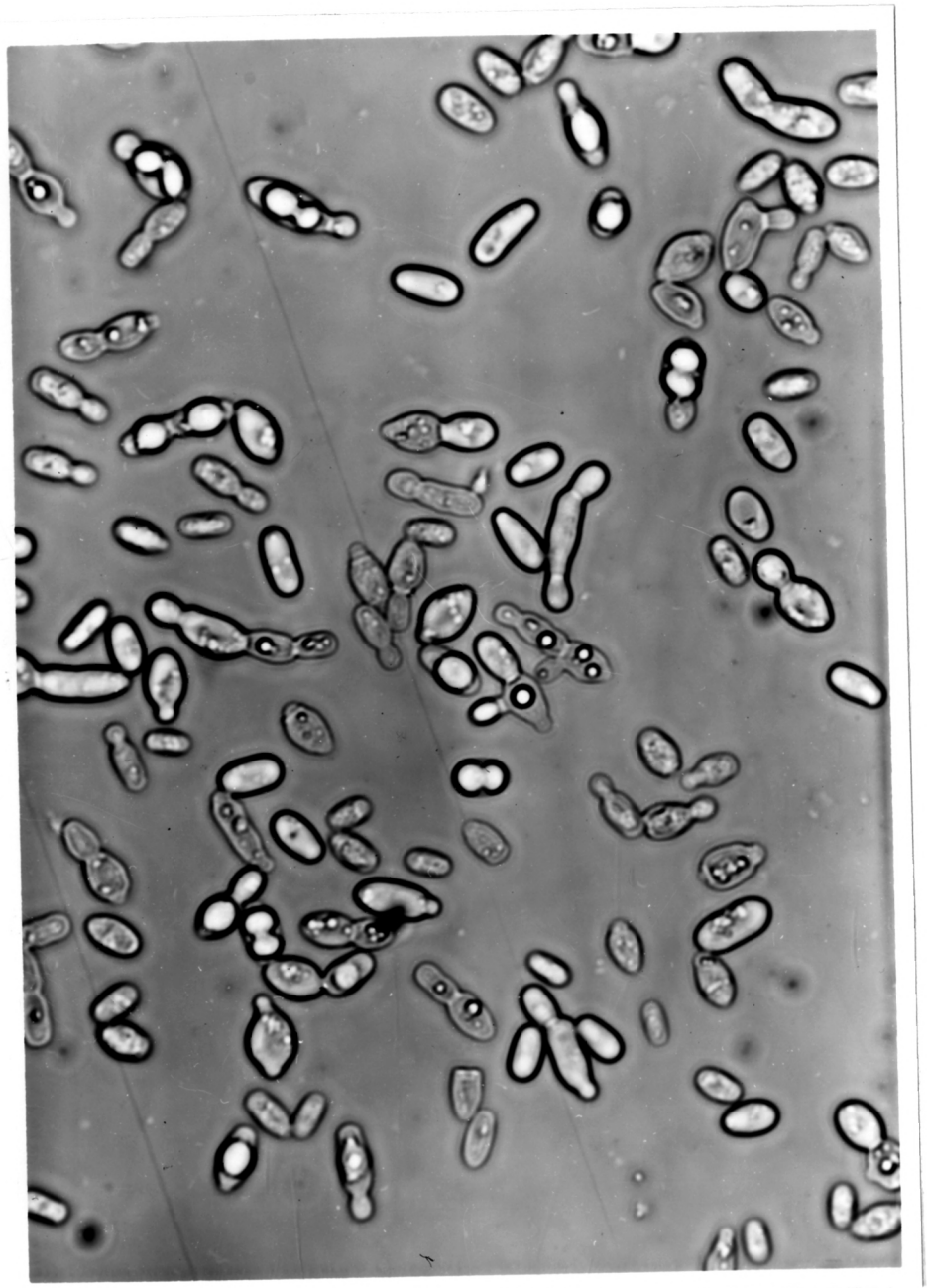


PLATE VI

Asci and ascospores of S-778 on V-8 juice agar slant with 1 % yeast extract after 2 months at 25°C. Magnification 1900.

PLATE VI

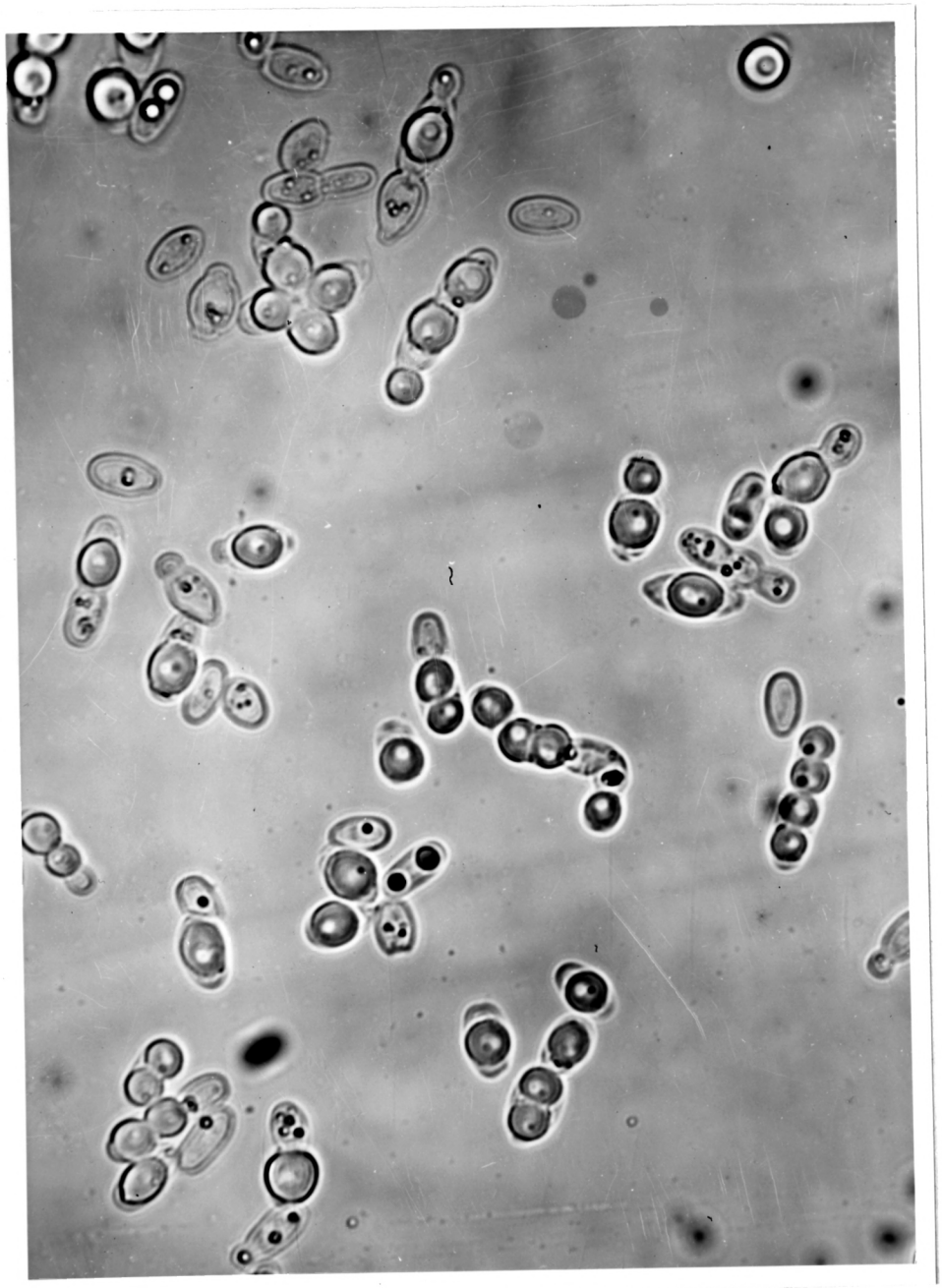
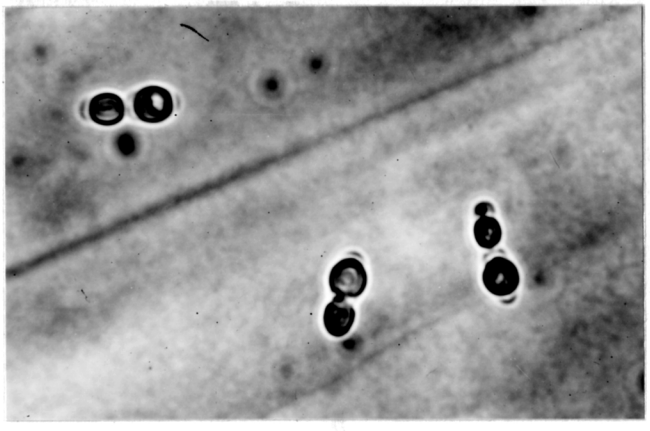


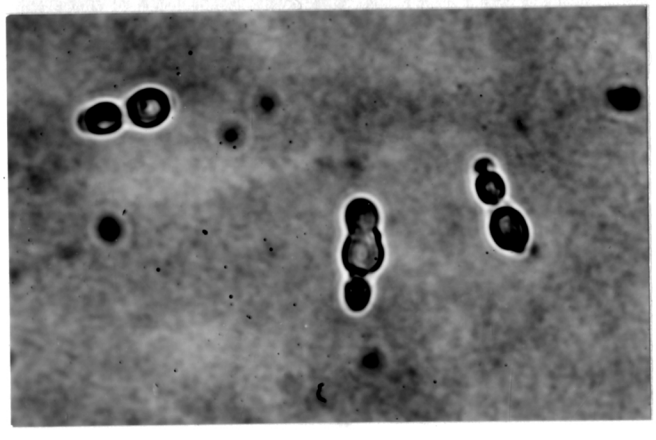
PLATE VII

Spore germination of S-778 on slide cultures at room temperature. The following is a series of micrographs taken at time intervals after inoculation as indicated a, 0 time ; b, 14 hours ; c, 16 hours ; d, 19 hours ; e, 22 hours ; f, 36 hours. Magnification 1600.

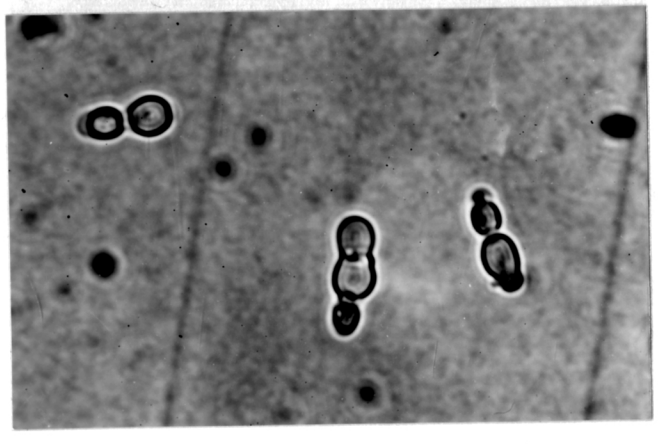
PLATE VII
(continued)



a

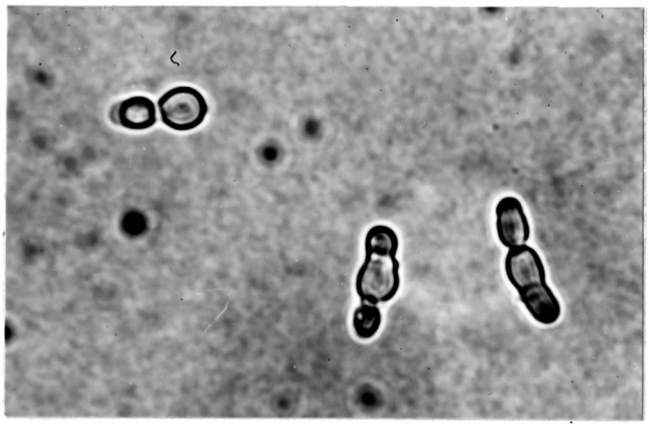


b

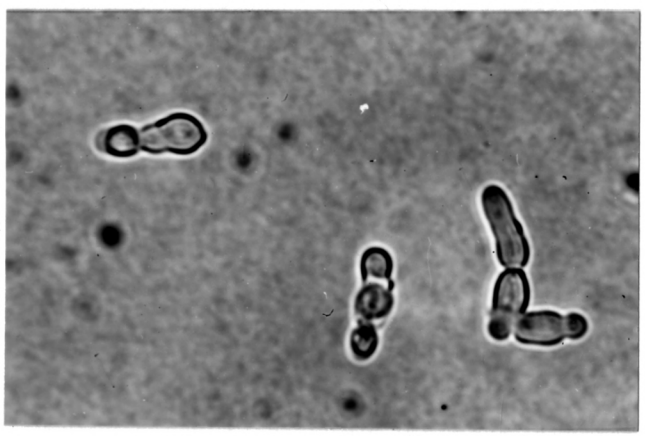


c

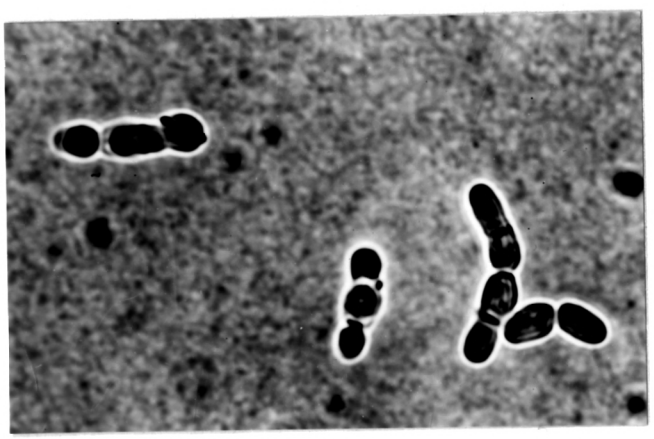
PLATE VII



d



e

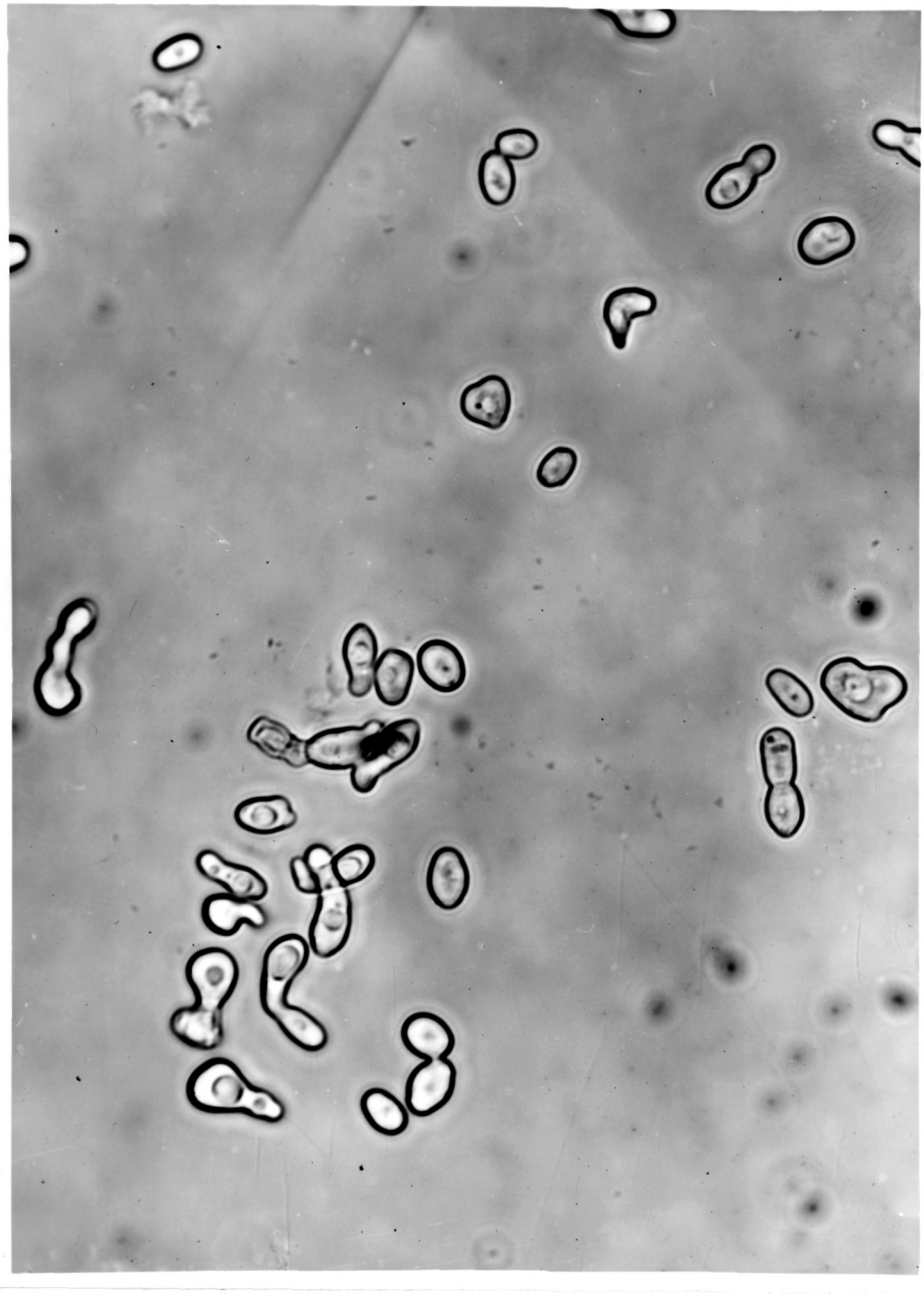


f

PLATE VIII

Cell conjugation observed one week after spores were transferred onto malt extract-yeast extract agar slants and incubated at 25°C. Magnification 1500.

PLATE VIII



but did not assimilate any of the following carbon compounds : lactose, sucrose, maltose, raffinose, starch, l-arabinose, l-rhamnose, mannitol, melezitose, inositol, inulin, cellobiose, ducitol, salicin, adonitol, melibiose, xylose, trehalose, erythritol, ∝-methyl glucoside, ethanol, succinate, and glycerol. Likewise, all four strains fermented glucose and galactose with the production of gas, but did not ferment sucrose, maltose, or lactose.

Additional physiological tests have shown that the organism was unable to grow at moderate osmotic pressure (10 % NaCl plus 5 % glucose) was unable to grow at 37°C, and liquified gelatin after 24 days at 25°C. Starch was not found in the cultures and no esters were detected in the culture medium. The organism was able to grow at PH 4 and PH 7 but limiting PH values were not determined.

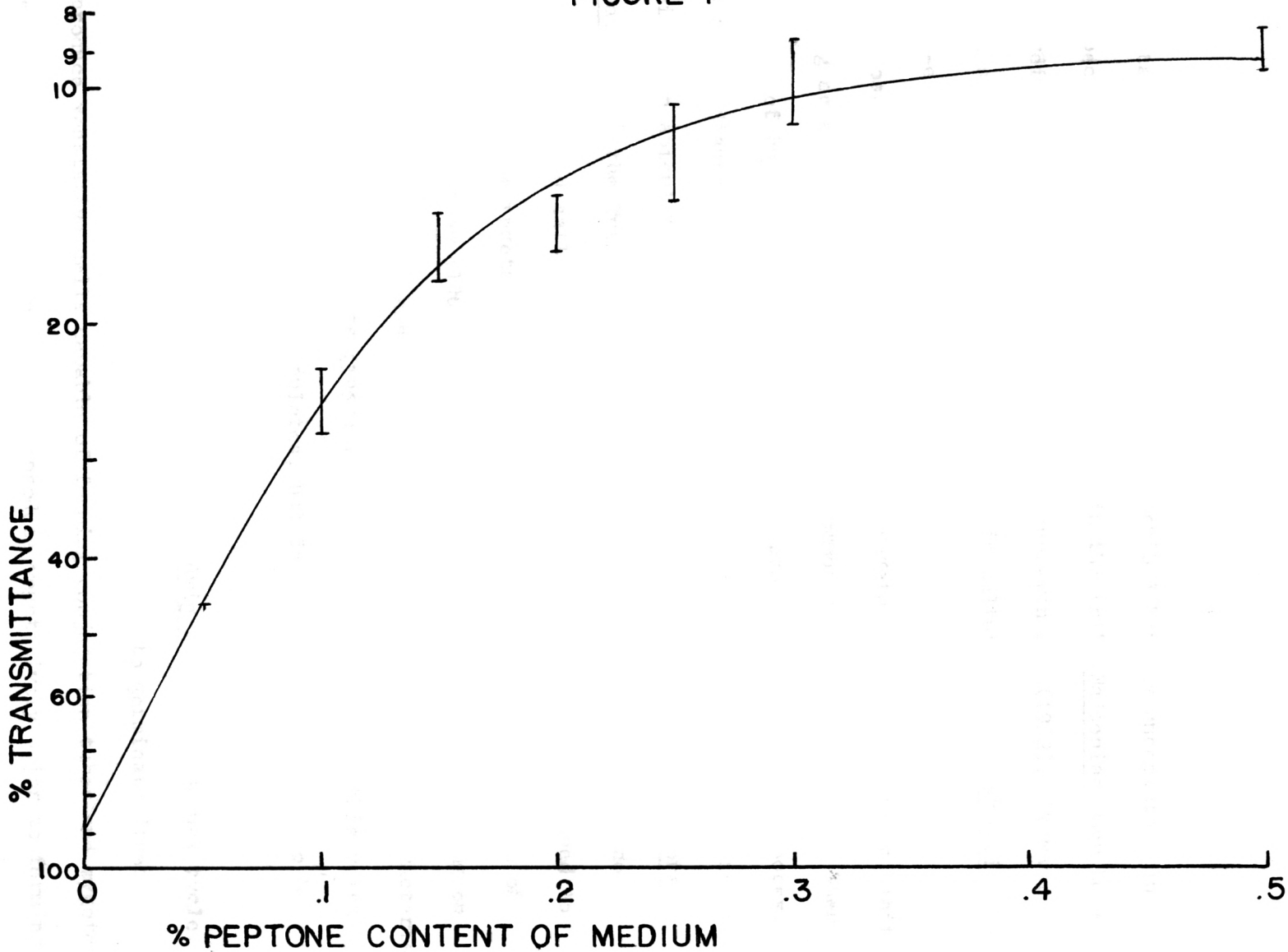
Known cultures of Saccharomycodes ludwigii have no special nutritional requirement, i.e., they grow on Nitrogen Base plus glucose and on Carbon Base plus ammonium sulfate (NH_4SO_4). The cultures under study did not grow on Nitrogen Base plus glucose unless appropriate amounts of peptone, yeast extract, casamino acids, peptone hydrolysate, yeast hydrolysate, or lactalbumin hydrolysate were added. Slow and imcomplete development took place on Carbon Base plus casamino acids, NH_4SO_4, and added amounts of tryptophane, methionine and histidine. A mixture of amino acids found in casamino acids plus Nitrogen Base and glucose did not fill their requirement. Purines and pyrimidines plus Nitrogen Base and glucose also did not support growth of these strains.

Response of these cultures to various concentrations of peptone is shown graphically in Figure I. With Nitrogen Base plus glucose as the basal medium, the cultures showed increasing growth with increased peptone concen-

FIGURE I

The dependence of peptone on growth of the four strains in N-base-glucose medium supplemented with various amounts of peptone was measured with a Spectronic 20 photocolorimeter after 2 days at 25°C.

FIGURE 1


8
9
10
20
40
60
100
% TRANSMITTANCE
0
.1
.2
.3
.4
.5
% PEPTONE CONTENT OF MEDIUM

trations ranging from 0.01 % to 0.15 %.

DISCUSSION

The taxonomic position of the yeast described here is uncertain. In many respects it resembles Saccharomycodes in the tribe Nadsoniae when keyed out according to the scheme of Lodder and Kreger-van Rij (1952). However, there are some obvious differences between this culture and S. ludwigii, the only well-established species in the genus.

The most striking characteristic of the vegetative cells is their habit of budding on a broad base. This is not a common property in yeasts. When this manner of budding occurs in yeasts which produce round, smooth spores, it is almost diagnostic of Saccharomycodes. However, Saccharomycodes is definitely an apiculate yeast, as are the other two genera Hanseniaspora and Nadsonia in the Tribe Nadsoniae. The cultures described here do not have what one would call apiculate cells. They are more sausage-shaped to oval. Further, the vegetative cells are definitely smaller than those of S. ludwigii. Wickerham (1963) has confirmed the observation of budding on a broad base. However, he has not made detailed studies of other characteristics of these cultures. Phaff (1963) is inclined to think that this yeast should be included among the apiculates, but feels that it may have to be placed in a new genus.

The cultures in question differ from S. ludwigii in their sexual cycle. Instead of the spores fusing in the ascus, they seem to germinate directly and produce vegetative cells capable of forming conjugation tubes and zygotes. Cells resulting from this fusion may reproduce vegetatively prior to forming ascospores. Presumably then, the normal vegetative cells are either haploid or diploid, but this was not investigated by genetic means. Such a fundamen-

tal difference in sexual reproduction would seem to preclude the placing of these cultures in the genus Saccharomycodes.

The absence of ascospores in culture S- 782 can be explained by assuming that it is an isolate derived from a single haploid vegetative cell and unable to form ascospores in the absence of a complementary mating type. This might be tested by isolating haploid non-sporulating strains from sporulating cultures and combining them with S-782 (Wickerham, 1952). If ascospores were formed by such mixtures, the haploid nature of the vegetative cells would be confirmed. If this is true it would be further reason for separating these cultures from Saccharomycodes for the heterothallism of that genus is one of the few instances known among yeasts and is one distinguishing feature of the genus.

Culture S-782 has another characteristic which marks it as a possible haploid. The cells are noticeably smaller than those in the other three strains. This corresponds to what one observes in cultures of Saccharomyces. The normal diploid vegetative cells are large and vigorous while haploid cells form single ascospores whose progeny did not conjugate are smaller and less vigorous. (Lindegren, 1949)

The nutritional requirements of these strains are unusually complex for a yeast. Cultures fail to grow on Wickerham's Nitrogen Base or Carbon Base media supplemented with simple compounds of carbon or nitrogen. This is in spite of the fact that these basal media contain a full complement of mineral salts, nine B vitamins, and three amino acids (methionine, histidine, and trytophane). At Wickerham's suggestion (Wickerham, 1963), the basal medium was fortified with additional amounts of inositol, pantothenic acid and biotin. Still the cultures failed to grow.

It appears that the special nutritional requirement must involve some

type of nitrogenous compounds. Wickerham's Nitrogen Base plus glucose will support growth of the yeast when supplemented with peptone, yeast extract, casamino acids, acid hydrolyzed peptone, yeast hydrolysate and lactalbumin hydrolysate.

Apparently the nutritional situation is a complex one. Experiments with Carbon Base gave conflicting results, and Carbon Base supplemented to duplicate Nitrogen Base often did not yield the same amount of growth.

Likewise, ascospore formation seems to be linked to nutrition. Ascospores were observed only on V-8 juice medium supplemented with yeast extract. Even cultures on the rich malt extract-yeast extract medium of Wickerham did not produce ascospores.

SUMMARY

A yeast isolated from "overnight dill" pickle produced sausage-shaped, oval or elongate cells as a result of vegetative reproduction by bipolar budding on a broad base. The cells measured 2.8-4.4 μ x 3.8-10.0 μ (mean 3.5 x 5.7 μ). In liquid cultures a sediment formed after 24 hours. Cells showed conjugation after spore germination.

Sporulation was found only on Henrici's Vegetable Juice Agar slants. Spores were round and smooth, 1-2 spores per ascus with diameters ranging from 2.5 to 6.3 μ (mean diameter 4.5 μ). The germination of ascospores was not preceeded by conjugation.

Giant colonies appeared umbonate with smooth margins. No pseudomycelium formed on dalmau plates.

Fermentation and carbon assimilation patterns were the same : Glucose +, Maltose -, Galactose +, Saccharose -, and Lactose -.

An unknown growth factor which is a component of yeast extract and peptone was required in minute amounts.

ACKNOWLEDGMENT

I wish to express my sincere appreciation and gratitude to the faculty and staff of the Bacteriology Dept., Kansas State University, Manhattan, Kansas for their assistance and encouragement. I would like especially to thank Dr. A. F. Borg for his enduring interest and guidance throughout the course of the present work and to Dr. J. O. Harris for his suggestions and criticism of the manuscript.

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B. S., Taiwan Provincial College of Agriculture, 1959

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Bacteriology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1964

A single celled organism was isolated by A. F. Borg and J. L. Etchells from fermenting "overnight dill" pickles. Since this organism did not seem typical of known yeasts, this investigation was undertaken to study the morphological and cultural characteristics of the isolate.

The experimental methods and media used in the present studies were mainly based upon those recommended by L. J. Wickerham in his publication "Taxonomy of yeasts"(1951), and by J. Lodder and N. J. W. Kreger-van Rij in their book entitled "The Yeasts, a Taxonomic Study" (1952). Cell morphology, colony morphology and ascospores morphology and spore germination were observed. Physiological properties including the ability of the organism to ferment sugar, to assimilate carbon and nitrogen compounds, to grow at moderate osmotic pressure, to liquid gelatin, to produce starch, to produce esters and to grow at 37°C were tested. An effort was made to determine the nutritional requirements of the culture.

Cells of the organism were sausage-shaped, oval or elongate, vegetative reproduction by bipolar budding on a broad base. The cells measured 2.8-4.4 x 3.8-10.0 μ (mean 3.5 x 5.7 μ). In liquid cultures a sediment formed after 24 hours. Cells showed conjugation after spore germination.

Sporulation was found only on Herici's Vegetable Juice Agar slants among other media so far used. Spores were round and smooth, 1-2 spores per ascus with diameters ranging from 2.5 to 6.3 μ (mean diameter 4.5 μ). The germination of ascospores was not preceeded by conjugation.

Giant colonies were umbonate with smooth margins. No pseudomycelium formed on dalmau plates.

Fermentation and carbon assimilation patterns were the same : Glucose +, Maltose -, Galactose +, Saccharose -, and Lactose -.

An unknown growth factor which is a component of yeast extract and peptone was required in minute amounts.