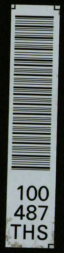

This is to certify that the

thesis entitled

Methanol Utilization by Yeasts

presented by

Ibrahim Saad Al-Mohizea

has been accepted towards fulfillment
of the requirements for

M.S. degree in Food Science

R. E. Stevenson

Major professor

Date 11/6/78

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MSU

METHANOL UTILIZATION BY YEASTS

By

Ibrahim Saad Al-Mohizea

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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MASTER OF SCIENCE

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ABSTRACT

METHANOL UTILIZATION BY YEASTS

By

Ibrahim Saad Al-Mohizea

The present study was carried out to investigate the characteristics of some methanol-grown yeasts. Six yeasts grown on methanol were isolated by means of a batch enrichment technique. One isolate, identified as a strain of Candida boidinii, was selected for further study; the isolate had temperature and pH growth optima of 28° C and 4 to 6, respectively. Although biotin enhanced growth, the growth proceeded slowly in vitamin-free medium.

The yeast grew in media containing up to 10% methanol. The duration of the lag phase was prolonged when methanol rather than glucose was used as the carbon source. The growth rate was retarded by high concentrations of methanol. The maximum cell dry weight (8.6 g/l) was obtained with 5% (v/v) methanol. Cells grown on from 1 to 4% methanol contained 41% protein and 4.8% nucleic acid (NA).

"IN THE NAME OF GOD,

MOST GRACIOUS, MOST MERCIFUL"

ACKNOWLEDGMENTS

The author would like to express his deep appreciation to his major professor, Dr. K. E. Stevenson, for his guidance, his very detailed and helpful reviews and for his constructive criticisms during the course of the laboratory work and during the preparation of this manuscript.

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Lastly, the author would like to pay tribute to his mother, his brother Mohammed, and his wife whose influence and encouragement were a source of much needed moral support.

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INTRODUCTION

Because of overpopulation and the scarcity of arable land, a shortage of food, particularly food high in protein, is anticipated, even in the so-called advanced nations (Han et al., 1971). This fact stimulated many scientists from all over the world to put forth a large effort exploring novel sources for food, especially protein-rich food. As a result of this interest, many approaches to the problem have been suggested, such as: leaf protein concentrate (Kohler and Knuckles, 1977), fish protein concentrate (Pigott, 1976) and single cell protein (SCP) (Mateles and Tannenbaum, 1968; Lipinsky and Litchfield, 1970, 1974; Tannenbaum and Wang, 1975; Litchfield, 1977). Of these products, SCP seems to be the more practical approach and the most promising source, at least in the near future (Lipinsky and Litchfield, 1974). Such sources of protein must fulfill a set of economic, nutritional, health and social criteria. The protein source has to be available and at a low cost, nutritionally valuable and free of any toxic compounds, and acceptable when used as food or as a food additive.

Many studies on SCP have been concerned with the

criteria cited above. Since raw material cost represents a major part of the total cost of SCP production (Wang, 1968), this aspect has been studied extensively. Investigations have shown the economic feasibility of utilizing many raw materials as possible substrates for SCP production (Kihlberg, 1972). One of these substrates, methanol has many advantages--enough to make it the substrate of choice for SCP production (Cooney and Levine, 1972). Methanol could be produced cheaply from natural gas by catalytic oxidation of methane, which would otherwise be flared, particularly in the Middle East. In a country like Saudi Arabia, which utilizes less than 20% of its daily 6×10^9 cubic feet production of natural gas with a methane content of 62.2% (Collins, 1976; Huval, 1976; Hatch and Matar, 1977), SCP production from methanol could potentially be produced in the near future.

It is for these reasons that I have been attracted to the study of methanol utilization by microorganisms. Yeasts have been selected as the microorganisms of choice in this study for their many advantages which will be discussed later.

The objective of this study was to review some work which has been done in this vital area, as well as to focus on the results of investigations which I have completed concerning growth of yeasts on methanol.

LITERATURE REVIEW

Historical Aspects

Yeasts were used by the ancient Egyptians more than 4,000 years ago (Phaff *et al.*, 1968). The yeast was used for leavening as much as 50 kinds of breads, by fermenting fruit juices, and for making foods palatable and nutritious (Hulse, 1974).

The possibility of growing yeast as a direct human food was first explored in Berlin by Delbruk *et al.* in 1910 (Scrimshaw, 1975). Thousands of tons of the yeast *Candida utilis*, formerly *Torula utilis*, were consumed in Germany during World War I and II as a meat substitute for different sectors of the population (Scrimshaw, 1975). Also, food yeast was incorporated into rations by the Russians and the Japanese during World War II (Scrimshaw, 1975). After that time and until the mid-60's, SCP did not appear as a likely source of food. The reason for that was the relatively rapid developments in improved methods of field crop production and pest control. In my opinion, these two were the major factors which contributed to the delay of development of SCP as a potential supplement to conventional protein sources. The situation has changed as

prices have risen due to the relative scarcity of protein sources and the growing demand which appeared as an inevitable result of the world population increase over a limited arable land. According to Senez (1972), the annual rate of population increase is about 2.5% in the so-called Third World and 1.2% in the so-called industrialized countries. With these rates of population increase, it is expected that world population will increase to seven billion in 2,000 A.D. It is for these reasons that many investigators over the world began to attach great significance to the production of SCP.

Methanol as a Novel Substrate for SCP Production

Since the raw material cost represents a major part of the SCP production, methanol has attracted many investigators as a substrate for SCP production. Many substrates have the potential to serve as substrates for the production of SCP, e.g. molasses (Bunker, 1963), agricultural wastes (Dunlap, 1975), cellulose (Han *et al.*, 1971), sauerkraut wastes (Hang *et al.*, 1972), food processing wastes (Church *et al.*, 1973), petroleum (Champagnat, 1965), ethanol (Goto *et al.*, 1973) and methanol (Cooney and Levine, 1972). Of these substrates, methanol seems to be the most promising due to the following features:

1. Methanol is completely miscible with water in all portions. This avoids three-phase problems and

the residual methanol is readily washed from cells (Cooney and Levine, 1972 and 1975; Gow et al., 1975).

2. It is readily available and can be produced at a low cost from a wide variety of hydrocarbon sources, ranging from methane to naphtha (Gow et al., 1975).
3. It is highly purified by distillation. Usually it is sold in a form which is 99.85% pure so the carryover of polycyclic aromatic compounds is minimized (Mehta and Pau, 1971).
4. It requires less oxygen for its metabolism by microorganisms and a lower cooling load than methane (Abbott and Clamen, 1973).
5. It is less explosive than methane-oxygen mixtures (Gow et al., 1975).
6. It is easy to handle and store (Cooney and Levine, 1972, 1975).
7. Its use by microorganisms is restricted which helps maintain a pure culture during fermentation (Cooney and Levine, 1975).

Yeast as the Microbe of Choice in the Production of SCP from Methanol

Yeasts have been selected for production of SCP for the following reasons:

1. Their ease of cultivation; they are not nutritionally fastidious.
2. Yeasts are not affected appreciably by changes in pH. Nearly all species can grow within a wide range of pH values (Phaff et al., 1968). This criterion helps yeasts to grow in different media and to overgrow bacteria, particularly at low pH.
3. Yeasts grow relatively rapidly.
4. They provide a high quality and quantity protein. The average crude protein content of yeast cells is usually from 45-50% of the dry weight.
5. They form a rich source of many of the B vitamins (Phaff et al., 1968; Bressani, 1968).
6. Recovery of yeast by either centrifugation or filtration is significantly easier and cheaper than recovery of bacteria due to the larger cell diameter of yeasts (Levine and Cooney, 1973).
7. Yeasts have been used for a long time as a food additive so they would be psychologically more acceptable than other microbes for human consumption.
8. Production of yeast is independent of climate, requires a small area and uses less water than most food processing plants (Kihlberg, 1972).

Methanol Utilization by Yeasts

Assimilation of methanol by bacteria is well known. In 1906, Sohngen reported for the first time that methanol was utilized by bacteria (Cooney and Levine, 1972). On the other hand, methanol was thought to be among the substrates which were not utilized by any yeast, particularly since Wickerham and Burton (1948) reported none of the strains they tested could utilize methanol. As a result, methanol has been neglected as a criterion in yeast classification.

Japanese investigators (Ogata *et al.*, 1969, 1970 a, b) reported for the first time that a strain of yeast, *Kloeckera sp.* No. 2201, could grow on methanol as the sole carbon and energy source. Some microbiologists insist that this yeast is a strain of *Candida boidinii* (Fukui *et al.*, 1975b). There have been relatively rapid increases in the amount of work done on methanol utilization by yeasts. This is clear from the increase in the number of yeasts reported to utilize methanol (Table 1) and also from research which has been done on the mechanism of methanol dissimilation.

Dissimilation of Methanol

Since the discovery of methanol utilization by yeasts, much interest has been devoted to the dissimilatory

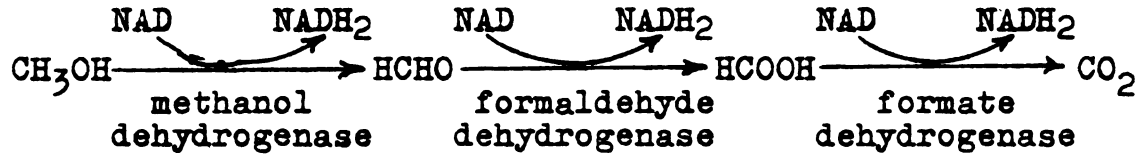
Table 1. Yeasts Identified as Methanol Utilizers.

Yeast	Species	Specific code	Reference
Candida	parapsilosis	IFO 0585	Okumura et al. (1970)
	N - 16		Fujii and Tonomura (1972)
	N - 17		
	methanolica		Oki et al. (1972)
	boidinii	CBS 2428	Hazeu et al. (1972)
	boidinii		Sahm and Wagner, (1972)
	alcomigas		Uragami et al. (1973)
Hansenula	wickerhamii	NRRL-YE-4743	Okumura et al. (1970)
	wickerhamii	CBS 4703	
	capsulata	CBS 1993	
	glucozyma	CBS 5766	
	henricii	CBS 5765	Hazeu et al. (1972)
	minuta	CBS 1708	
	nonfermentans	CBS 5764	
	philodendra	CBS 6075	
	polymorpha	CBS 4732	
	polymorpha	NRRL-Y-7560	Levine and Cooney (1973)
Kloeckera	sp. No. 2201		Ogata et al. (1969)
Pichia	halophila	CBS 2028	Okumura et al. (1970)
	pastoris	CBS 704	
	pastoris		Kato et al. (1974)
	pinus	CBS 744	Hazeu et al. (1972)
	trehalophila	CBS 5361	
Rhodotorula			Asano et al. (1972)
Saccharomyces	H-1		Fujii and Tonomura (1972)
	metha-nonfoams		Uragami et al. (1973)
Torulopsis	methanolovescens		Oki et al. (1972)
	glabrata		Asthana et al. (1971)
	nemodendra		
	nitratophila		Hazeu et al. (1972)
	pinus		
	methanofloat enukii		Uragami et al. (1973)
	methanophila		

Table 1.--Continued.

Yeast	Species	Specific code	Reference
	methanosorbosa		Yokote et al. (1974)
	methanodomercqii		Fujii and Tonomura (1972)
	M-1		

pathway of methanol. The pathway for dissimilation of methanol by bacteria has been studied extensively and proceeds as follows (Kaneda and Roxburgh, 1959; Large and Quayle, 1963; Lawrence et al., 1970; Quayle, 1972):



Tani et al. (1972 a, b) reported for the first time the isolation of a flavin-dependent alcohol oxidase from yeasts grown on methanol. They found no evidence for the presence of the hydrogen acceptor NAD. The enzyme was responsible for catalyzing the transformation of methanol to formaldehyde and peroxide.

Fujii and Tonomura (1972) studied the overall oxidation of methanol to carbon dioxide by yeast. In their work with Candida N-16, they have also been able to isolate an alcohol oxidase. They examined the enzyme activity in cells grown on glucose and methanol and found the enzyme was inducibly formed in response to the presence of methanol. Nevertheless, the enzyme had a broad specificity for

alcohols such as methanol, ethanol, n-propanol, n-butanol and n-amyl-alcohol. The same authors also found that when a pure form of alcohol oxidase was used to catalyze the oxidation of methanol, one mole of oxygen was consumed as one mole of formaldehyde was produced. In experiments using cell extracts, one mole of O_2 was consumed as two moles of formaldehyde were produced. This suggested the involvement of catalase in the oxidation of methanol to formaldehyde. Apparently, catalase reacted with hydrogen peroxide which was produced by the alcohol oxidase. Later, Roggenkamp et al. (1975) confirmed this assumption when they found that both enzymes were present in the same location, the peroxisome. Similar results were obtained by Fukui et al. (1975 a, b).

A formaldehyde dehydrogenase has been found to mediate the conversion of formaldehyde to formate, while a formate dehydrogenase was found to be responsible for the oxidation of formate to CO_2. Both enzymes require NAD (Fujii and Tonomura, 1972; Fukui, et al., 1975 a, b; Sahm et al., 1975).

Until recently, nothing was known about the location of assimilatory and dissimilatory enzyme systems involved in the methanol metabolism in yeast cells. However, van Dijken et al. (1975 a, b), Fukui et al. (1975 a, b), and Sahm et al. (1975) found, independently, that yeast cells grown on methanol, when examined by electron microscopy,

contained microbodies, while cells grown on glucose did not have or had very small microbodies. Roggenkamp et al. (1975) found that the microbodies of methanol-grown yeasts, characterized by their enzyme complement, were peroxisomes. Alcohol oxidase and catalase have been confirmed to be located in these structures (Roggenkamp et al. 1975).

On the other hand, formaldehyde dehydrogenase and formate dehydrogenase were found in the cytoplasmic fraction (Sahm et al., 1975; Fukui et al., 1975 a, b).

Nutritional Value of Methanol Grown Yeasts

Since SCP is intended to compete economically with protein from conventional resources, it has to have compatible characteristics. According to Cooney and Levine (1972), high protein, low nucleic acid, low carbohydrate, low lipid content and balanced content of amino acids are desired in sources of SCP. Despite the increase in the number of yeasts which can grow on methanol, little data are available on the composition of methanol-grown yeasts.

Data on the protein content of yeasts grown on methanol are within the range of protein content of yeast cells grown on conventional substrates. Protein quality usually is determined in terms of the amino acid profile. In general, the amino acid profiles of yeast cells grown on methanol (Table 2) compare favorably with the standards set

Table 2. Essential Amino Acids in Some Microorganisms Grown on Methanol.

Amino Acid	a FAO ref.	b *Kloeckera* sp N. 2201	c *Candida boidinii*	d *Hansenula polymorpha*	e TM 20	f *Ps. aeruginosa*
	g of amino acid per g of protein					
Isoleucine	4.2	5.1	3.78	5.1	3.7	3.57
Leucine	4.8	7.1	5.37	3.34	6.7	5.62
Lysine	4.2	7.5	6.01	8.1	5.3	4.88
Methionine	2.2	0.7	0.86	1.45	1.81	2.00
Phenyla-lanine	2.8	4.0	3.39	4.76	4.18	2.85
Threonine	2.8	5.1	4.42	5.17	4.52	3.81
Trypto-phane	1.4	--	--	--	--	0.74
Valine	4.2	5.3	4.59	6.21	5.85	4.34

[a]Jones (1974).

[b]Ogata *et al.* (1970).

[c]Sahm and Wagner (1972).

[d]Levine and Cooney (1973).

[e]Haggstrom (1969).

[f]Gow *et al.* (1975).

by the Food and Agricultural Organization of the United Nations (FAO) except for deficiencies in methionine and tryptophane (Jones, 1974). Single-cell protein is commonly deficient in methionine (Cooney *et al.*, 1975). However, bacteria have relatively balanced amino acid composition

(Haggstrom, 1969; Rosenzweig and Ushio, 1974; Dostalek and Molin, 1975).

Methanol-grown yeasts contain a fairly low content of nucleic acid (3-7%) compared with yeasts grown on other substrates. This is ascribed to the slow growth rate exhibited by yeast during growth on methanol. It is well known that the nucleic acid content of yeasts is proportional to the growth rate (Kihlberg, 1972).

MATERIALS AND METHODS

Microorganisms

Methanol-utilizing yeasts were isolated from natural sources, using enrichment techniques. In addition, sixty-seven yeast strains from the Food Microbiology Laboratory at Michigan State University were tested for their ability to assimilate methanol as the sole source of carbon and energy.

Isolation of Yeasts

Samples were obtained from various fruits (pineapples, apples, oranges, strawberries, cactus and pears), vegetables (tomatoes, lettuce and cabbage), and soils (fruit garden soil, vegetable garden soil and greenhouse soil). A suspension was prepared from each sample by blending with distilled, deionized water.

Enrichment techniques were used to isolate methanol-assimilating yeasts from these samples. The enrichment experiments were carried out in 500-ml Erlenmeyer flasks containing 100 ml of the isolation medium. The medium (YNB-methanol broth) contained 0.67% yeast nitrogen base (YNB)

(Difco Laboratories, Detroit, Michigan), 1% (v/v) methanol, and 0.5% KH_2PO_4. Yeast nitrogen base medium was prepared according to Difco manual (1953). Methanol was aseptically, added to the medium. The pH was, initially, adjusted to pH 4 by adding 1N HCl; pH readings were taken by a pH meter (Model 7, Corning Scientific Instruments, Medfield, Mass.).

A 2-ml quantity of each sample suspension was added to each flask containing YNB-methanol broth; the flasks were incubated for seven days in a gyrotary shaker (Model G-25, New Brunswick Scientific Co., New Brunswick, New Jersey) developing 200 rpm in the dark at 25° C. One ml of this culture was added to 100 ml of the same medium containing 0.1% glucose, and the flask was incubated for four days; one ml of this subculture was transferred to 100 ml of YNB-methanol broth and the culture incubated for three days. Two successive subcultures were made in YNB-methanol broth if visible growth was present. All the cultures were observed periodically using a light microscope. A culture which showed growth in the last subculture was presumed to be a methanol-assimilating yeast; this presumptive result was confirmed by streaking on a plate containing YNB-methanol plus 2% agar. Cultures which grew were subcultured on YNB-methanol agar plates in order to obtain pure cultures. Methanol assimilation was further confirmed by running control tests, using either the same isolate and the same

conditions except that methanol was not added, or using Candida steatolytica and C. utilis, which do not utilize methanol and adding methanol as the sole carbon source.

The methanol-utilizing isolates were maintained on YMPG agar slants (yeast extract, 0.3%; malt extract, 0.3%; peptone, 0.5%; glucose, 1.0%; agar, 2.0%; pH 5.5), and subjected to further studies.

Identification of the Isolates

The taxonomic studies were conducted in accordance with the methods described by Lodder (1970).

Screening Test

The yeasts were maintained either on Gorodkowa agar or YMPG-agar slants. Sixty-two yeast cultures were isolates from various natural sources, and they have not been classified. The remainder were:

Candida steatolytica Y-31
Candida utilis Y-39
Hansenula anomala Y-60
Pichia sp. Y-5
Rhodotorula glutinis

Screening test was made by growing the yeasts either on YNB-methanol agar plates or in YNB-methanol broth tubes. The tubes were incubated on a rotary drum (Model G-6, New

Brunswick Scientific Co., New Brunswick, New Jersey), operating at 6 rpm. Both plates and test tubes were incubated at room temperature (23° C) for 1-2 weeks.

Growth Studies

Yeasts were precultured in YMPG broth for 18 hours at 25° C. Then, cells were harvested by centrifugation and washed twice with sterile, distilled, deionized water. The cells were suspended in sterile, distilled, deionized water and used as inocula for growth experiments.

In all growth studies, except those to determine vitamin requirements, the medium was the same for isolation except that the methanol concentration and pH were varied. Vitamin-free yeast base (Difco) was used to study vitamin requirements and was prepared according to the manufacturer's instructions except that initial pH was adjusted to 5.5 instead of 4.5.

All growth experiments were carried out in 500-ml Erlenmeyer flasks containing 100 ml of media. The flasks were inoculated with a cell suspension and incubated at the appropriate temperatures in a New Brunswick gyrotary shaker, developing 200 rpm. The initial pH was adjusted in all growth experiments by adding 1N NaOH or 1N HCl.

Cell concentrations were measured with a spectrophotometer (Spectronic 20, Bauch and Lomb, Rochester, New York) at a wave length of 600 nm. A standard curve was

prepared by plotting optical density (O.D.) vs. cell dry weight (g/l).

Analytical Techniques

To determine dry weight, cells were harvested by centrifugation at 9000 x g for 10 min. and washed twice with deionized water. The washed cells were dried in pre-dried, tared aluminum weighing dishes for 16 hr. at 100° C. The weight of the dried pellets was used to determine the dry cell weight in g/l (AOAC, 1975; Levine and Cooney, 1973).

Protein was determined by two methods. A modified biuret method (Herbert et al., 1971) was used: 2-ml aliquots of washed cell suspension containing 1-5 mg of cell dry weight per ml broth were transferred to 5-ml centrifugation tubes; 1 ml of 3N NaOH was added to each tube; the tubes were placed in a boiling water bath for 5 min. and then cooled; 1 ml of 2.5% $CuSO_4$ was added to each tube, the tube was shaken thoroughly and allowed to stand at room temperature for 5 min. and centrifuged. A set of standard protein solutions (2, 4, 6 and 8 mg standard protein/tube) were prepared and treated in a similar manner. The optical densities of the supernatant from the cell suspensions and protein standards were read against the reagent blank in a spectrophotometer (Spectronic 20, Bauch and Lomb) using a wave length of 555 nm. A standard curve was drawn

by plotting O.D. vs protein concentration.

For the micro-Kjeldahl method, the following modifications of the AOAC (1975) procedure described by Shannon and Stevenson (1975a) was used: 4-ml aliquots of a digestion mixture containing 5g $CuSo_4$ $5H_2O$, 5g SeO_2 and 500 ml conc. H_2SO_4 was added to a micro-Kjeldahl flask containing approximately 0.1 g of dried cells. When the samples cleared during heating, the sides of the flask were rinsed with deionized water, 1 ml of 30% H_2O_2 was added and heating was continued for another hour.

The total nucleic acids content of the cells was estimated by the method described by Levine and Cooney (1973). According to this method, a 2-ml volume of culture broth, containing 1 g of cell dry weight/l was centrifuged, washed with 3 ml of cold, distilled water and centrifuged. The supernatant was saved and the pellets were extracted with 3 ml of 0.5 N perchloric acid (PCA) at 0° C for 30 min., centrifuged, and the supernatant was saved. The pellets were extracted with 0.5 N PCA at 70° C for 20 min., centrifuged, and the supernatant was saved. The absorbance at 260 nm of the wash and two PCA extracts were measured on a Beckman DB-G spectrophotometer (Beckman Inst., Fullerton, California). The total nucleic acid content was calculated by assuming an absorptivity of 32 ml/mg-cm for the nucleic acids as determined by Ohta et al., (1971) in a study with Candida utilis.

Experimental Design

Six strains of yeast were isolated which utilized methanol. These yeasts were subjected to preliminary investigations concerning growth rate, dry cell weight and average cell size when grown on a methanol-containing medium. Based on these preliminary investigations (data not shown), one strain, isolated from pineapple, was selected for further studies. Figure 1 outlines the main experiments which were conducted to characterize this isolate.

None of the sixty-seven yeast strains from the Food Microbiology Laboratory culture collection were able to utilize methanol. Therefore, further experiments were not conducted with these strains.

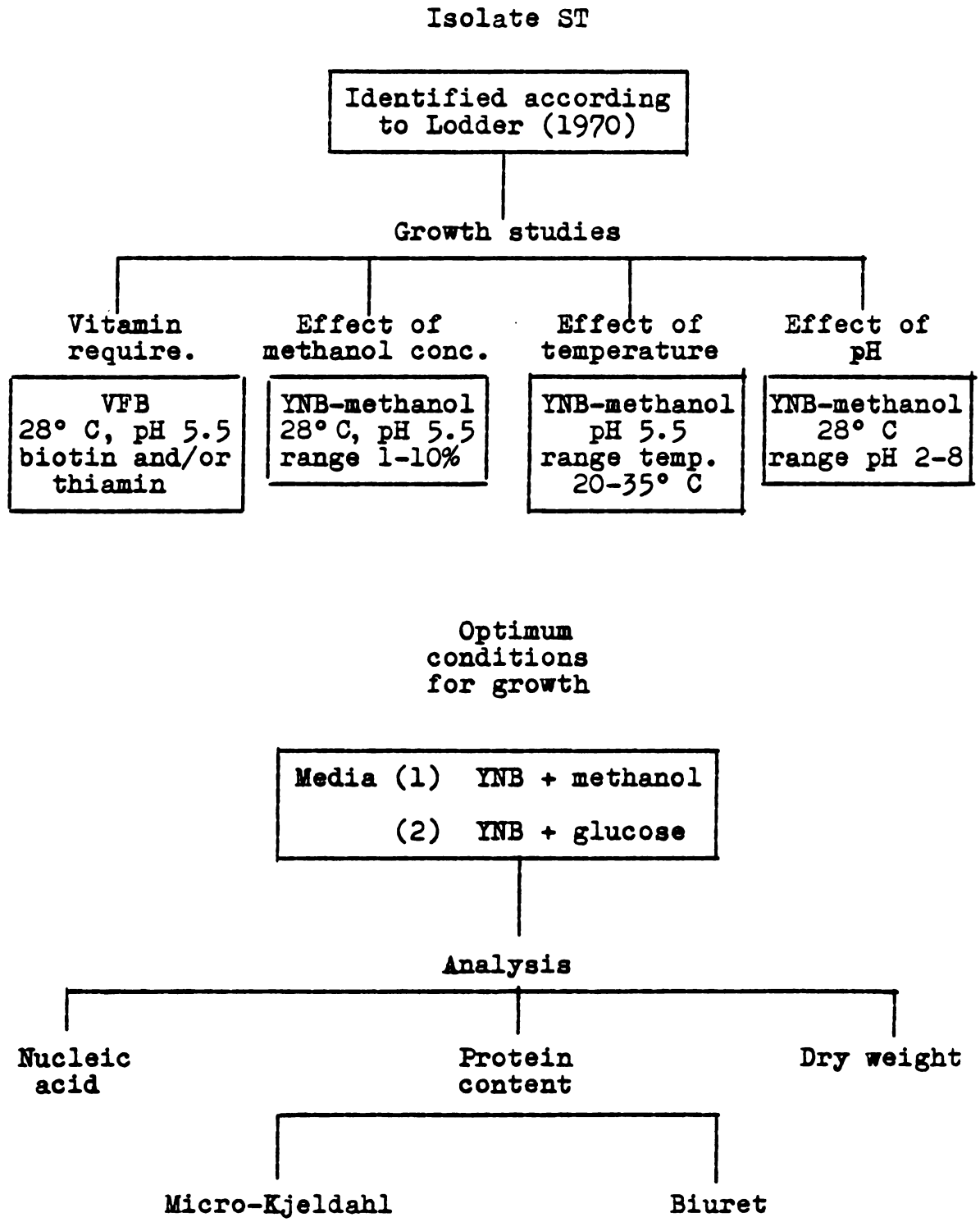


Figure 1. A schematic diagram of the main experiments.

RESULTS

Taxonomic Studies

Morphology

Growth at 25° C in YMPG broth: After incubation for three days, cells were oval to long oval (3-6) x (6-10)μ, and occurred singly or in pairs; a sediment was formed. A pellicle was formed after one day and became creeping after 2-3 days.

Growth at 25° C on YMPG agar: After 5-7 days, the streak culture was raised slightly, slightly wrinkled (umbilicate after 15 days), shiny, butyrous and had complete margins. The margins were fringed after 10 days.

Dalmau plate and slide culture on YMPG: A well developed pseudomycelium was formed after incubation for 2-3 days at 25° C. Blastospores were formed after 4-5 days. True mycelia were not observed.

Reproduction

Vegetative reproduction: Proceeds by multilateral budding.

Sporulation: Absent on both Gorodkowa and V-8 media after 1, 2, 3, 4 and 9 weeks.

Physiological Characteristics

Fermentation: Only glucose was fermented.

Assimilation of carbon source:

Glucose +
Galactose + (very weak)
Sorbose -
Sucrose -
Maltose -
Cellibiose -
Trehallose -
Lactose -
Melibiose -
Raffinose -
Inulin -
Soluble starch -
Xylose -
Arabinose -
Rhamnose -
Ethanol +
Mannitol +
Salicin -
Inositol -
Methanol +

Assimilation of $NaNO_3$: +

Vitamin requirements: None. However, growth was stimulated by biotin.

Sodium chloride tolerance: Up to 10%

Gelatin liquification: --

Optimum temperature: 28° C

Maximum temperature: 35° C

Optimum pH: 4-6

Source: Pineapple

According to the scheme of Lodder (1970), the characteristics of this yeast strain were in a good agreement with those of C. boidinii, thus it has been identified as C. boidinii ST.

Growth Characteristics

Effect of Temperature

A temperature of 28° C was optimum for growth (the highest specific growth rate) of C. boidinii ST (Figure 2). Using 1% methanol as the carbon source at pH 5.5 and 28° C, the specific growth rate was 0.165 hr^{-1} which corresponds to a generation time of 4.2 hours.

The maximum temperature for growth (after which the growth was no longer detected) was 35° C. The lag phase was rather prolonged while the maximum cell concentration was unaffected when the isolate was grown at 25° C, whereas at 30° C a prolonged lag phase and lower maximum cell concentration were observed as shown in Figure 2.

Effect of pH

The organism grew well over a wide range of pH (from 3.0 to 7.0) with an optimum pH for specific growth rate in the range from 4 to 6 (Figure 3). Slow growth was detected at both pH 2.5 and 7.5.

Vitamin Requirements

Biotin was found to enhance growth. A concentration of 20 µg/l was required to give the maximum growth-enhancing effect. However, growth proceeded slowly in vitamin-free broth (Figure 4).

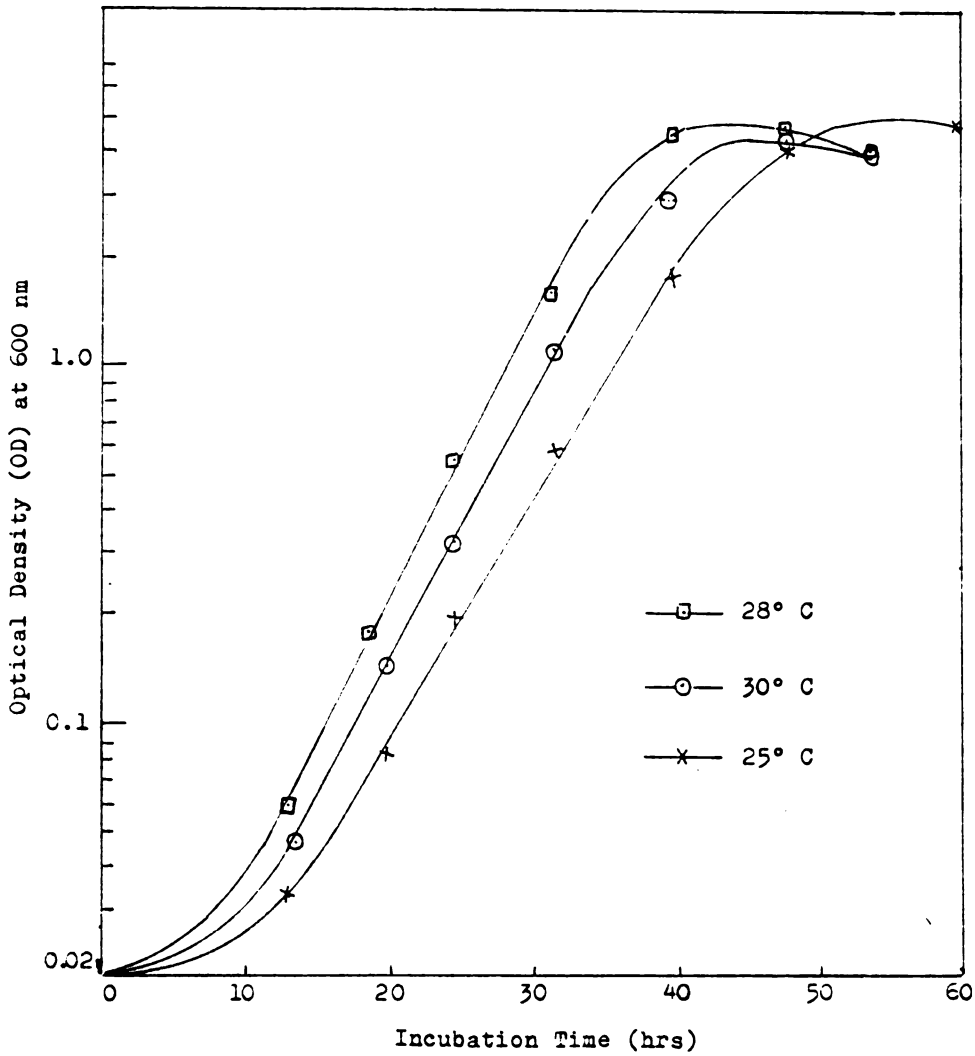


Figure 2. Effect of temperature on the growth of Candida boidinii 3T in a medium containing 1% (v/v) methanol at pH 5.5.

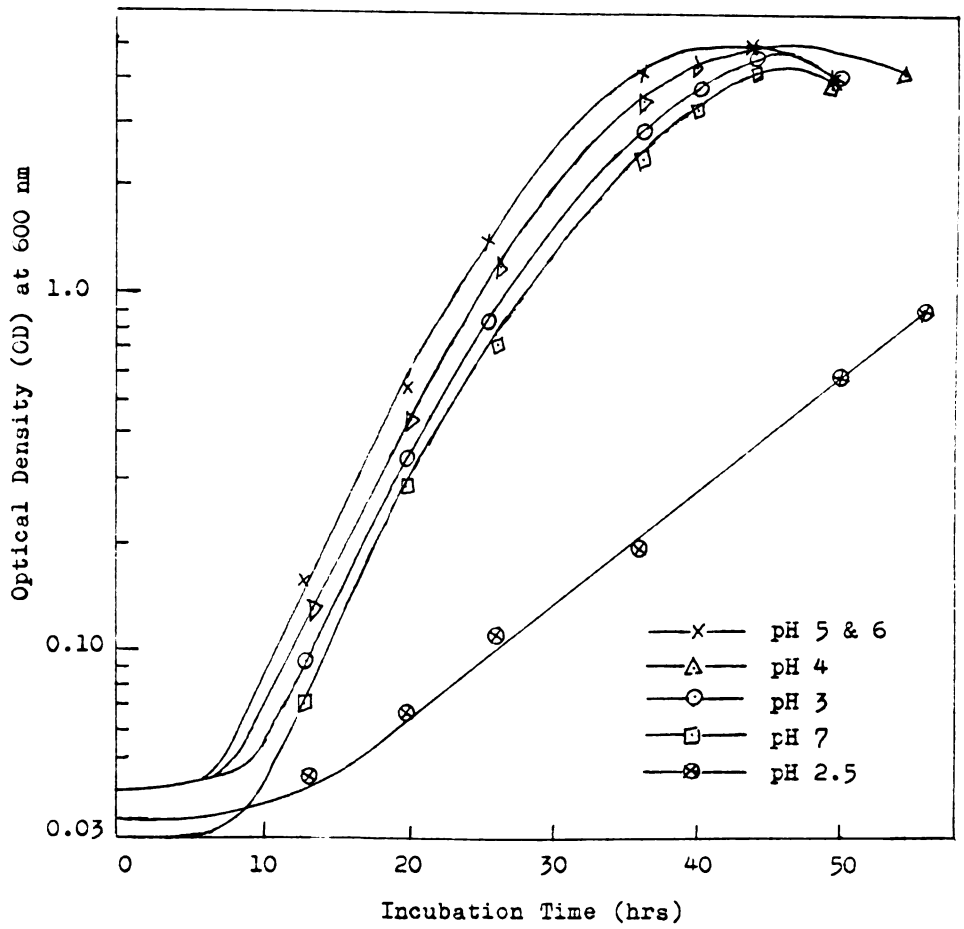


Figure 3. Effect of pH on growth of Candida boidinii ST at 28° C in a medium containing 1% (v/v) methanol.

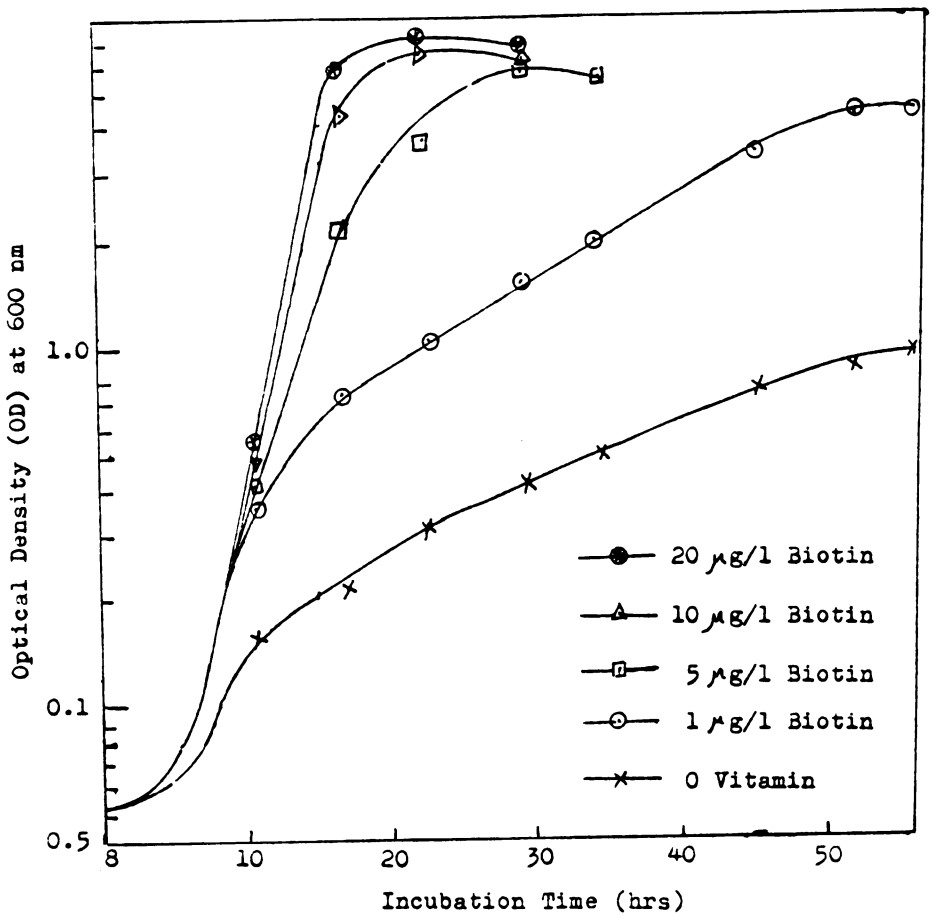


Figure 4. Effect of biotin on growth of Candida boidinii ST in a vitamin-free broth at 28° C and pH 5.5.

Effect of Methanol Concentration

A prolonged lag phase was associated with using methanol as the sole source of carbon; the extent of the lag phase was variable and, generally, increased with an increase in the concentration of methanol (Table 3).

The specific growth rate was appreciably affected by methanol concentration. The growth rate decreased as the concentration of methanol was increased above 0.5%. At $\leq$ 0.5% methanol, the highest specific growth rate was obtained (Table 3).

Cell concentration was expressed in terms of optical density. The optical density was found to have a linear relationship with cell dry weight (Figure 5). The final cell concentration increased as the initial methanol concentration increased, reaching its maximum value at a methanol concentration of approximately 5%. Increases in methanol concentrations above 5%, up to 10%, resulted in decreases in the final cell concentration. At methanol concentrations above 10%, growth was no longer detected.

The growth yield (g of cell dry weight/g of substrate) followed a different pattern. With initial methanol concentrations of $\leq$ 0.7%, the growth yield increased as the methanol concentration increased. At methanol concentrations above 0.7%, growth yield decreased as the initial methanol increased. The maximum growth yield of 0.39 was obtained with a methanol concentration of 0.7% (Table 3).

Table 3. The Growth Parameters of Candida boidinii ST in YNB + Methanol Broth Containing Various Methanol Concentrations.

Initial conc. of methanol (ml/l)	Lag phase duration (hrs)	Generation time (hrs)	Specific growth rate (hr^{-1})	Dry cell weight (g/l)	Growth yield (g D.C. wt./ g sub.)
3	6-8	3.6	0.193	.8	.33[a]
5	8-10	3.6	0.193	1.4	.35
7	8-10	3.6	0.193	2.1	0.38
10	8-10	4.2	0.165	2.7	0.34
20	11	4.8	0.144	4.1	0.26
30	13	6.3	0.110	5.8	0.24
40	19	7.8	0.090	7.1	0.22
50	b	b	b	8.6	0.21
60	b	b	b	8.1	0.17
70	b	b	b	7.5	0.13

[a]In calculating growth yield, methanol lost by evaporation was ignored.

[b]The growth curves were so complicated to allow calculating these parameters.

When glucose was substituted for methanol, there was a relatively short lag phase. In addition, the generation time was as short as one hour, and the final cell concentration was slightly higher than when methanol was used as the sole carbon source (Figure 6 and Table 4).

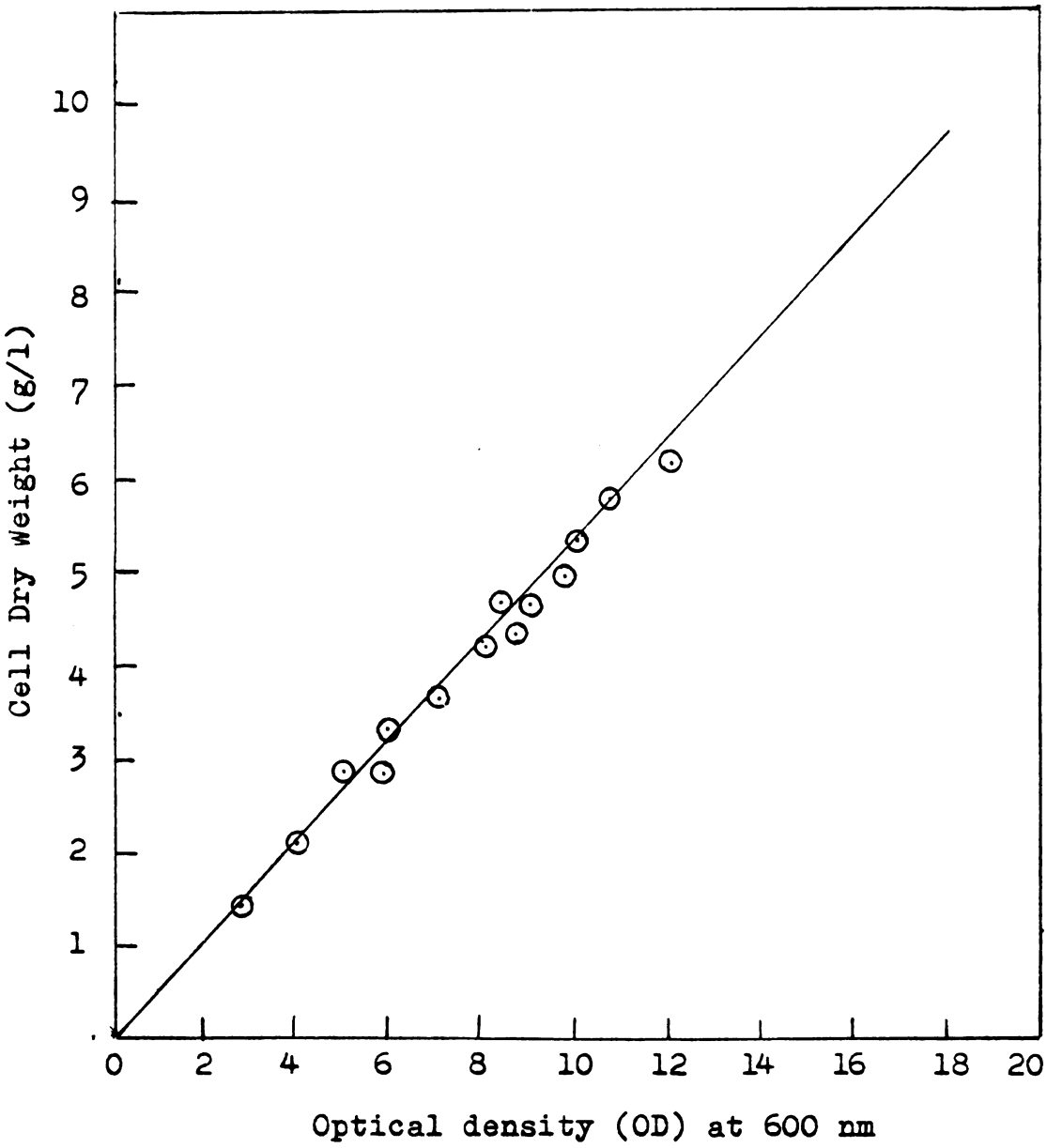


Figure 5. Standard curve showing the relationship between cell dry weight and optical density (OD) at 600 nm.

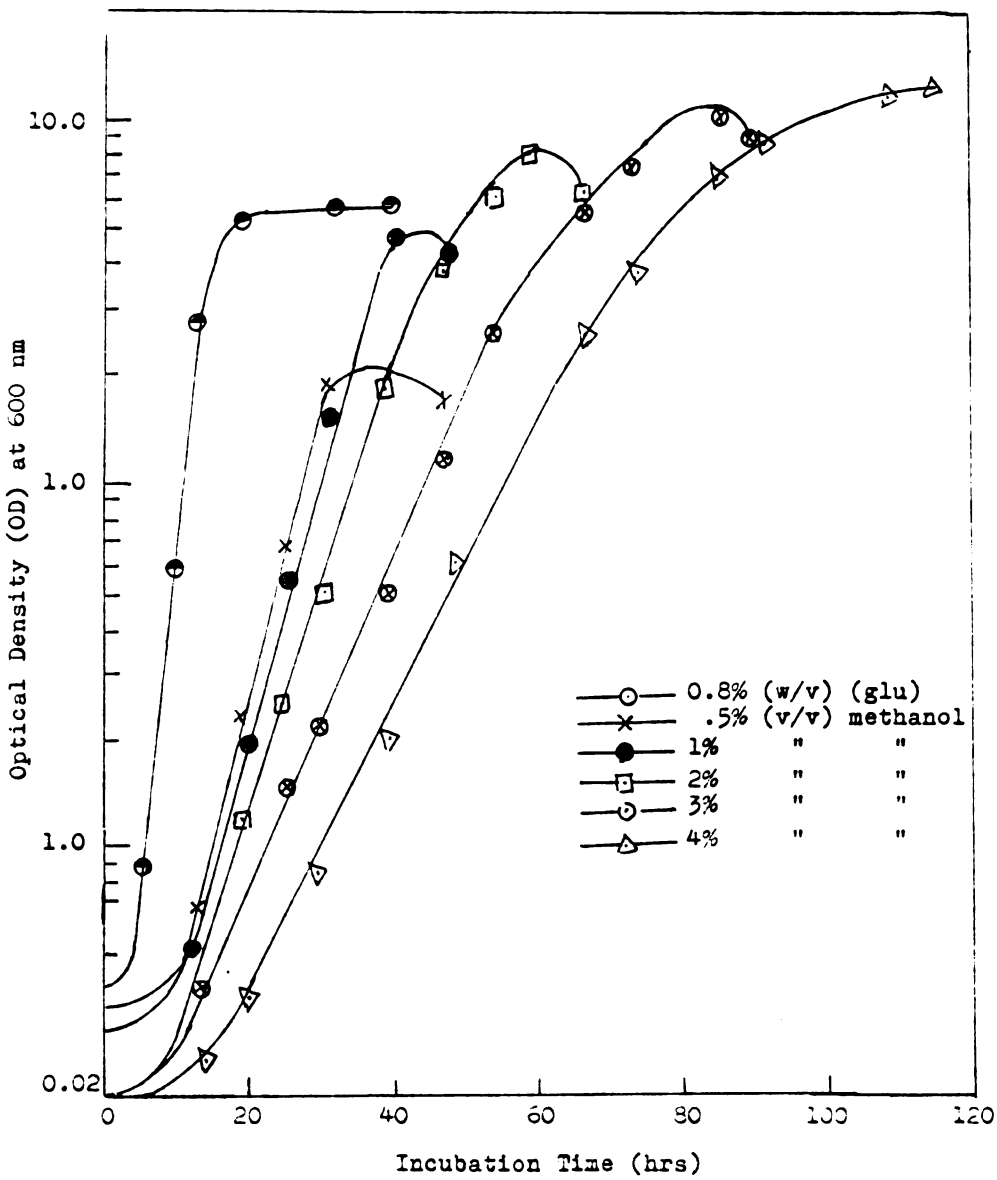


Figure 6. Effect of initial concentration of methanol on growth of Candida boidinii ST at pH 5.5 and a cultivation temperature of 28° C.

Table 4. Comparison of Growth Parameters of Candida boidinii ST Grown on Glucose or Methanol.

	Initial conc. of glu g/l	Lag phase duration (hrs)	Generation time (hrs)	Specific growth rate (hr^{-1})	Dry cell weight g/l	Growth yield (g D.C. wt/ g Sub.)
Glucose	8	4	1	.69	3.2	.40
Methanol	8	8 - 10	4.2	0.165	2.7	.34

Macromolecular Composition

Protein Content

The true protein content of C. boidinii ST grown on methanol or glucose as the sole sources of carbon was 41%, while the crude protein was 47-48% (Table 5). There was no significant difference in the protein contents of cells grown on methanol or glucose.

Table 5. Protein and Nucleic Acid Content of Cells of Candida boidinii ST Grown on Methanol and Glucose.

Carbon Source	Crude Protein %	True Protein %	% Nucleic Acid (NA)	% NA / Cr. Protein
Methanol	47	41	4.8	10.2
Glucose	48	41	5.2	10.8

Nucleic Acid Content

The total nucleic acid content was approximately 4.8% of dry cell weight when cells were grown on methanol. When the cells were grown on glucose, a slightly higher nucleic acid content (5.2% of dry cell weight) was obtained (Table 5).

DISCUSSION

Enrichment techniques with minimal media are widely used for isolating microorganisms from different natural sources. For this investigation, Difco yeast nitrogen base (YNB) was used in the minimal medium since it contained all the necessary growth factors which are required by the most fastidious yeasts, and in addition it contained ammonium sulfate which is utilized by all yeasts as a nitrogen source. Methanol was the only carbon source added to the initial culture while glucose (0.1%) was added to the first subculture to stimulate the growth of the methanol-utilizing yeasts recovered during the initial enrichment procedure. Methanol was the only carbon source in subsequent subcultures. By this mechanism, six strains of methanol-utilizing yeasts were isolated. The assimilation of methanol by the isolates was confirmed by streaking on YNB-methanol agar and by running control experiments using nonmethanol-utilizing yeasts.

According to Lodder (1970), methanol-utilizing yeasts are ubiquitous; they were isolated from soil, fruit, vegetables, flowers, insects, tree barks . . . etc. Furthermore, Hazeu et al. (1972) related the high incidences of isolation of methanol-utilizing yeasts from environments

rich in compounds such as lignin and pectin to the presence of methoxy compounds which might have enhancing effects on methanol-utilizing yeasts. In our study, our isolates came mostly from immature fruits and vegetables since these were the primary sources used. The results of this investigation support the above hypothesis by Hazeu et al. (1972) since the isolates came from samples that were rich in methoxy-containing substances.

The isolate ST was selected for further investigations since preliminary experiments indicated that this isolate could grow efficiently on a wide range of methanol concentration. The fermentation and assimilation patterns, and the morphological and physiological characteristics of this isolate were in good agreement with those of Candida boidinii. However, there were slight differences in the assimilation of galactose. vanUden and Buckley (1970) described C. boidinii as unable to utilize galactose and by having biotin as a growth-enhancing vitamin. Therefore, this slight difference was deemed insufficient to classify this isolate as a new species of Candida resembling C. boidinii, and instead it was identified as a new strain of C. boidinii. Ramerez in 1953 and Santamaria in 1958, however, reported the weak assimilation of galactose by some strains of C. boidinii (Lodder, 1970). Sahm and Wagner (1972) isolated a methanol-utilizing yeast which was identified as a strain of C. boidinii which required biotin for

growth. These slight differences among the strains within the species could be ascribed to the effect of natural habitat.

One of the purposes of this study was to establish the optimum conditions for growth and investigate the effect of methanol concentration on growth of our isolate C. boidinii ST.

Temperature is an important parameter which affects the growth of any microorganism. In general, any microorganism has three important temperatures; the minimum, the optimum and the maximum for growth. The last two temperatures are very important in SCP production. Candida boidinii had an optimum temperature (28° C) similar to that found for another strain of Candida boidinii by Sahm and Wagner (1972). A rather prolonged lag phase and a lower growth rate were observed when cells were grown at 25° C or 30° C. These results were expected since the two temperatures were below and above the optimum temperature of growth, respectively (Brock, 1974). The reason for the low final concentration of cells when grown at 30° C may be explained by the fact that methanol is a toxic substance. Hence, at a temperature higher than the optimum, the toxicity becomes more apparent (Chalfan and Mateles, 1971), or it might be ascribed to the increased cell maintenance (Cooney and Levine, 1975).

Recently, several investigators have described other methanol-utilizing yeasts. Oki et al. (1972) isolated a new

yeast, C. methanolica, which resembled C. boidinii; the optimum temperature of this yeast was in the range of 23° to 33° C. Tozuka (1975) also isolated a new methanol-utilizing yeast which was identified as closely resembling C. boidinii, and the optimum temperature of this yeast was 28.5° C and the maximum was 40° C. Of the yeasts, which can utilize methanol, only a few can grow well at elevated temperatures. In this regard, Levine and Cooney (1973) isolated a strain of H. polymorpha which can grow well at 37° C. The significance of this criterion becomes important when SCP production is carried out in tropical and subtropical areas (Mateles, 1968) where the cost of cooling comprises a major part of the total cost of production.

Temperature of cultivation also may play an important role in determining the nucleic acid content of the yeast. In a study conducted by Alroy and Tannenbaum (1973) on the influence of the cultivation temperature on the macromolecular composition of C. utilis, they have found that by increasing the cultivation temperature from 15 to 37.5° C, while other conditions were constant, the nucleic acid content decreased markedly (from 7.94% to 4.36%).

Yeasts grow readily over a wide range of pH. The isolate C. boidinii ST grew from pH 2.5 to 7.5. Although there was no significant difference in growth rate within the optimum pH (4-6) range, pH 5.5 was chosen arbitrarily for subsequent experiments in this investigation. Sahm and Wagner (1972) found that C. boidinii could grow over a wide

range of pH (2-9). The difference between the pH ranges for these two strains are slight and may be due to the effect of the natural habitat from which they were isolated or due to the differences in the cultural conditions. The optimum pH range of the isolate used in this investigation provides a mechanism for inhibition of many bacteria, especially when coupled with relatively high methanol concentrations. This inhibitory effect might be helpful in the production of SCP since these operations are usually conducted under nonaseptic conditions. The pH of the medium plays an important role in the protein and the amino acid content of the cells. According to Yamada *et al.* (1968) in a study with *Candida tropicalis* grown on hydrocarbons, the protein content declined with increases in pH of the cultivation medium from 4 to 8, while the content of essential amino acids reached a maximum at pH 6; the yield of dried cells reached a maximum at pH 7. However, the ability of methanol-grown yeasts to grow over a wide range of pH could be utilized efficiently to manipulate SCP production, i.e., production at a low pH could be utilized to obtain a high content of crude protein and/or to inhibit bacteria.

Yeasts vary widely in their vitamin requirements, i.e., some yeasts grow in vitamin-free media whereas other yeasts have an absolute requirement for certain vitamins. According to Phaff *et al.* (1968), biotin is the vitamin most commonly required by yeasts. In the present study, the

isolate ST required biotin for rapid growth, while growth proceeded slowly when grown in a vitamin-free medium. This result may be ascribed to the fact that some yeasts have the ability to synthesize slowly their vitamin requirements (Phaff et al., 1968).

Under optimum conditions, pH 5.5 and 28° C, the strain of C. boidinii used in this investigation grew over a wide range of initial methanol concentrations (up to 10%) in comparison to several other methanol-utilizing yeasts. Sahm and Wagner (1972) reported that another strain of C. boidinii was inhibited by 5% (v/v) methanol. Asthana et al. (1971) isolated a strain of the yeast T. glabrata which was inhibited by methanol concentrations higher than 3% (v/v). Yokote et al. (1974) isolated a yeast which was classified as T. methanosorbosa. This strain could grow in up to 5% (v/v) methanol and it grew well at 1% methanol. The value of the relatively high tolerance of yeasts to methanol is related to the fact that high concentrations of methanol could be used to produce SCP. This method can efficiently inhibit many extraneous organisms since methanol is toxic to many organisms, especially when present at relatively high concentrations.

The initial methanol concentration had an effect on lag phase, growth rate, growth yield and cell concentration. The lag phase was prolonged, even if the inocula were taken from cultures in the exponential phase. The extent to which the lag phase was prolonged was related to the

initial methanol concentration.

The lag phase may be regarded as a period of adaptation where specific enzymes (adaptive enzymes) are formed under the influence of the substrate (Monod, 1949). The reasons for the relatively long lag phase which was associated with methanol utilization could be ascribed to the fact that methanol is slightly inhibitory to the microorganism. This assumption was supported by the extension of lag phase in relation to the initial methanol concentration.

The specific growth rate (μ) was markedly affected by the initial methanol concentration. Growth was retarded by relatively low methanol concentrations. This result came out as a deviation of the Monod model of growth:

$$\mu = \mu_{max} \frac{C}{S + C}$$

where μ and μ_{max} are the specific, and maximum specific growth rate, respectively. C is the concentration of an essential nutrient. S is the concentration of this nutrient at which the rate is one-half the maximum. According to this equation, the specific growth rate is linearly proportional to the concentration of an essential nutrient at relatively low concentrations. After a certain concentration, the specific growth rate reaches the maximum, i.e., $\mu = \mu_{max}$ and is no longer affected by the concentration of the nutrient. In this study the specific growth rate decreased with higher concentrations of methanol as shown

in Figure 6. Similar results were reported in other studies. Levine and Cooney (1973) reported the inhibition of H. polymorpha DL-1 in shake flask experiments by methanol concentrations greater than 1% and in chemostat experiments by methanol concentrations greater than 0.5%. Asthana et al. (1971) in a study on the effect of methanol concentration on growth of T. glabrata found that growth was inhibited by high concentrations of methanol. However, in a study on the effects of methanol and other substrates on the growth of C. methanolica and T. methanolovescens, Goto et al. (1976) found that as long as growth occurred the specific growth rate remained unaffected by the methanol concentrations, i.e., the specific growth rate was independent of methanol concentration.

Cell concentration and the growth yield were also affected by the initial methanol concentration. The results indicate that cell concentration increased as the initial methanol concentration increased to 5% (v/v) where the maximum cell concentration was reached. Higher methanol concentrations resulted in a decline in cell concentration and, subsequently, in cell dry weight. This decline was probably due to the inhibitory effect of high methanol concentrations.

The growth yield (g cell dry weight/g substrate) is an important parameter, particularly where the commercial production of SCP is desired since it measures the

efficiency of converting the substrate to cells. The growth yield which was obtained for C. boidinii ST (0.39) was within the acceptable range for yeast. In comparison to other methanol-grown yeasts, this isolate had a higher growth yield than that obtained by Sahm and Wagner (1972) for another strain of Candida boidinii (0.29), and it is similar to the growth yield (0.37) obtained by Levine and Cooney (1973) for H. polymorpha. However, higher growth yields were reported by Asthana et al. (1971) for T. glabrata (0.40 - 0.57) and by Goto et al. (1976) for C. methanolica (0.545) and T. methanolovescence (0.512). The wide discrepancy in the reported growth yields may be due to various factors related to the organism itself or to the techniques applied in the cultivation, i.e., whether continuous or batch cultures were used, whether pH was controlled or not, and whether the culture was aerated or not.

Under normal conditions of cultivation, protein forms the major component of yeast cell. Yeast, however, have been reported to contain crude protein in the range of 40 - 60% of cell dry weight (Stewart, 1975). The protein content of our isolate (41% and 47%, true and crude protein, respectively) when grown on methanol falls within the acceptable range for yeasts grown on conventional substrates. There was no significant difference in the protein content between methanol-grown cells and glucose-grown cells when grown under the same conditions. In comparison to another methanol-grown yeast, Sahm and Wagner (1972) obtained only

about 35% crude protein for C. boidinii. Higher protein contents for methanol-grown yeasts were reported by other workers. Yokote et al. (1974) reported a crude protein content of T. methanosorbosa in the range of 47.4 - 51.1% while Levine and Cooney (1973) obtained 46% (biuret protein) for H. polymorpha, and Ogata et al. (1969 and 1970b) reported 45% crude protein for Kloeckera sp. no. 2201. So, regard to the protein content, C. boidinii ST compares well with other methanol-grown yeasts. On the other hand, working with other substrates, Reiser (1954) obtained up to 55% crude protein for C. utilis grown on potato starch wastes. However, Vananuvat and Kinsella (1975) reported 42% crude protein for S. fragilis when grown on crude lactose, and Shannon and Stevenson (1975b) obtained 44.3% crude protein for C. steatolytica when grown on brewery wastes. The wide variation in protein contents of yeasts was influenced by the strain, the cultivation conditions, substrate on which the yeasts were cultivated and finally by the analytical techniques.

The high nucleic acid content of single-cell protein (SCP) is one of the major problems which limits its use. In general, yeasts have a high nucleic acid content, varying from 8 to 25% of the crude protein content (Sinskey and Tannenbaum, 1975). The phenomenon of high nucleic acid content seems to be normal for organisms which have a relatively rapid growth (Scrimshaw, 1975). The nucleic acid content of C. boidinii ST was found to be in the lower

limit of the range reported for yeasts. This result is similar to those reported by other researchers working with methanol-grown yeasts. Yokote et al. (1974) reported total nucleic acids were 2.82 - 3.36% of cell dry weight for T. methanosorbosa, and Levine and Cooney (1973) found nucleic acids were 5 - 7% of cell dry weight of H. polymorpha. Ogata et al. (1969 and 1970b) reported a total nucleic acid content 5 - 7% for Kloeckera sp. no. 2201. On the other hand, yeasts grown on other substrates generally were reported to have higher nucleic acid contents. Vananuvat and Kinsella (1975) reported the total nucleic acid content of crude lactose-grown S. fragilis was 12% of the cell dry weight. Castro et al. (1971) reported total nucleic acids to be in the range of 7.5 - 9.0% of cell dry weight for C. utilis grown on glucose.

The relatively low nucleic acid content of methanol-grown yeasts probably is related to the fact that nucleic acid content is usually proportional to the growth rate. Knowing this and the fact that high concentrations of methanol retard the growth rate as shown previously and in other studies (Asthana et al., 1971; Cooney and Levine, 1972; Levine and Cooney, 1973), the low nucleic acid content can be ascribed to the relatively low growth rate of methanol-grown yeast. However, more research needs to be done on the effect of methanol concentration on NA content.

The nucleic acid content of SCP can be reduced to the extent that a relatively large quantity could be

ingested by humans without undue hazard. Several methods have been suggested for the reduction of the nucleic acid content of yeasts. These methods include: controlling the cell growth rate (Kihlberg, 1972), chemical extraction of the cell RNA (Kihlberg, 1972), disruption of cells (Dunell and Lilly, 1975), degradation of cell RNA by exogenous enzymes (Castro *et al.*, 1971), degradation of cell RNA by endogenous enzymes (Maul *et al.*, 1970) and, finally, attempts to produce mutants with low RNA contents (Sinskey and Tannenbaum, 1975).

SUMMARY AND CONCLUSIONS

In 1969, it was first reported that yeasts can utilize methanol as the sole carbon source. Since that time, a considerable amount of research has been done on this subject. The present study was undertaken to investigate the characteristics of methanol-grown yeasts which serve as a possible source of protein. Sixty-seven strains of yeast were tested for their ability to utilize methanol as the sole source of carbon. As a matter of fact, none of these strains could utilize methanol. However, six methanol-utilizing yeasts were isolated from natural sources by means of a batch enrichment technique. One isolate, identified as a strain of Candida boidinii, was selected for further study. The isolate had temperature and pH growth optima of 28° C and 4 to 6, respectively. Although biotin was found to enhance growth, the growth proceeded slowly on vitamin-free methanol. The yeast grew in media containing up to 10% methanol. The duration of the lag phase was prolonged when methanol, rather than glucose, was used as the sole carbon source. The growth rate was retarded by high concentration of methanol. The maximum cell dry weight (8.6 g/l) was obtained with 5% (v/v) methanol. The protein content of the isolate was 41% and

the total nucleic acid (NA) content was 4.8% of cell dry weight.

When glucose was substituted for methanol, a short lag phase and generation time were obtained. While there was no significant difference between methanol-grown cells and glucose-grown cells in the protein content, the glucose-grown cells had higher nucleic acid contents.

In conclusion, methanol-grown cells of C. boidinii ST exhibited relatively long lag phase, low growth rate and low nucleic acid content. The isolate appeared to be suitable for SCP production from methanol. Taking into account the cost of raw materials, methanol compares favorably with the other substrates which could be utilized for SCP production. However, before establishing SCP production from methanol, using the isolate C. boidinii ST, a continuous culture study would be necessary.

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ملخص البحث

إنتاج بروتين من خميرة منماة على بيئة تحتوي على غول الخشب

اعداد

ابراهيم سعد المهيزع

تعاني البشرية من نقص خطير في المواد الغذائية الغنية بالبروتين، الأمر الذي حدى بكثير من العلماء الى استحداث مصادر جديدة، علاوة على تنمية المصادر التقليدية للبروتين. استغلال الطاقة الهائلة لدى الأحياء الدقيقة (بكتيريا، فطريات وطحالب) يعتبر من المصادر الجديدة التي تبشر بخير، ذلك أنها تتكاثر بسرعة هائلة منتجة خلايا تحتوي على ٤٠-٦٠٪ بروتين (من المادة الجافة). هذا مع العلم انها تنمو على مواد خام رخيصة، لاسيما مصدر الكربون. غول الخشب (الكحول الميثايلي) من المواد التي أثبتت الدراسات المبدئية جدواها الاقتصادية - خاصة في المملكة العربية السعودية حيث توجد امكانيات هائلة لانتاجه من الغاز الطبيعي.

في هذا البحث تمت دراسة امكانية تنمية الخميرة على غول الخشب كمصدر للكربون والطاقة، بغرض انتاج البروتين. بدأ البحث باجراء دراسات اولية على بعض المحفوظات بمختبر ميكروبيولوجيا الأغذية بجامعة ولاية متشجان لمعرفة امكانية نموها على هذا الغول، الا انه ثبت ان ليس من بينهن خميرة واحدة استطاعت أن تنمو على غول الخشب. لهذا السبب تم عزل ست خمائر من مصادر مختلفة (تربة، خضار وفواكه). بعد اجراء دراسات أولية على هذه الخمائر تم انتخاب واحدة من اجل اجراء دراسات أطول عليها.

نتائج الدراسات التقسيمية لهذه الخميرة تشير الى أنها سلالة جديدة من كانديدا بويدينياي.
اما نتائج الدراسة حول الظروف المثلى للنمو فتشير الى انها تفضل درجة الحرارة ٢٨° م والاس الهيدروجيني من ٥ الى ٦، فيتامين البايوتين لازم للنمو السريع بتركيز ٢٠ ميكروجرام/لتر، الا انه لم يكن من بين عوامل النمو. هذا ولقد تبين ان هذه الخميرة بامكانها أن تنمو على بيئة تحتوي على غول خشب لغاية ١٠٪ (ح/ح). أما المحصول الأعظم (٨.٦ جم/لتر) فقد وجد عندما كان تركيز الغول ٥٪ (ح/ح). هذا وتجدر الاشارة الى أن الغول تسبب في اطالة دور السكون ... وابطاء معدل النمو النسبي، الأمر الذي لا يزال محيراً. من تحليل للخلايا تبين أنها تحتوي على ٤٧٪ بروتين (كلداهل) و ٤.٨٪ احماض نووية.

من ما سبق يتبين لنا أن غول الخشب مادة مثالية لانتاج البروتين أو حتى الأحماض الأمينية أو الفيتامينات والأنزيمات من الخميرة، وان المملكة العربية السعودية بانتاجها الضخم من الغاز الطبيعي (٦ × ١٠^{٩} م.م./يومي) وبمشاريعها الجبارة لاسالة الغاز الطبيعي (والذي يحتوي على ما يزيد على ٦٠٪ ميثان) بامكانها ان تكون منتجاً رئيسياً للبروتين الذي يمكن أن يدخل في تركيب الأعلاف المركزة للحيوانات - أو حتى للانسان.