

INDUCTION OF FERMENTATION IN THE
OBLIGATELY PSYCHROPHILIC YEAST,
LEUCOSPORIDIUM STOKESII

by

Donald Steven Lucas

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN MICROBIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my
direction by Donald Steven Lucas
entitled Induction of Fermentation in the Obligately
Psychrophilic Yeast, Leucosporidium stokesii
be accepted as fulfilling the dissertation requirement for the
degree of Doctor of Philosophy

Harmond G. Saindani
Dissertation Director

13 May '77
Date

As members of the Final Examination Committee, we certify
that we have read this dissertation and agree that it may be
presented for final defense.

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Harold J. Jeter

9 May '77
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16 May 1977

Final approval and acceptance of this dissertation is contingent
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SIGNED:

Donald S. Lucas

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ABSTRACT

The obligately psychrophilic yeast, Leucosporidium stokesii, was subjected to various substrates, temperatures, and atmospheric conditions in order to determine their effect on the induction of fermentation and fermentative enzymes. Induction of fermentation required oxygen limitation, a fermentable sugar, and temperatures at or below 20 C. Fermentation was induced in glucose, sucrose, and galactose weakly in maltose and xylose, but not in arabinose. In addition, although the rate of fermentation decreased with lower temperatures, the rate of induction of fermentation was inversely proportional to temperatures below 20 C.

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) are inducible enzymes which increased in activity corresponding to the induction of fermentation. Induction of PDC required oxygen limitation and temperatures at or below 20 C. ADH induction, on the other hand, was not sensitive to 21 C and in addition, was induced in ethanol medium aerobically indicating that it functions in the aerobic assimilation of ethanol. The stability of preformed PDC was also examined. The stability of this enzyme to 35 C heat was inversely proportional to the degree of purification of the enzyme. The possibility of stabilization due to substrate binding was examined and discussed as a possible mode of enzyme protection in whole cells. In addition reduced fermentation in whole cells heated at 35 C was due to impairment of

permeability of substrate rather than inactivation of fermentative enzymes as has been previously speculated.

INTRODUCTION

The process of fermentation was discovered long before man began recording history. For centuries the prevailing opinion regarding fermentation was that changes which occur in decomposing material were due primarily to the action of the oxygen in the air, and the process was thought to be purely a chemical one. In 1837 Latour and Schwann independently showed that yeasts are living organisms and that they cause the formation of alcohol by the fermentation of sugars as they grow in sugar solutions. Pasteur provided the world with convincing evidence of this phenomenon and defined fermentation as "la vie sans air".

Since the discoveries of Latour, Schwann and Pasteur, much work has been done concerning fermentation in yeast. However, in part because of their economic importance, practically all of the work thus far has concerned the industrial fermentative yeasts, specifically Saccharomyces cerevisiae. The concentration on this species has been so complete that authors often simply refer to "yeasts" when speaking of S. cerevisiae.

Since 1929 it has been known (14) that the decomposition of glucose by Saccharomyces occurs fermentatively via ethyl alcohol. This phenomenon has been called the "reverse Pasteur effect," and later the "Crabtree effect" (15). Investigating its occurrence in yeasts, De Deken (15) found the effect in 60 percent of the 25 species examined, belonging to 12 genera. The effect was present in members of

the genus Saccharomyces, but it was not established in members of the genus Candida.

The growth of S. cerevisiae in aerated glucose-containing media is considered to be glucose-ethanol diauxic growth. During the first exponential growth phase, glucose is primarily metabolized to ethanol. Maxon and Johnson (25) have reported that this process is not influenced by a strong increase in aeration. The second exponential growth phase, which follows an often distinct lag phase, begins when the sugar of the medium is almost used up. During this phase, growth is dependent on the degree of aeration and occurs at the expense of the previously formed ethanol.

It seems that in Saccharomyces there is a bias towards fermentation as the main mode of obtaining energy. Growth becomes oxidative only at the point when the carbon source finally limits the growth rate, providing oxygen is available. Therefore, oxygen is not the sole factor regulating aerobic metabolism, but the quantity and quality of the metabolizable energy source are also of value.

Respiratory mutants, known as petite mutants, have been produced by acriflavine treatment in several genera of Crabtree positive yeasts (8), but apparently this phenomenon does not occur in Crabtree negative yeasts or in obligately aerobic yeasts. Bulder (9) has proposed that such mutants in respiratory yeasts are lethal and thus not expressed. Kot et al. (22) have produced acriflavin-induced mutants of Candida albicans which are deficient in cytochromes aa_3

and b. These mutants, although impaired in respiratory functions, are capable of limited growth in glucose.

Henry, Hamiade-Deplus, and Nyns (19) have demonstrated that C. lipolytica is insensitive to cyanide poisoning when the cells are either in stationary phase or aerated in the resting state. Cyanide-insensitivity has also been demonstrated in the psychrophilic Candida species P-25, an obligate respiratory yeast (27). Since cyanide inhibits the function of the cytochrome system, it has been proposed that an alternate respiratory chain coexists with the normal respiratory chain in the mitochondria. The cyanide-insensitivity can be prevented by incubation in cyclohexamide, at 0 C, or under anaerobic conditions. These facts suggest that some form of induction involving new protein synthesis is responsible for the insensitivity.

Differences in respiratory and fermentative yeasts appear to be substantial. McClary and Bowers (26) have shown that glucose concentrations as low as 2 percent repress the formation of mitochondria as well as respiration in the highly fermentative yeasts, but these effects were not apparent in obligately aerobic yeasts. C. utilis, a petite negative yeast, will grow micro-aerobically (oxygen concentration less than one thousandth of that of saturated medium), and under these conditions the formation of unsaturated fatty acids is decreased (2, 7). Kellerman, Biggs, and Linnane (21) reported decreased synthesis of mitochondrial cytochromes at low but not high oxygen tensions, whereas Moss et al. (28) and Rickard et al. (34) have found increases in mitochondrial cytochromes under micro-aerobic conditions.

In addition, in step-down experiments from high oxygen tension to low oxygen tension there is a lag which results in fermentative metabolism. Rogers and Stewart (35) have shown that C. parapsilosis grows oxidatively under aerobic conditions and fermentatively in micro-aerobic (0.2 mM oxygen) continuous culture. Increases in glucose of up to 250 mM do not alter this pattern.

From the preceding data it appears that oxidation of glucose by respiratory yeasts is an oxygen dependent process which is unaffected by glucose concentration and which can be insensitive to cyanide. Fermentation of glucose to ethanol also seems to be dependent upon oxygen concentration but in a negative way. This appears to be a reverse of the fermentative process in Saccharomyces.

The process of transformation from oxidation to fermentation in respiratory yeasts is separated by a lag phase. During the lag phase the cells are apparently changing their metabolic processes, probably due to the synthesis of fermentative enzymes which have been de-repressed. The actual factors which initiate this de-repression have not been identified, but some investigators have suggested that oxygen may be involved in the biosynthetic pathways (28).

This study was initiated to investigate the induction of fermentation in the obligately psychrophilic yeast, Leucosporidium stokesii. The factors examined include the effect of the presence or absence of oxygen, fermentation of sugars other than glucose, and temperature within and above the range of growth for this organism. In

addition, assays for specific fermentative enzymes were performed and the thermal stability of these enzymes was examined.

MATERIALS AND METHODS

Cultivation Media

The media used in this investigation consisted of a rich complex medium, yeast extract and glucose (YAD), and a minimal chemically defined medium, yeast nitrogen base (YNB). YAD is prepared by combining 10.0 g glucose, 10.0 g yeast extract (Difco Laboratories), 1.0 g KH_2PO_4 , and 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in one liter of double distilled water. YNB is prepared in a 10X concentration by adding 6.7 g yeast nitrogen base (Difco Laboratories) to 100 ml double distilled water. This solution is filter sterilized and held at 4 C in an amber bottle until needed. The final medium is prepared by combining, under aseptic conditions, 1 volume YNB to 9 volumes of 67 mM KH_2PO_4 buffer containing 1.8 or 2.0 percent of a desired carbon source. When necessary, the media were solidified with 1.5 percent agar (Difco Laboratories).

Organism and Cultural Conditions

The organism used in this study was isolated in 1964 from an Antarctic snow sample by Sinclair and Stokes and was designated as Candida species, P-16 (39). More recently the organism was reclassified and named Leucosporidium stokesii (17). This yeast has the following cardinal growth temperatures: a maximum of 20 C, an optimum of 15 C, and a minimum of less than 0 C. Larkin and Stokes (23) have reported growth at -7 C for this organism, and since L. stokesii grows

optimally below 20 C, this yeast has been classified as an obligate psychrophile by Stokes (41).

The yeast was cultivated in the following manner. Cells were transferred from stock YAD slants into Erlenmeyer flasks containing YNB plus glucose broth and incubated aerobically at 15 C using a reciprocating water bath shaker (100 rpm, New Brunswick), cooled by a Frigid-Flow Water Bath Circulator (New Brunswick). A medium to flask ratio of 1:5 was used to insure adequate aeration. A minimum of three transfers were made from the mid-exponential phase of growth prior to experimentation. The final subculture was routinely taken from a culture grown to 100 Klett units¹ (Klett-Sommerson Photoelectric Colorimeter, model 800-3) using a red (no. 66) filter. An inoculum to medium ratio of 1:100 was used in all experiments.

In some experiments the cells were grown anaerobically. Anaerobic conditions were obtained by flushing the flasks and media with 99.99 percent dry nitrogen gas. The flushing was continued for 15 minutes while the flasks were agitated at the incubation temperature. After flushing, the flasks were sealed with a rubber stopper and parafilm. To compensate for CO₂ gas produced by fermentation, the flasks were vented with a rubber hose which extended into a water reservoir. A solution of ergosterol and Tween 80 in ethanol (1) was added to the medium in some cases.

Cultures used for anoxygenic induction of fermentation were grown to 50 to 100 Klett units and harvested by centrifugation

1. One Klett unit equals 0.002 O D units.

(3500 x g, 25 C), washed twice in double distilled water and re-suspended in an equal volume of 67 mM KH_2PO_4 containing 50 μM glucose per ml. The flasks were then purged with nitrogen for 15 minutes and sealed as described. The cultures were incubated for 4 to 24 hours at 15 C and cells were harvested for manometric or enzymatic assays.

In some experiments the organism was grown in a fermentor (New Brunswick, model M-19). The culture was agitated at 200 rpm and aerated at a rate of one volume of air per volume medium per minute. For anaerobic conditions the culture was purged with nitrogen gas for 15 minutes. The fermentor vessel was sealed during the incubation.

Induction studies were carried out in temperature controlled water baths. The baths were cooled by circulation of chilled 50 percent ethylene glycol through copper coils and temperature regulation (± 0.25 C) was achieved using thermoregulators (Bronwill, model 27559-E). Cell suspensions were purged with and incubated under an atmosphere of nitrogen gas.

Manometric Measurements

Oxidation and fermentation of substrates by cells suspended in water or 67 mM KH_2PO_4 at temperatures ranging from 0 C to 25 C were studied using standard manometric techniques (43). A Gilson Differential Respirometer model GR-14 was used. Each flask contained 1.9 or 2.0 ml of cell suspension (6.1 mg cell dry weight per ml) in the main compartment. For respiration, 0.2 ml of 30 percent KOH plus an accordian-folded strip of Whatman #1 filter paper was placed in the center well. For studies involving respiration or fermentation of

pyruvate, 0.1 ml of 0.2 N succinic acid was placed in the main compartment in order to lower the pH for pyruvic acid assimilation. Substrate (25 to 500 μ M in 0.5 ml) was tipped in from the side arm following temperature equilibration to give a total volume of 2.5 ml. The gas phase was air for oxidation and nitrogen gas (99.99 percent) for fermentation. Inhibitors including iodoacetic acid, cyclohexamide, and parahydroxymercurobenzoate were added in a concentration of 1.0 mM per ml in some experiments. Data are expressed in most cases as oxygen or carbon dioxide quotients, i.e., QO_2 equals microliters O_2 taken up/mg cell dry weight/hour and QCO_2 equals microliters CO_2 given off/mg cell dry weight/hour. In all cases the quotients are corrected for endogenous metabolism by subtracting the respective endogenous values.

Chemical Assays

The concentrations of glucose, ethanol and ammonia in growth media were analyzed in some experiments. Duplicate 5.0 ml samples were filtered through glass fiber filters (Whatman, GFC 2.4 cm) and the filtrates were frozen at -20 C in stoppered vials until used. Glucose levels were determined by the glucose oxidase-peroxidase method (6). Ethanol levels were measured using alcohol dehydrogenase (13). Ammonia was determined using the modified Conway micro-diffusion dish method (29).

Enzyme Assays

Pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) were assayed in either crude cell free extracts or partially purified

cell free extracts (12, 20). Crude cell free extracts were prepared in the following manner. Cultures were harvested by centrifugation (3500 x g, 4 C), washed twice with cold distilled water, and resuspended in 33 mM KH_2PO_4 to 0.05 volumes of the original culture. The cells were disrupted using a pre-cooled (4 C) French Pressure Cell at 9,000 to 12,000 psi. One pass at this pressure was sufficient to disrupt 85 to 95 percent of the cells as verified by direct counts using a Neubauer counting chamber. The disrupted cell suspension was centrifuged (13,000 x g, 0 C) for 15 minutes to remove remaining whole cells and cell walls. The supernatant was recentrifuged (27,000 x g, 0 C) for 30 minutes to remove membranes and mitochondria. The remaining supernatant was used for the crude cell free extract and was held in an ice bath at 0 C until used (not longer than one hour) or frozen at -20 C.

Partially purified PDC was prepared as follows. Sufficient $(\text{NH}_4)_2\text{SO}_4$ was added to the crude extract held at 0 C to produce a 25 percent solution. This solution was held at 0 C for 30 minutes with agitation followed by centrifugation (27,000 x g, 0 C). The precipitate was discarded. Additional $(\text{NH}_4)_2\text{SO}_4$ was added to the supernate to produce a 55 percent solution. This mixture was agitated at 0 C for one hour. The resulting precipitate contained 95 percent of the PDC activity and was harvested by centrifugation (27,000 x g, 0 C) and frozen at -20 C. The enzyme is stable for several months at this temperature and can be used as necessary by resuspending the precipitate in 33 mM KH_2PO_4 .

Levels of the enzymes are expressed in units of specific activity (units of enzyme per mg protein) as described by Colowick and Kaplan (12). Protein levels were determined by the method of Lowry et al. (24).

Radioisotope Methods

Aerobic or anaerobic uptake of either glucose-u- ^{14}C or glucose-1- ^{14}C by resting cells was carried out in Warburg flasks. Each flask contained 2.0 ml of a cell suspension in 67 mM KH_2PO_4 and 0.5 ml of 500 mM glucose containing 0.1 μC of ^{14}C -glucose (47.8 $\mu\text{C}/\mu\text{M}$). At various time intervals 0.5 ml samples were removed from the flasks and cells were harvested by filtration (Gelman, 0.45 μ). The cells were washed twice with 2.0 ml of double distilled water and placed into 10 ml of scintillation counting fluid. The scintillation fluid contained 4.0 g BBOT (2,5-bis (5-t-butylbenzoxazolyl (2'))thiophrene), 80.0 g naphthalene, 400 ml ethylene monomethyl ether, and 600 ml toluene. To compensate for $^{14}\text{CO}_2$ evolution, the center wells of some flasks contained 0.5 ml ethanolamine to trap CO_2 . One-tenth ml of the ethanolamine was added to 10 ml of scintillation fluid and $^{14}\text{CO}_2$ radioactivity was measured using a Packard Tricarb Liquid Scintillation spectrophotometer, model 3320.

Statistical Evaluation of Data

Data were subjected to various distribution statistics and to an analysis of variance to assess their significance based on the 0.01 level of probability. Analyses and computations were done using a

program kindly provided by Dr. L. M. Kelley, Department of Microbiology, The University of Arizona, and a Control Data Computer Model 6400, University of Arizona Computer Center.

RESULTS

Analysis of Growth Characteristics Under Various Growth Conditions

The aerobic growth curve for L. stokesii in YNB supplemented with 10 mM glucose at 15 C is shown in Fig. 1. The generation time of the organism under these cultural conditions was approximately 8 hours. The maximum stationary phase was reached in 48 hours. Cessation of growth at this time is accounted for by disappearance of glucose from the culture medium. Ethanol was not detected in the culture filtrates.

The aerobic growth curve of L. stokesii cultivated in YNB supplemented with 100 mM glucose at 15 C is shown in Fig. 2. The generation time of the organism at this glucose concentration was approximately 8 hours. As indicated 10 mM of glucose provided sufficient carbon and energy to raise the population density to 100 Klett units as noted above. The utilization of an additional 10 mM of glucose increased the density by another 100 Klett units to 200. At this point the rate of growth decreased and the culture shifted to a fermentative state as indicated by the production of ethanol. Both the rate of glucose utilization and the rate of growth decreased during fermentation. The level of ethanol increased until all of the glucose was consumed. At this point the ethanol level reached a maximum and began to decrease until it too was consumed. A final maximum stationary population of approximately 400 Klett units was attained.

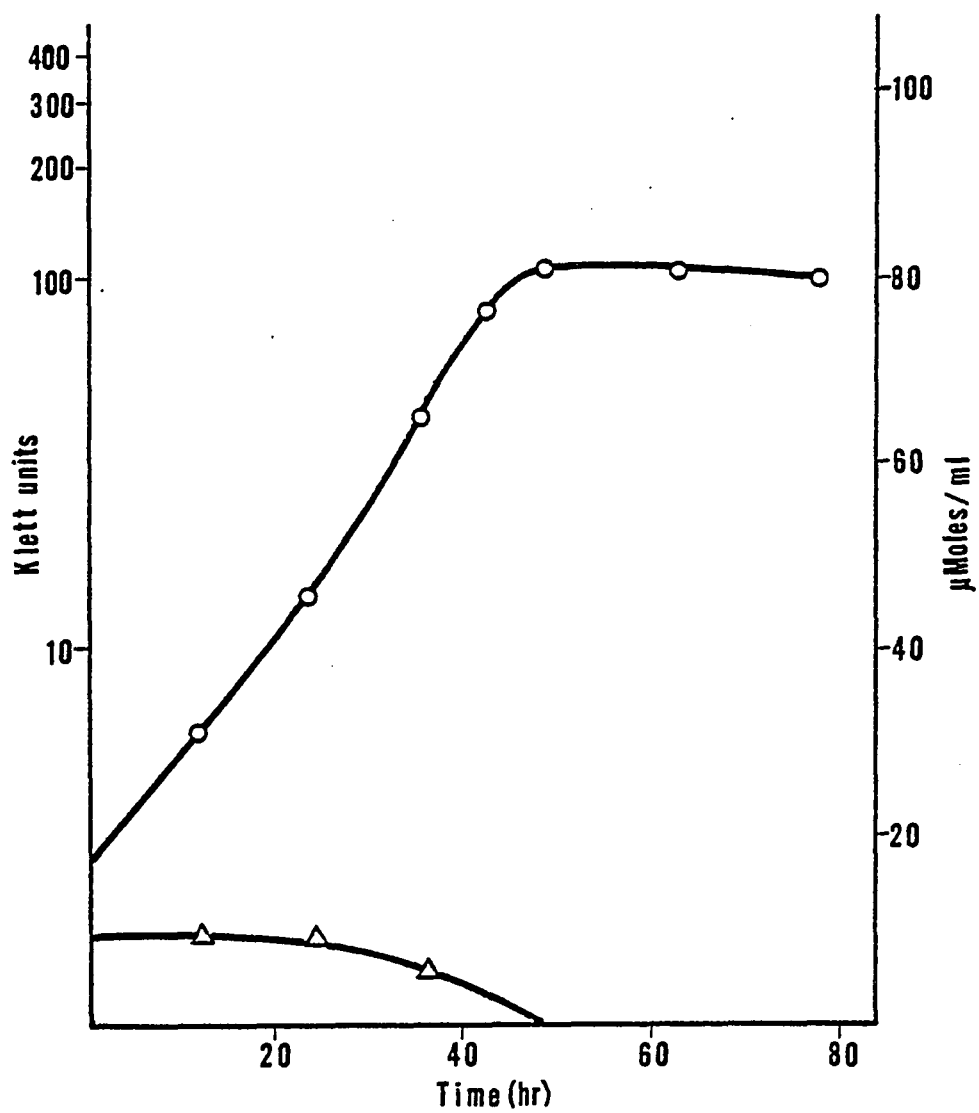


Fig. 1. Aerobic Growth of *L. stokesii* in YNB Plus 10 μ M/ml Glucose at 15 C

Kletts (O); Glucose (Δ)

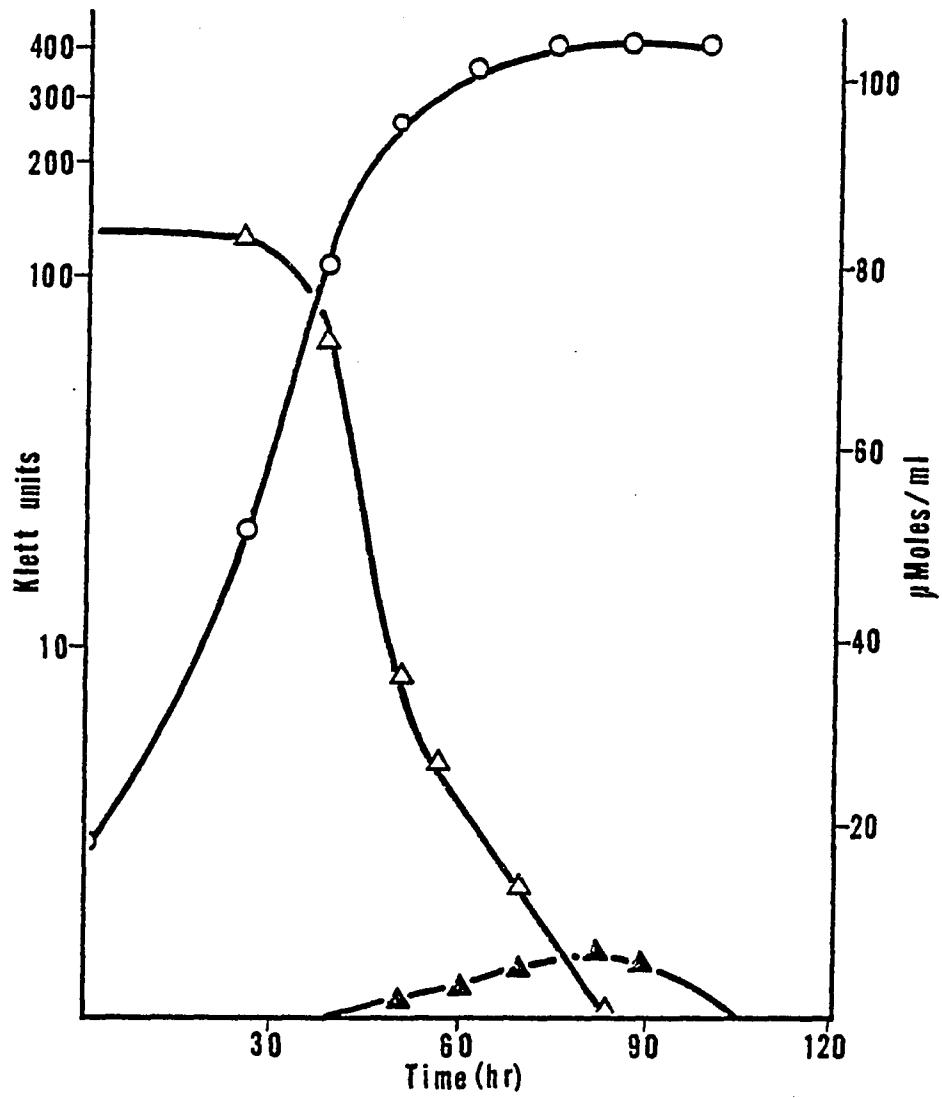


Fig. 2. Aerobic Growth of *L. stokesii* in YNB Plus 100 μ M/ml Glucose at 15 C

Kletts (O); Glucose (Δ); Ethanol (\blacktriangle)

The anerobic growth curve of L. stokesii in YNB plus 10 mM glucose at 15 C is shown in Fig. 3. The generation time of the organism under these conditions was approximately 18 hours, over two times longer than the aerobic generation time. The population reached a maximum of 25 Klett units after 66 hours incubation. Twenty mM of ethanol were produced but not assimilated since all of the ethanol produced remained in the medium even after 150 hours incubation.

The anaerobic growth curve of L. stokesii in YNB plus 100 mM glucose at 15 C is shown in Fig. 4. The generation time of the organism under these conditions was also approximately 18 hours. A maximum stationary phase (125 Klett units) was reached at 148 hours incubation. One hundred ten mM of ethanol were produced. Ethanol, first detected in the medium after 36 hours incubation, reached a maximum at 160 hours incubation. At this time glucose was depleted from the medium. Ethanol was not assimilated by the organism and remained in the medium.

Ergosterol and Tween 80 have been shown to stimulate anaerobic growth of fermentative yeasts (1). Because of this anaerobic growth curves of L. stokesii in YNB plus 100 mM glucose and YNB plus 100 mM glucose supplemented with ergosterol and Tween 80 were ascertained. Results are shown in Fig. 5. Although the maximum stationary populations attained in both cultures were approximately the same, the rates of growth differed slightly.

From the data presented in Fig. 2, L. stokesii appears to shift to a fermentative type metabolism in media containing excess glucose even under aerobic conditions. In order to test the possibility that

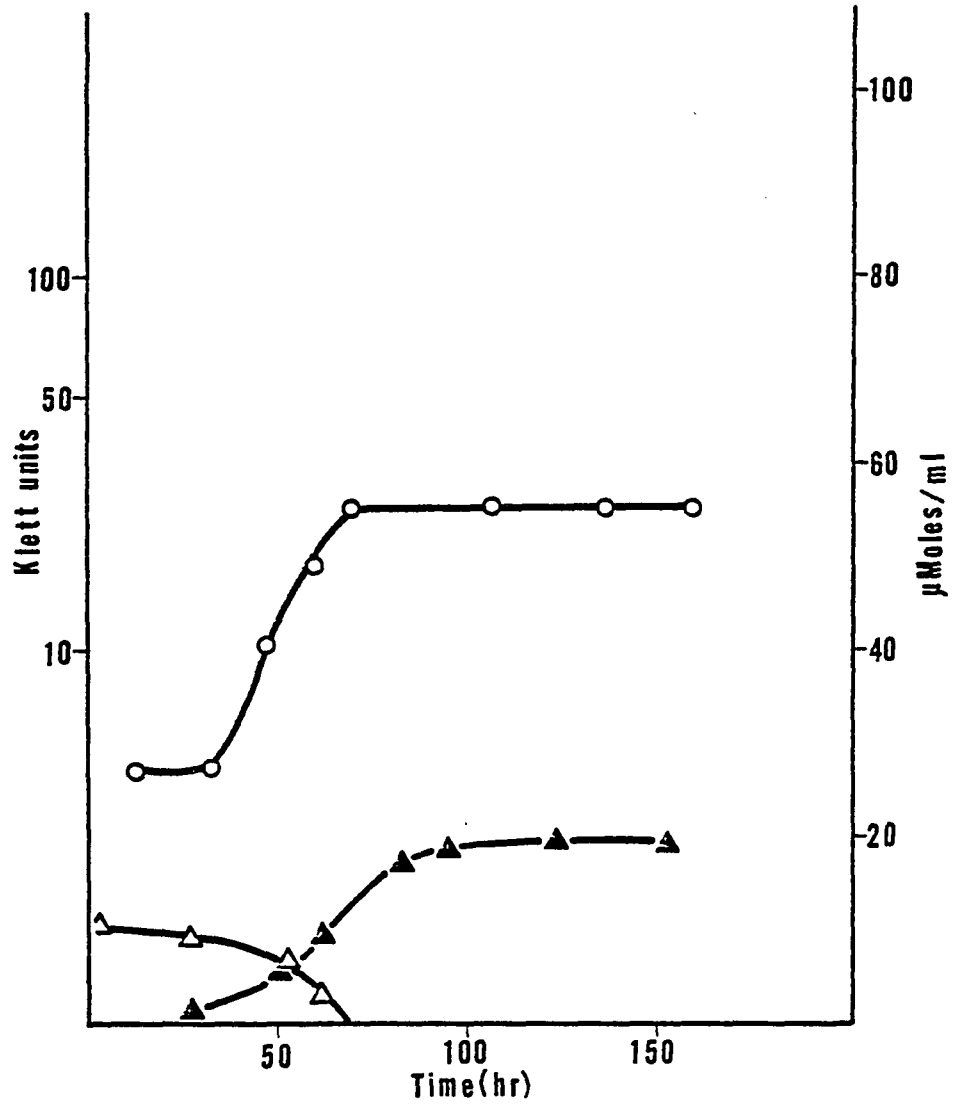


Fig. 3. Anaerobic Growth of *L. stokesii* in YNB Plus 10 μM/ml Glucose at 15 C

Kletts (O); Glucose (Δ); Ethanol (▲)

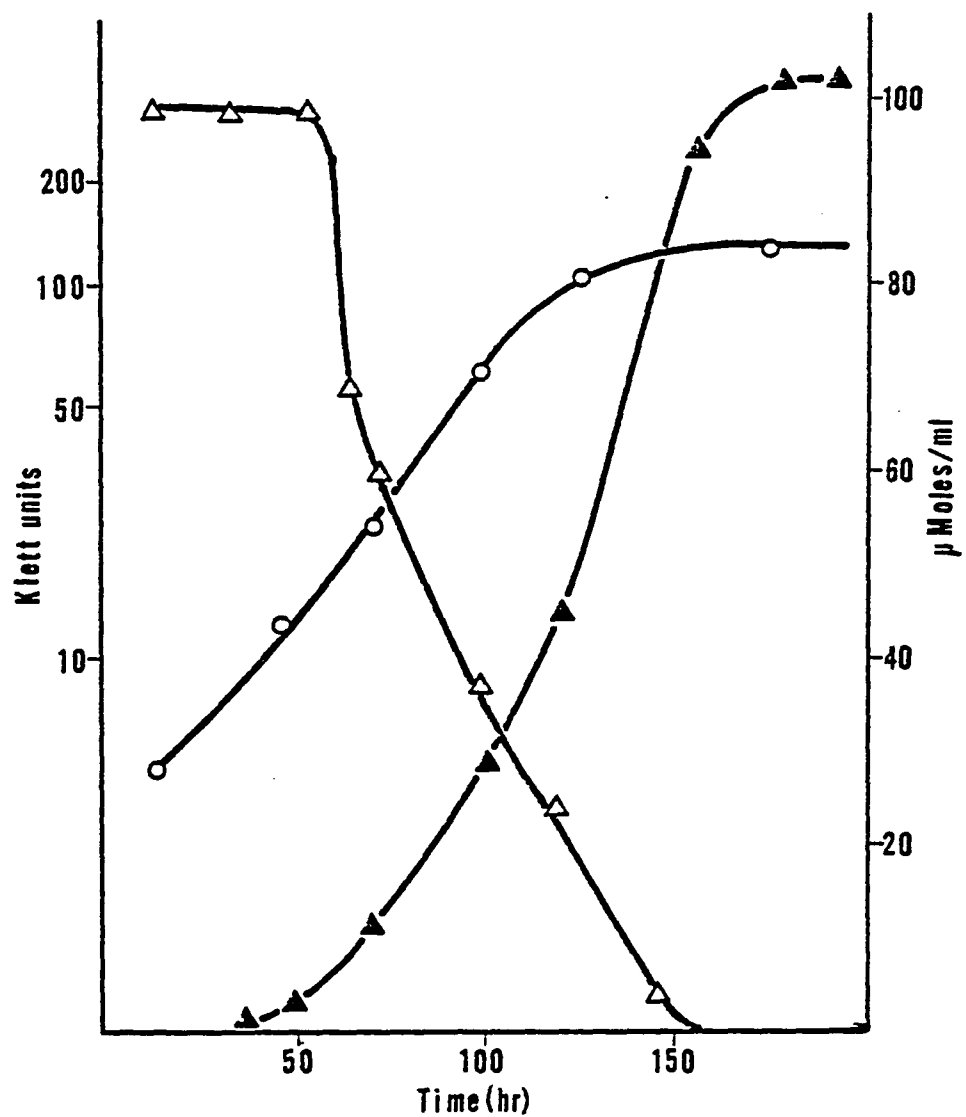


Fig. 4. Anaerobic Growth of *L. stokesii* in YNB Plus 100 μM/ml Glucose at 15°C

Kletts (O); Glucose (Δ); Ethanol (▲)

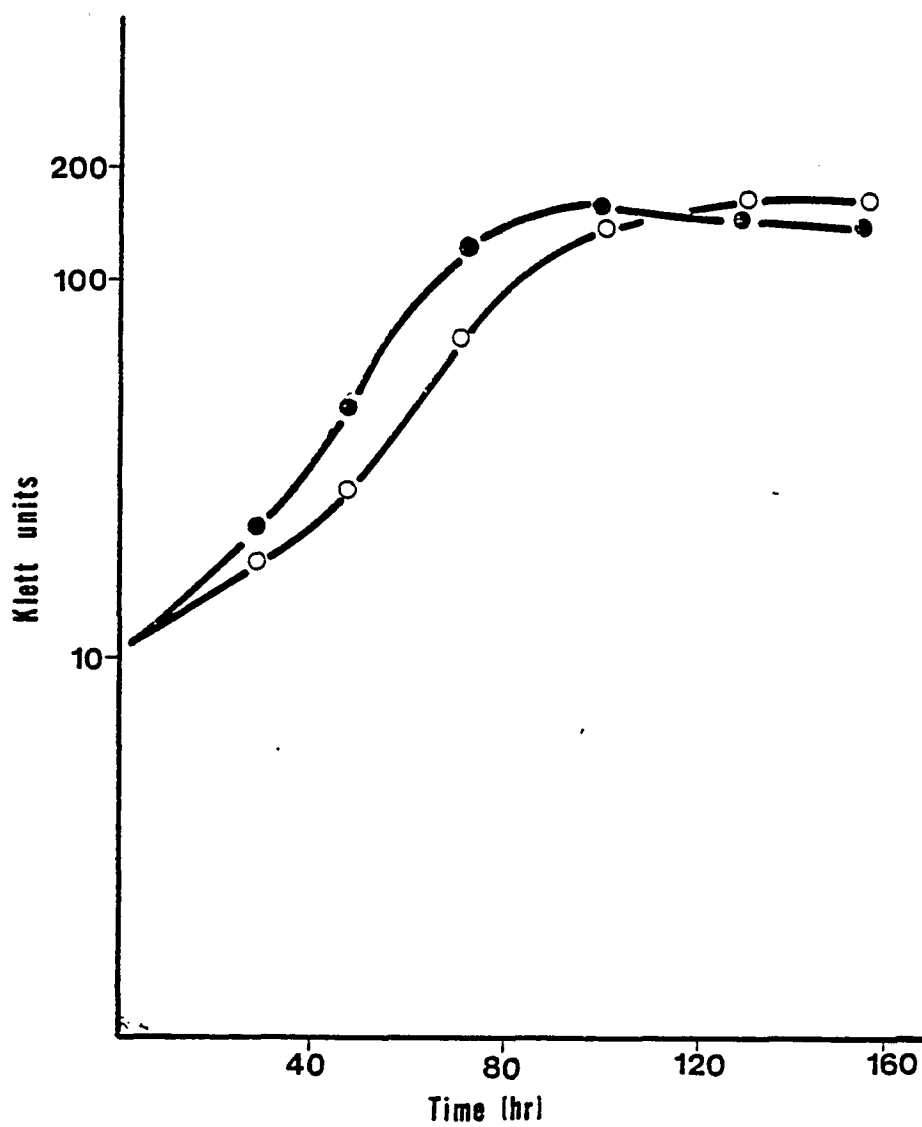


Fig. 5. Anaerobic Growth at 15 C of *L. stokesii* in YNB Plus Glucose and in YNB Plus Glucose Supplemented with Ergosterol and Tween 80

Glucose (●); Tween 80 (○)

limiting oxygen induced this shift from oxidative to fermentative metabolism, the following experiment was carried out. L. stokesii was cultivated in YNB supplemented with 100 mM glucose in a fermentor under controlled aeration. Data are shown in Fig. 6. During the first 60 hours of aerobic cultivation the generation time was approximately 8 hours and glucose utilization was at a maximum. At this time aeration was discontinued for 30 hours (as indicated by the arrows). During this period both the rates of growth and glucose utilization decreased. Ethanol was produced and reached a maximum at 90 hours. At this point aeration was resumed and the growth rate increased as did the rate of glucose utilization. The accumulated ethanol was assimilated and completely disappeared from the medium at 110 hours incubation.

Effects of Incubation Conditions on the Induction of Fermentation

Effect of Media and Aeration on Induction of Fermentation

In order to examine the conditions which affect the oxidative and fermentative capabilities of the yeast, the QO_2 and QCO_2 of resting cells were studied after four hour anaerobic incubation periods in various media. The results of these experiments are shown in Table 1. The yeast retained the ability to oxidize both glucose and pyruvate after incubation under all of the conditions tested. Fermentation of both glucose and pyruvate, represented by QCO_2 , was demonstrated only after the cells had been incubated under anaerobic conditions in the presence of glucose. For example, when cells were incubated

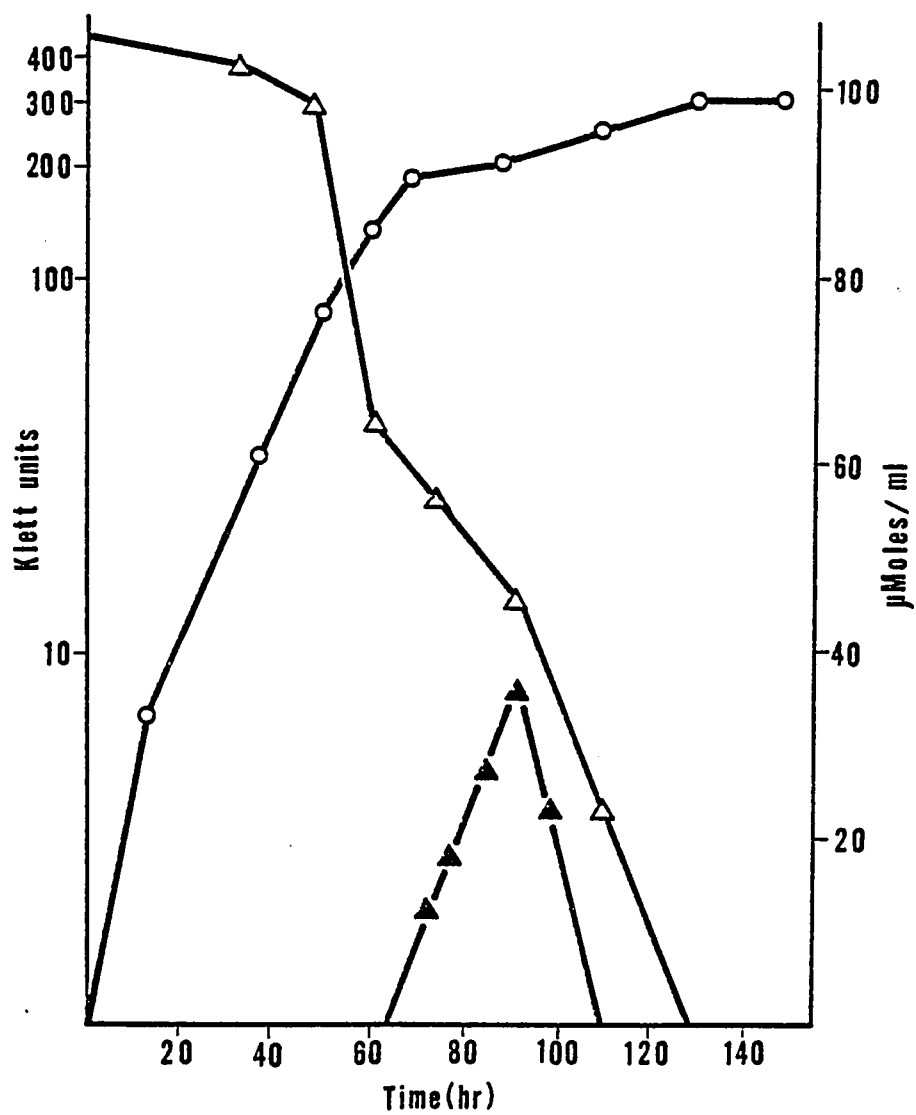


Fig. 6. Growth of *L. stokesii* in a Fermentor with Controlled Aeration at 15 C

Kletts (O); Glucose (Δ); Ethanol (▲)

Table 1. Anaerobic Induction of Fermentation in Different Media at 15 C

Conditions of Induction	QO_2		QCO_2	
	Glucose	Pyruvate	Glucose	Pyruvate
Aerobic: Glucose + YNB	50	51	0	0
Anaerobic: 67 mM KH_2PO_4	53	47	0	0
Anaerobic: YNB only	65	37	0	0
Anaerobic: Glucose	57	45	72	57
Anaerobic: Glucose + YNB	80	44	114	85

aerobically in complete medium or anaerobically in media lacking glucose, induction did not occur. Furthermore it is evident that some compound of YNB, possibly the nitrogen source, stimulated fermentative activity.

In order to further examine the process of induction, the yeast was treated with three metabolic inhibitors during anaerobic induction in buffered glucose at 15 C. The results of these experiments are shown in Table 2. Untreated, T_0 cells did not ferment glucose or pyruvate. After four hours anaerobic incubation glucose supplemented cultures showed a typical induction of fermentation. Cells treated with actidione, a selective inhibitor of cytoplasmic protein synthesis (36) were not induced to ferment. Respiration by these cells, however, was not affected. Iodoacetic acid, an inhibitor of glycolysis, reduced by approximately one-half both the oxidation and fermentation of glucose. On the other hand, the oxidation and fermentation of pyruvate was only slightly decreased. Parahydroxymercuribenzoate, which affects enzyme function, completely destroyed the cells ability to respire or ferment either glucose or pyruvate.

Kinetics of Induction of Fermentation

In order to examine the kinetics of the induction process, early exponential phase cells from an aerobically growing culture were harvested, washed twice, and resuspended in phosphate buffer to a density of 450 Klett units. These cells were placed in Warburg flasks and purged with nitrogen on a Gilson Differential Respirometer at 15 C. Glucose was added from the side arm to give a final concentration of

Table 2. Effect of Inhibitors on Induction of Glucose Fermentation at 15 C

Cell Treatment	QO ₂		QCO ₂	
	Glucose	Pyruvate	Glucose	Pyruvate
None (control)	56	62	0	0
Anaerobic: Glucose	48	47	45	56
Anaerobic: Glucose Plus Actidione ^a	80	61	0	0
Anaerobic: Glucose ^a Plus Iodoacetate ^a	28	35	22	45
Anaerobic: ^b Glucose Plus PCMB ^b	0	0	0	0

^aAdded in a concentration of 1 mM/ml.

^bParahydroxymercuribenzoate added in a concentration of 1 mM/ml.

100 $\mu\text{M}/\text{ml}$ and, the amount of carbon dioxide gas evolved was recorded at 15 minute intervals for 5 to 6 hours. The results of this process are shown in Fig. 7. During the first hour of incubation no carbon dioxide was produced. During the second and third hours carbon dioxide evolution increased and reached a maximum rate after 3 hours incubation.

Since the density of the resting cell cultures used in the manometric experiments exceeded the density of normal stationary phase cultures, it was of interest to determine what, if any, difference concentration could play in the induction process. Three cell concentrations were used in an induction experiment (100, 200, and 450 Klett units). The results of this experiment are shown in Table 3. The QCO_2 of the three concentrations varied by only 5 percent. Moreover, the difference between the cultures at 100 Klett units and 450 Klett units was only 2 percent.

Table 3. Effect of Cell Concentration on Anaerobic Induction of Glucose Fermentation at 15 C

Cell Concentration	QCO_2
100 Kletts	10.0
200 Kletts	9.5
450 Kletts	9.8

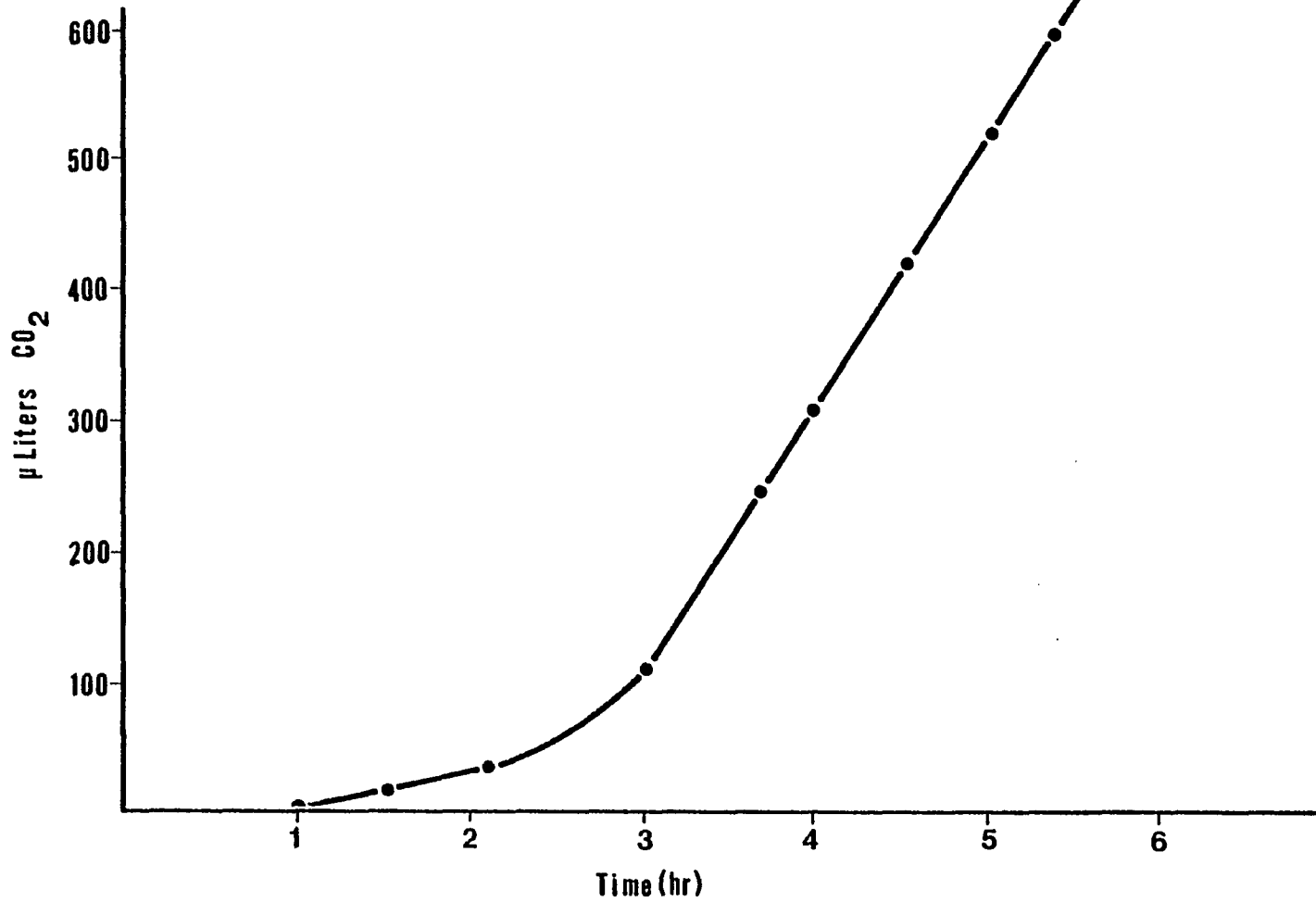


Fig. 7. Kinetics of Induction of Fermentation of *L. stokesii* in 100 μM/ml Glucose

Anaerobic Uptake of ^{14}C -Glucose at 25 C

Although L. stokesii grows optimally at 15 C and has a maximum growth temperature of 20 C, fermentation of glucose by resting cells is optimal at 25 C (39). In order to provide evidence that L. stokesii can assimilate glucose at an enhanced rate at 25 C the following experiment was carried out. Resting cells were incubated anaerobically in the presence of glucose-1- ^{14}C at 15 C and 25 C respectively. Samples were removed at various time intervals, cells were collected by filtration, and radioactivity was determined. As can be seen from data in Fig. 8, the rate of uptake of ^{14}C -glucose was greater at 25 C than at 15 C. Moreover, three times more glucose was assimilated at 25 C than at 15 C.

Induction and Fermentation of Sugars Other than Glucose

Cultures anaerobically induced in glucose were also tested for their ability to ferment other sugars. Aerobically grown early exponential phase cells were anaerobically induced in glucose for four hours, harvested, washed twice, and finally suspended in phosphate buffer to a density of 450 Klett units. Fermentation of sucrose, galactose, maltose, xylose, and arabinose was measured. The results of this experiment are shown in Table 4. As indicated, glucose-induced cells ferment glucose readily. Of the five additional sugars tested, only two, sucrose and galactose were fermented. Maltose, xylose, and arabinose were not fermented. In addition, the yeast was tested for fermentation of each sugar after anaerobic induction in that sugar and

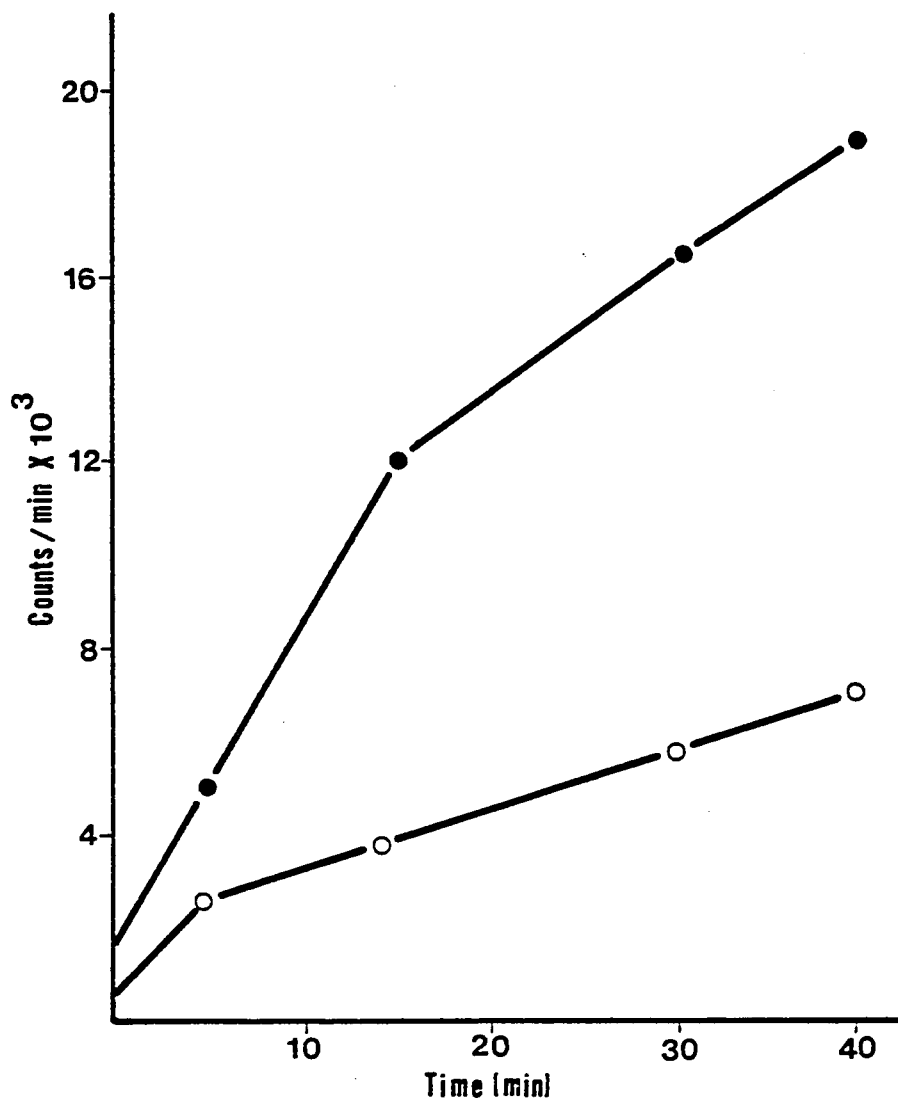


Fig. 8. Anaerobic Uptake of Glucose-1-¹⁴C by *L. stokesii* at 15 C and 25 C

15 c (○); 25 c (●)

Table 4. Fermentation of Various Sugars by Glucose-Induced
L. stokesii

Substrate Fermented	QCO ₂
Glucose	32
Sucrose	8
Galactose	19
Maltose	0
Xylose	0
Arabinose	0

for fermentation of glucose. The results of these experiments are shown in Table 5. Sucrose-induced cells were capable of fermenting glucose as well as sucrose. Similar results were obtained with galactose-induced cells. Maltose-induced cells were incapable of fermenting glucose but were weakly capable of fermenting maltose. Xylose-induced cells show a weak fermentation of glucose but were incapable of fermenting xylose. Arabinose-induced cells could ferment neither glucose nor arabinose.

Effect of Temperature on Induction of Fermentation

The effect of temperature on anaerobic induction of fermentation by glucose in L. stokesii is shown in Fig. 9. Aerobically grown, early exponential phase cells were harvested, washed, and fermentation was induced as described in Warburg flasks attached to the Gilson Respirometer. Induction was carried out at the following temperatures: 0 C, 5 C, 10 C, 15 C, 20 C, and 21 C. Twenty-one C was selected since it is very close to the maximum growth temperature. Cells were allowed to equilibrate at each respective temperature, glucose was tipped in, and carbon dioxide evolution was measured. When the maximum rate of fermentation was achieved as indicated by 5 linear points at 15 minute intervals, the water bath temperature was rapidly adjusted to 25 C and fermentation was measured at this later temperature for an additional hour. Since the rate of fermentation is optimal at 25 C, fermentative activity at this temperature would be an indirect measure of the amount of enzyme(s) formed at the induction temperature. Results are shown in

Table 5. Induction of Sugar Fermentation by Sugars Other than Glucose

Inducer	Substrate	QCO ₂
Sucrose	Glucose	17
	Sucrose	26
Galactose	Glucose	10
	Galactose	8
Maltose	Glucose	0
	Maltose	5
Xylose	Glucose	5
	Xylose	0
Arabinose	Glucose	0
	Arabinose	0

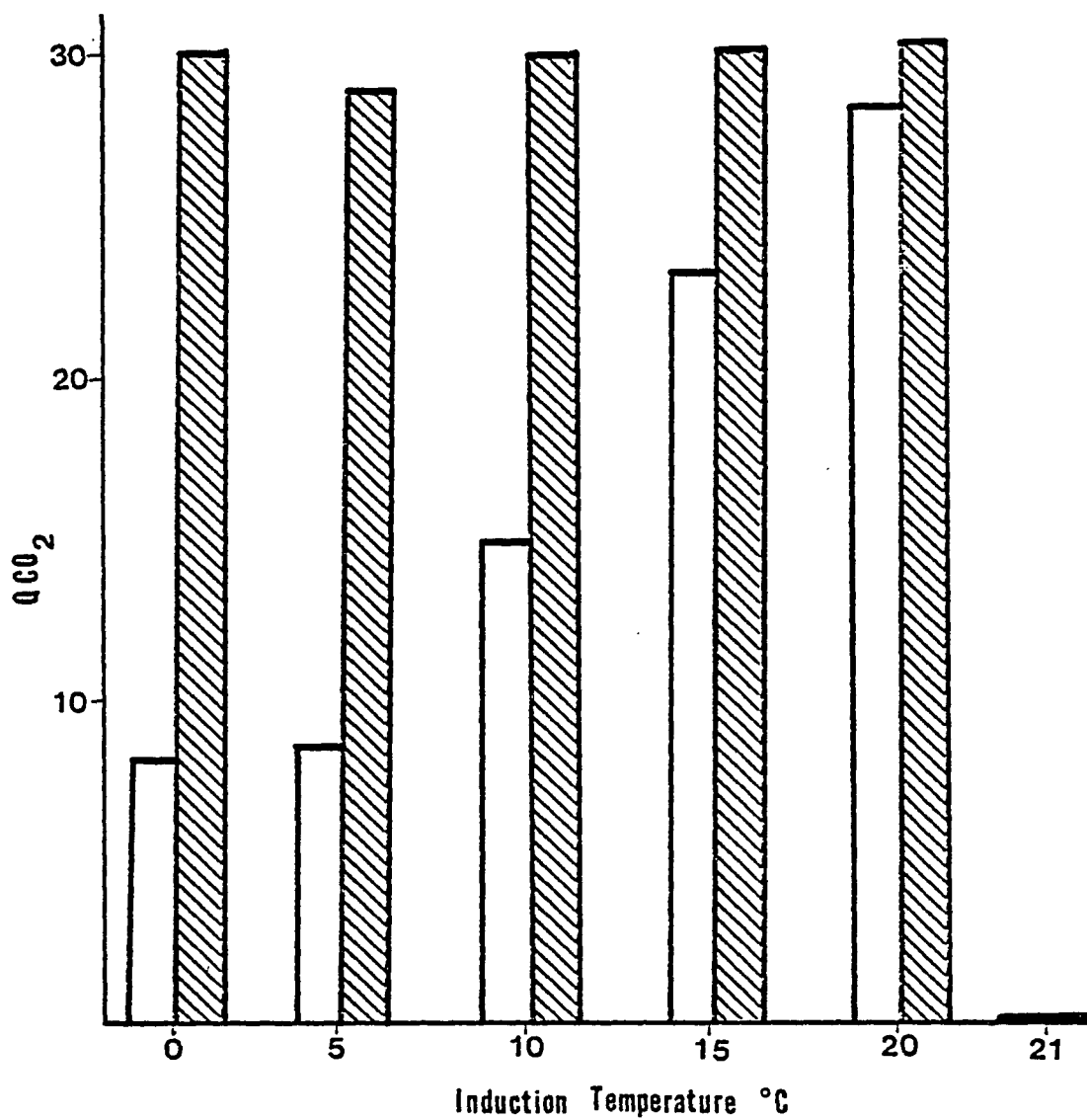



Fig. 9. Effect of Temperature on Induction of Fermentation

QCO₂ at induction temperature 


QCO₂ at 25 C 

Fig. 9. As indicated the QCO_2 values at 0 C and 5 C were approximately the same, but at temperatures between 5 C and 20 C the QCO_2 was doubled with each increase of 10 degrees in temperature corresponding to a Q_{10} effect. For example, the QCO_2 at 10 C was 14 and that at 20 C was approximately 28. When shifted to 25 C, the QCO_2 values were the same irrespective of the initial induction temperature. This suggests that although rates of fermentation at temperatures below 25 C were lower, the levels of fermentative enzymes were approximately the same. No induction occurred at temperatures above 20 C. Moreover, inspection of the data obtained at each temperature suggest an inverse relationship between the induction temperature and the rate of induction. For each temperature tested the time required to reach the maximum fermentative activity (indicated by the third linear point) was found and plotted against the induction temperature. These data are shown in Fig. 10. As can be seen from this graph, the rate of induction of fermentation is inversely proportional to temperature at temperatures between 0 C and 20 C.

Cell Free Assays of Fermentative Enzymes

Levels of Fermentative Enzymes During the Growth Cycle

The induction of fermentation implies the induced synthesis of fermentative enzymes. Since the first enzyme in the ethanol branch of glycolysis is pyruvate decarboxylase (PDC), the level of this enzyme was assayed in cells harvested during the growth cycle. Aerobically grown cells were harvested at various time intervals during the growth

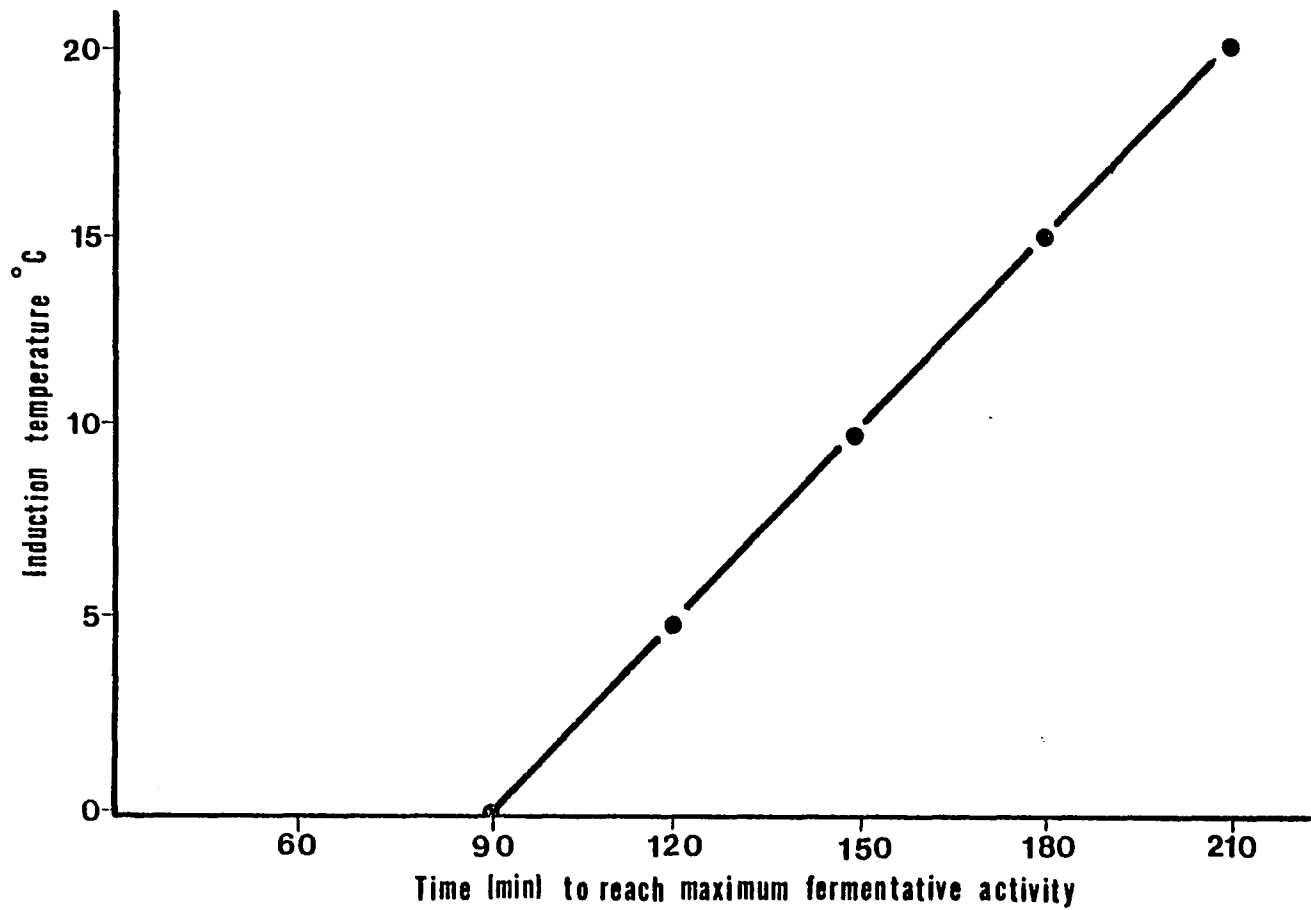


Fig. 10. Effect of Temperature on the Rate of Induction of Fermentation in *L. stokesii*

cycle and cell free extracts were assayed for PDC activity (Fig. 11 and see Fig. 2 for growth curve). During the early exponential growth phase (50 Klett units) no PDC activity was evident. During late exponential and early stationary phases (150 and 220 Klett units respectively) the specific activity of PDC increased to 4 and 5 respectively. PDC activity in cell free extracts prepared from stationary phase cells (300 Klett units) had a specific activity of 12.

In order to ascertain the effect of aeration on the induction of fermentation and fermentative enzymes, L. stokesii was cultured in a fermentor in which aeration could be controlled. Cells were harvested at 12 hour intervals after the culture had reached mid-exponential phase. Respiration and fermentation of glucose by whole cells was measured, PDC and alcohol dehydrogenase (ADH) in cell free extracts were assayed, and specific activities of these enzymes were calculated. Results are shown in Table 6. After 36 hours of aerobic growth, the culture had attained a density of 100 Klett units. Resting cells respired but were unable to ferment glucose. Also, a low level of ADH activity was evident and no PDC was detected in the cells. After 12 hours of anaerobic growth, the density had increased by only 50 percent. The QO_2 decreased slightly but there was a marked increase in QCO_2 to 165. Moreover, the specific activity of PDC was 123 and the specific activity of ADH increased approximately fifty-fold to 295. The culture was then shifted back to aerobic conditions for an additional 24 hours. Two samples were removed at 12 hour intervals. During the first 12 hour aerobic growth period the population density doubled. Although

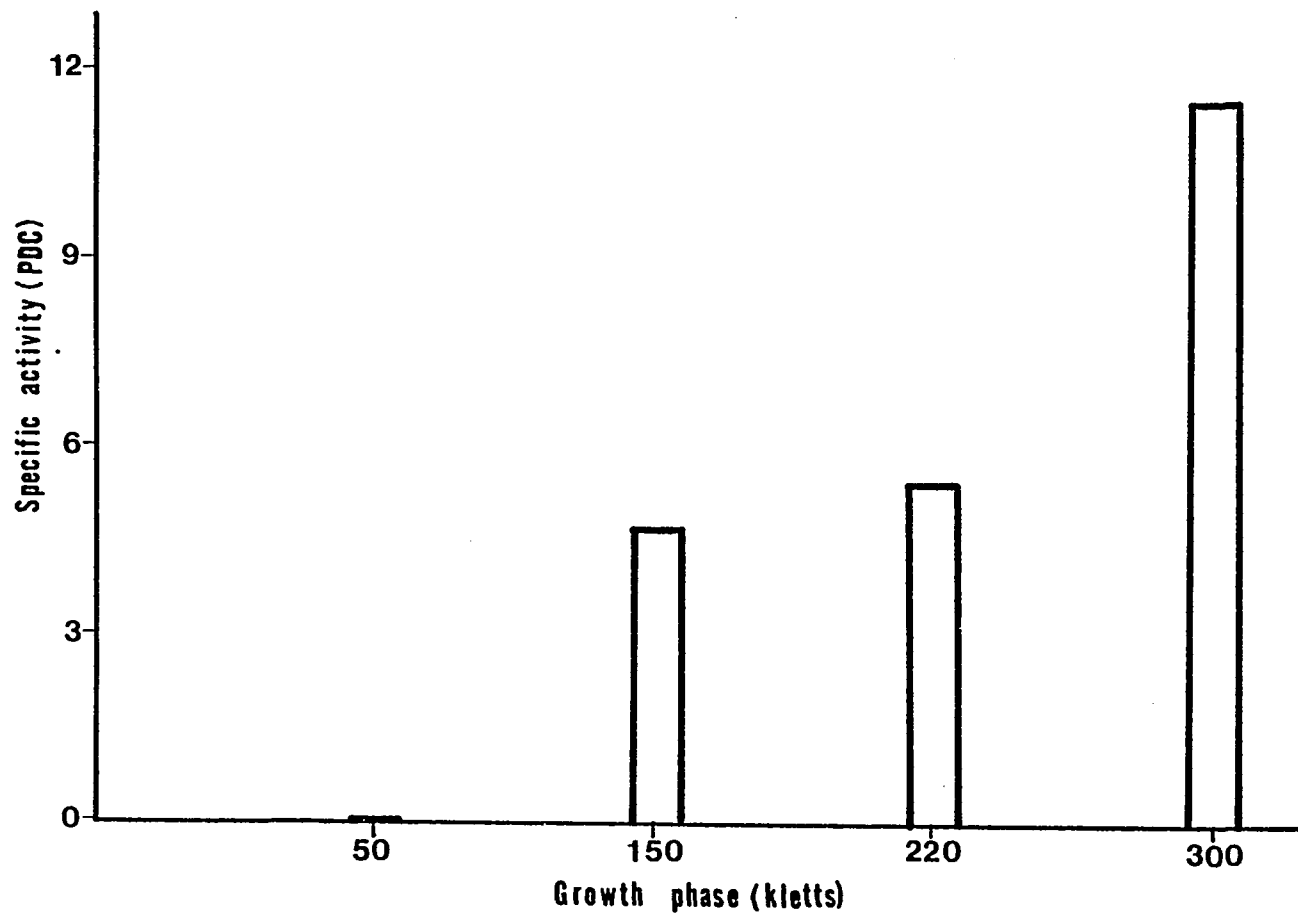


Fig. 11. PDC Activity of Cell Free Extracts Prepared from Cells Harvested at Various Growth Phases

Table 6. Growth, Respiration, and Fermentation by L. stokesii During Controlled Aeration in a Fermentor at 15 C

Total Time (hr)	Incubation Conditions	Kletts	QO ₂ Glucose	QCO ₂ Glucose	Specific Activity ADH	Specific Activity PDC
36	Aerobic	100	92	0	6	0
48	12 Hours Anaerobic	150	87	165	295	123
60	12 Hours Aerobic	300	85	63	142	7
72	24 Hours Aerobic	400	57	15	205	9

there was little change in QO_2 , QCO_2 decreased by 62 percent. Moreover, the specific activity of PDC decreased by 94 percent, i.e., from 123 to 7, and the specific activity of ADH decreased by 50 percent from 295 to 142. The reduction of both the QCO_2 and the specific activity of ADH and PDC can be explained in part by dilution of these activities by growth under non-inducing conditions. The marked loss in PDC activity, however, may be further explained by an active removal of the enzyme. During the final 12 hours of aerobic growth, the population increased to 400 Klett units (stationary phase). At this time the QO_2 decreased to 57, a loss of approximately 40 percent, and the QCO_2 decreased to 15, a loss of 75 percent activity. The specific activity of PDC increased slightly and the specific activity of ADH increased by 44 percent from 142 to 205. The marked increase in specific activity of ADH during the final 12 hours of growth suggested that this enzyme might also be active in the aerobic assimilation of ethanol. To test this possibility, the levels of ADH and PDC were assayed in cell free extracts prepared from cells cultured both aerobically and anaerobically in YNB plus ethanol medium. For purposes of control the yeast was also cultivated under these conditions in YNB-glucose medium. The results of this experiment are shown in Table 7. Cells used for inoculating the various media were checked initially for specific activity of ADH and PDC. As indicated, the specific activity of ADH was 2.5 and PDC was 0. After 12 hours anaerobic cultivation in YNB plus ethanol, the specific activity of ADH decreased to 1.5 and PDC remained at 0. After 12 hours incubation in YNB plus glucose, however,

Table 7. Induction of ADH and PDC in Ethanol Plus YNB at 15 C

Treatment of Cells	Specific Activity	
	ADH	PDC
None (Control)	2.5	0
Anaerobic in Ethanol	1.5	0
Anaerobic in Glucose	205.0	40
Aerobic in Ethanol	25.5	0
Aerobic in Glucose	2.2	0

there was a marked increase in the specific activity of both enzymes. After 12 hours aerobic incubation in YNB plus ethanol, the specific activity of ADH increased ten-fold and the specific activity of PDC was 0. After 12 hours aerobic incubation in YNB plus glucose, the specific activity of both enzymes did not change from those initially present in the T_0 cells. The increased specific activity of both enzymes observed in anaerobic YNB plus glucose can be explained in part by the role played by these enzymes in glycolysis and subsequent metabolism of pyruvic acid to ethanol. The increase in specific activity of ADH in the aerobic YNB plus ethanol culture, on the other hand, can be explained in part by the possible role played by this enzyme in the aerobic assimilation of ethanol. Supporting this possibility is the fact that this yeast can be cultivated in media having ethanol as the sole carbon source.

Effect of Temperature on Induction of PDC and ADH

The previous data suggested that PDC and ADH are inducible enzymes which reach maximum specific activity when L. stokesii is incubated in glucose under anaerobic conditions. It is also evident from the data shown in Fig. 9 that induction of fermentation is inhibited above 20 C. In order to ascertain the effect of temperature on the induction of PDC and ADH, young L. stokesii cells were induced anaerobically in buffered glucose at 15 C, 20 C, and 21 C for 12 hours. Cells were harvested after 12 hours incubation, cell-free extracts were prepared and PDC and ADH were assayed. The results of this experiment

are shown in Fig. 12. Cells used as inoculum for this experiment were initially examined for specific activity of PDC and ADH for purposes of control. As indicated there was no PDC activity and only slight ADH activity. A maximum specific activity of both ADH and PDC was produced in the culture incubated at 20 C (325 and 85 respectively). The extracts prepared from cultures incubated at the optimum growth temperature (15 C) exhibited slightly lower specific activities. ADH activity in extracts prepared from 21 C cultures was markedly reduced. No PDC activity was detected.

Thermal Stability of Preformed Enzyme

The data thus far presented show that once formed, enzymes, in particular PDC, are stable when exposed to temperatures of 25 C. Induction of PDC, however, is sensitive to temperatures above 20 C. These data confirm and extend those of other investigators (18, 39). Heat sensitivity of PDC at various stages of purity, however, has not been investigated thoroughly. For this reason the following experiments were performed. Thermal stability of fermentative enzymes in whole cells was investigated as follows. Young (50 Klett units) aerobically grown cultures of L. stokesii were anaerobically induced to ferment glucose at 15 C. Cells were harvested, washed, and finally suspended in 67 mM KH_2PO_4 . The suspension was heated for one hour at 35 C. Samples were taken at 20 minutes and fermentation of glucose was measured at 25 C. In addition, crude cell free extracts were prepared from cells harvested at each time period and the levels of PDC and ADH were assayed. As shown by the data in Fig. 13, the specific activity

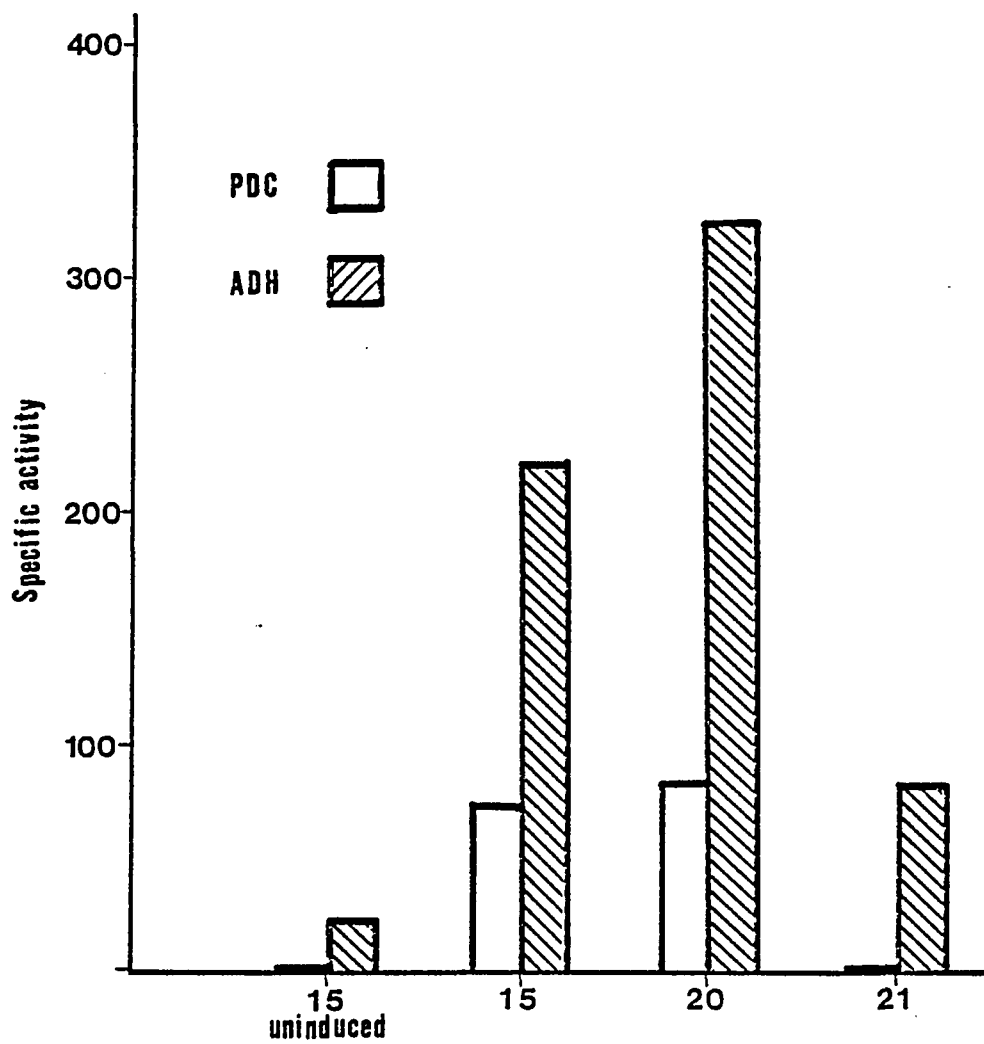


Fig. 12. Effect of Temperature on Anaerobic Induction of PDC and ADH

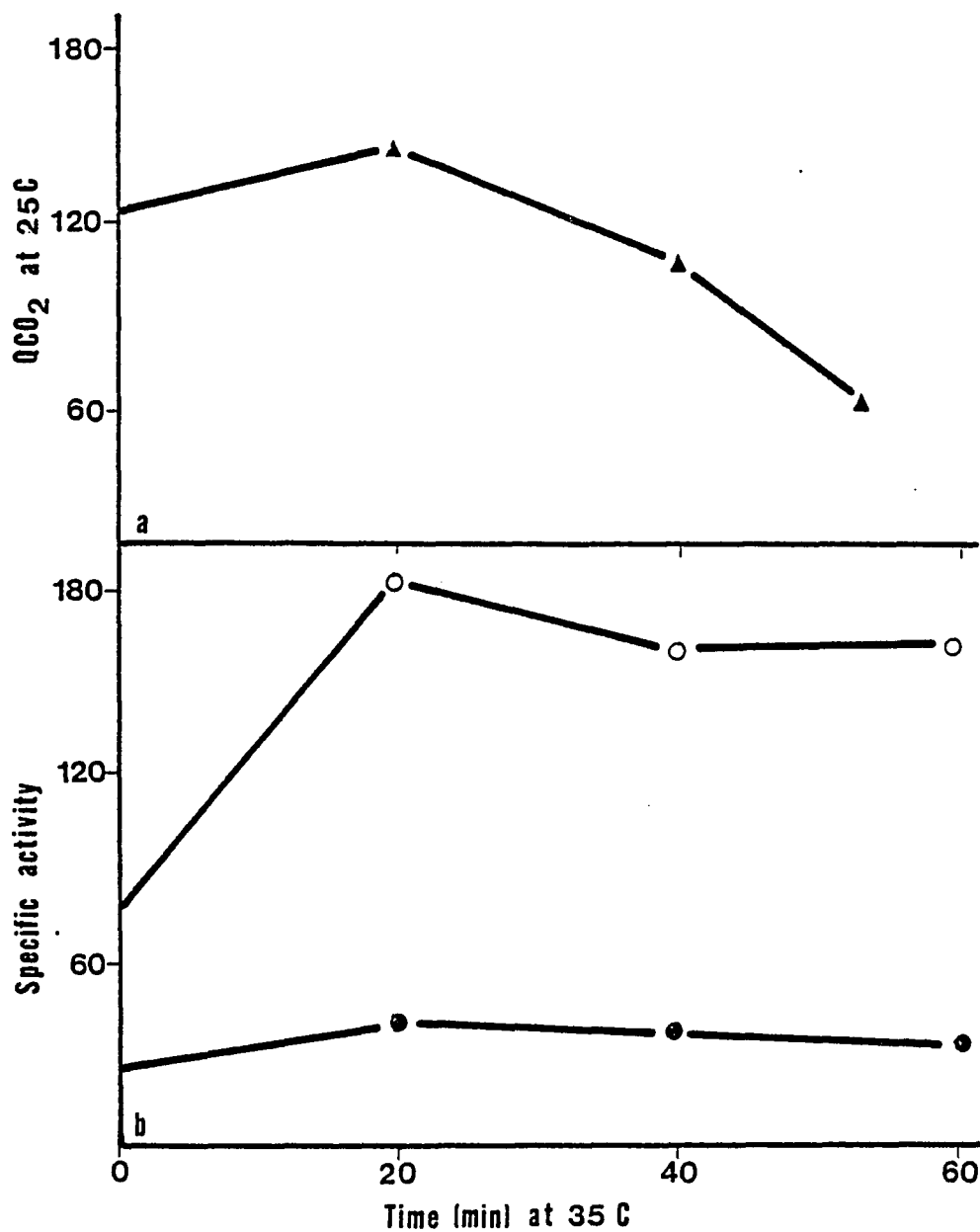


Fig. 13. Effect of 35 C on: a. QCO₂ in Whole Cells and b. PDC Activity and ADH Activity from Treated Whole Cells

PDC Activity (●); ADH Activity (○); Whole Cells (▲)

of PDC remained unchanged after one hour at 35 C. The specific activity of ADH, however, almost tripled within 20 minutes and remained constant over the remaining 40 minutes. In contrast, fermentation of glucose by whole cells showed a different pattern. Although there was a slight increase in the QCO_2 after 20 minutes exposure to 35 C, QCO_2 decreased thereafter and only 50 percent of the initial activity remained after 60 minutes.

Since a reduction in QCO_2 in whole cells held at 35 C cannot be explained by a loss of fermentative enzymes, an experiment was developed to determine the effect of 35 C on the anaerobic uptake of glucose-u- ^{14}C at 25 C. A young culture was anaerobically induced in glucose at 15 C before being placed at 35 C. Samples were taken at 20 minute intervals and assimilation of glucose-u- ^{14}C at 25 C was measured (Fig. 14). The counts per minute expressed are the sum of the counts found in the cells and in the $^{14}CO_2$ given off by the cells. Uptake of radioactive glucose by unheated T_0 cells was rapid for the first 6 minutes, and then decreased. The rate of uptake of glucose by cells heated 20 minutes at 35 C was similar. In contrast, the rate of uptake of glucose by cells heated 40 minutes and 60 minutes at 35 C was markedly reduced. These data suggest that the loss of ability of whole cells to ferment glucose when heated at 35 C is owing to the inability of the cells to assimilate glucose from the medium and not due to denaturation of either PDC or ADH. Moreover, PDC as well as ADH are stable for periods of up to 60 minutes in whole cells incubated at 35 C.

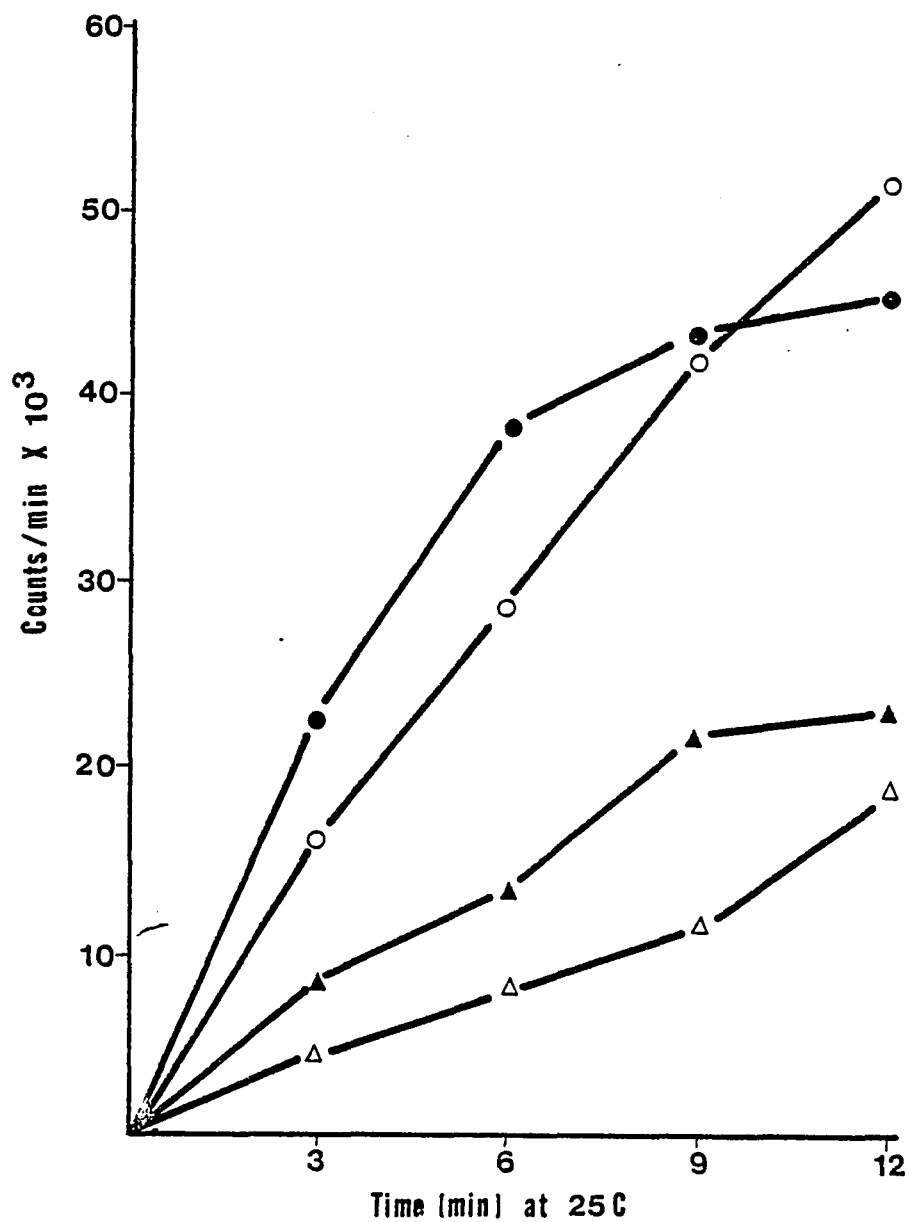


Fig. 14. Effect of 35 C on Uptake of Glucose-u- ^{14}C at 25 C

0 minutes at 35 C (●); 20 minutes at 35 C (○);
40 minutes at 35 C (▲); 60 minutes at 35 C (△)

Thermal stability of PDC in crude cell-free extracts prepared from anaerobically induced L. stokesii was determined as follows. Extracts were incubated at 0 C, 20 C, 25 C, and 35 C for varying periods of time. Samples were taken at 15 minute intervals and PDC was assayed. Data are shown in Fig. 15. Loss of PDC activity at 35 C was rapid. No activity was detected after 45 minutes heating. PDC activity in extracts incubated at 25 C were unaffected after 15 minutes, but after 30 minutes the rate of loss of activity was approximately the same as extracts incubated at 35 C. PDC activity in extracts incubated at 20 C and 0 C also decreased slightly. For comparative purposes PDC activity in partially purified extracts prepared from anaerobically induced L. stokesii were also examined for thermal stability (Fig. 16). Loss of activity of partially purified PDC was rapid and the rate of loss was directly proportional to temperature over the range 25 C to 35 C. At temperatures within the normal growth range of the yeast, i.e., 10 C, 15 C, 20 C, some loss of activity did occur over the 45 minute treatment. At 20 C, the maximum growth temperature, 40 percent of the activity was lost, while at 10 C and 15 C only 15 percent of the activity was lost after 45 minutes. The enzyme was stable at 0 C.

The data thus far show an inverse proportion of 35 C heat stability to degree of purification for PDC. For example, PDC activity of crude extracts prepared from whole cells of L. stokesii exposed to 35 C heat was stable for periods of one hour. In contrast, activity of partially purified PDC was destroyed within as little as 15 minutes

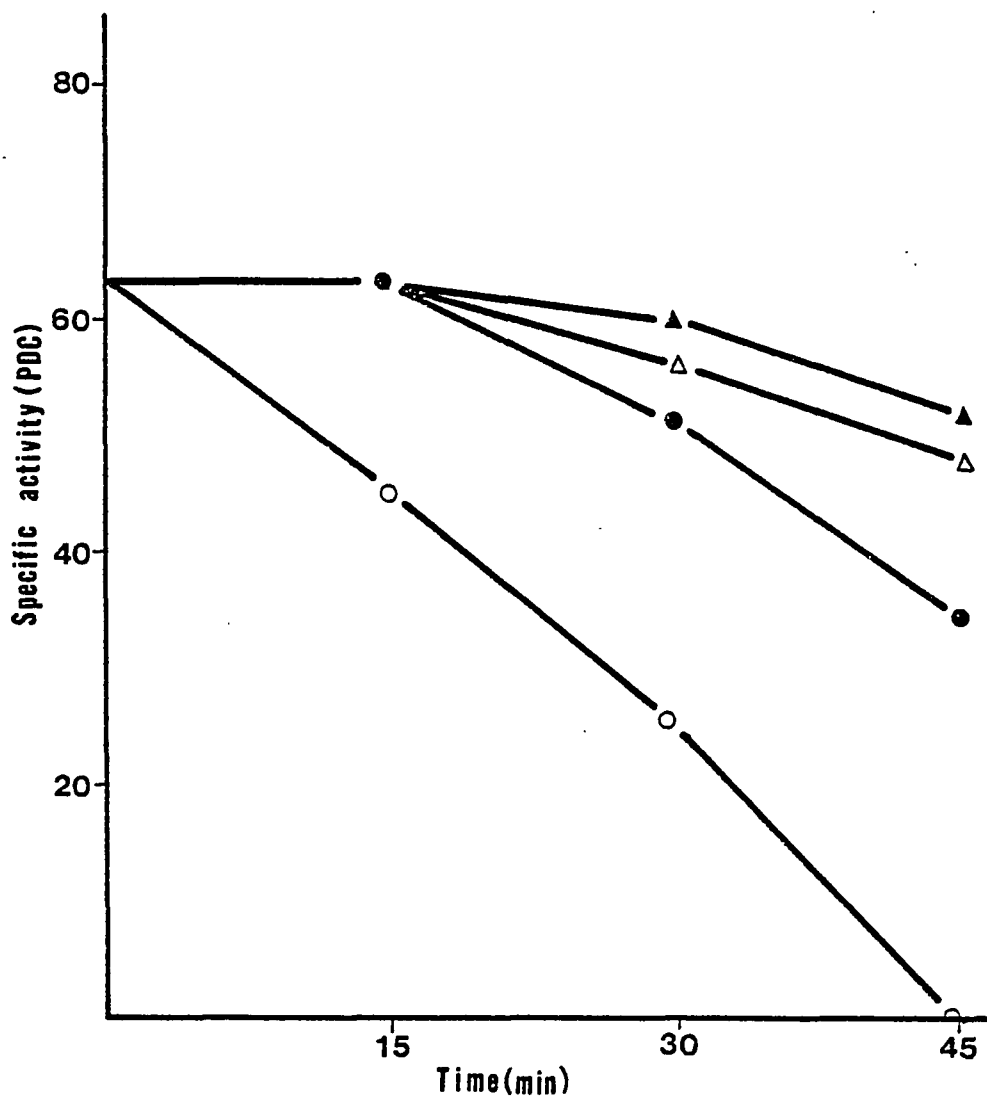


Fig. 15. Effect of Temperature on PDC Activity in Crude Cell-free Extracts of *L. stokesii*

0 C (▲); 20 C (△); 25 C (●); 35 C (○)

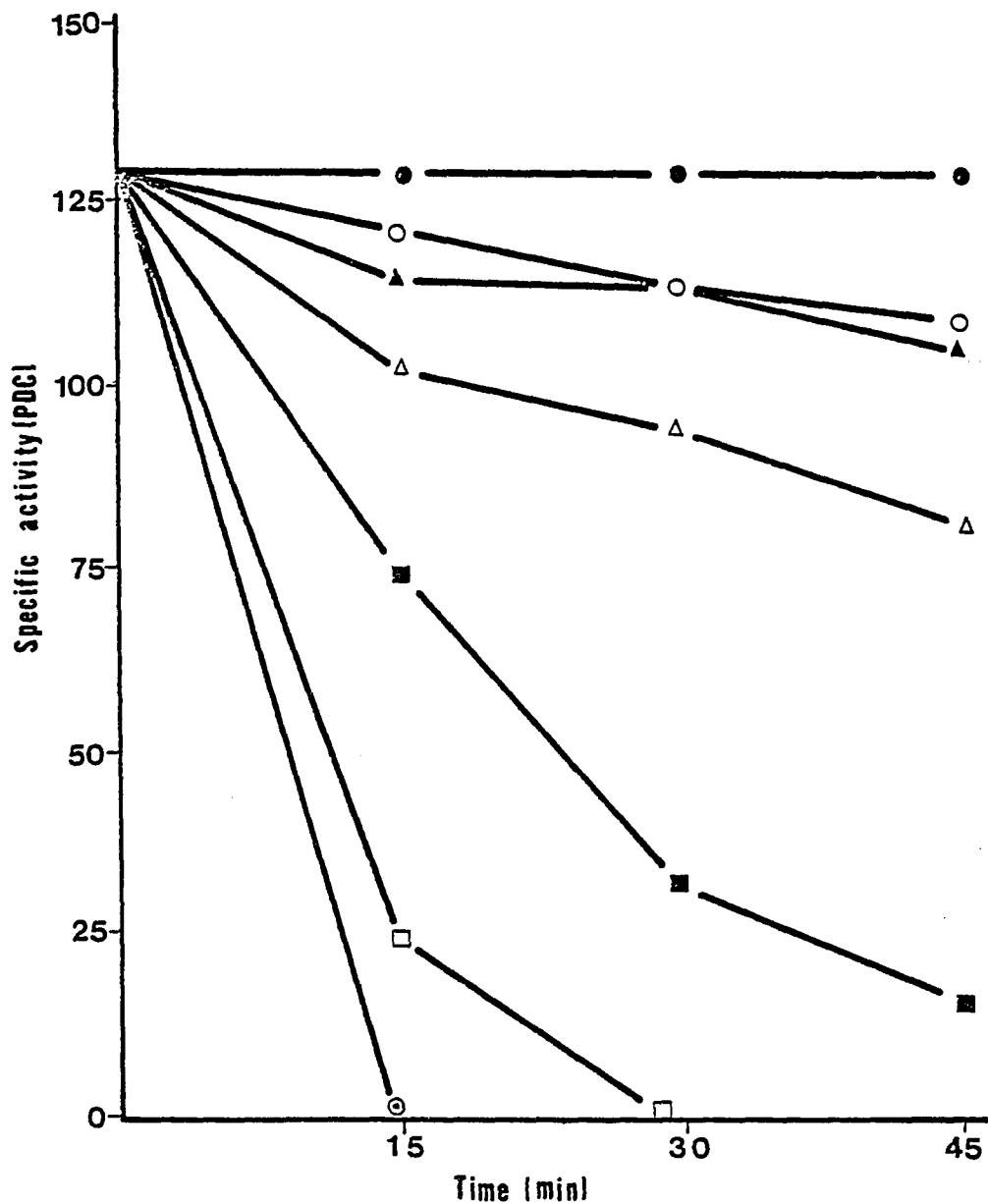


Fig. 16. Effect of Temperature on PDC Activity in Partially Purified Cell-free Extracts

0 C (●); 10 C (○); 15 C (▲); 20 C (△); 25 C (■);
30 C (□); 35 C (⊙)

exposure to 35 C. Since substrate stabilized enzymes have been shown to be more stable to heat (16), the possibility that this accounts at least in part for heat stability in whole cells was investigated. Partially purified extracts were prepared from young induced cells and were suspended in 0.1 M sodium pyruvate at pH 5.0. A control which contained partially purified extracts suspended in 33 mM KH_2PO_4 at pH 5.0 was included. Both suspensions were heated at 30 C and 35 C respectively for 45 minutes. Samples were taken at 15 minute intervals and PDC activity was determined. When partially purified extracts suspended in sodium pyruvate were exposed to 30 C heat for 45 minutes, there was little loss of initial activity. However, partially purified extracts suspended in KH_2PO_4 were rapidly denatured and no activity was detected after 45 minutes exposure (Fig. 17a). Results are similar for partially purified extracts exposed to 35 C heat with the following exception. Pyruvic acid protected the enzyme for 30 minutes. Activity then decreased and was not detected after 45 minutes (Fig. 17b). The enzyme suspended in KH_2PO_4 was completely denatured within 15 minutes at this temperature. These data support the contention that the enzyme is stabilized in whole cells most likely by substrate binding.

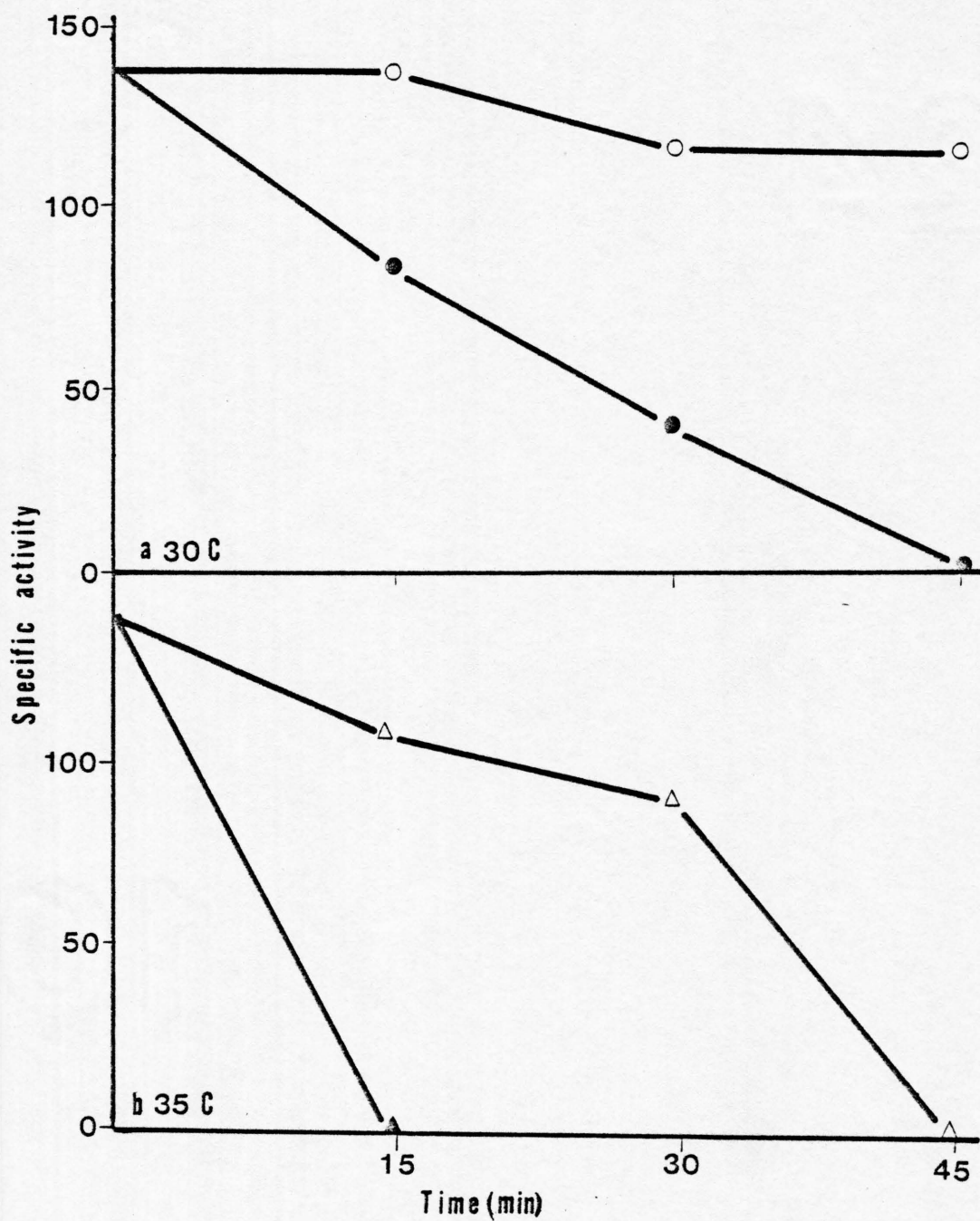


Fig. 17. Incubation of Partially Purified PDC at: a. 30 C in 0.1 M sodium pyruvate and in 33 mM KH₂PO₄, and b. 35 C in 0.1 M sodium Pyruvate and in 33 mM KH₂PO₄

30 C in 0.1 M sodium pyruvate (○); 33 mM KH₂PO₄ (●);
 35 C in 0.1 M sodium pyruvate (△); 33 mM KH₂PO₄ (▲)

DISCUSSION

The majority of the studies done thus far concerning fermentative enzymes of yeast have been carried out with species of the genus Saccharomyces, specifically Saccharomyces cerevisiae. When grown in high concentrations of glucose or other rapidly fermentable sugars, the synthesis of mitochondrial components of S. cerevisiae are inhibited, and aerobic growth under these conditions leads to the production of cells which show a fermentative pattern of metabolism. This impairment of respiration resulting from catabolite repression can be accounted for by a) the "reverse Pasteur effect" (inhibition of biosynthesis of respiratory enzymes), or b) the "Crabtree effect" (inhibition of activity of respiratory enzymes) (14, 15). The low QO_2 of intact cells grown with high concentrations of glucose is accompanied by a reduction in levels of activity of enzymes of the tricarboxylic acid cycle (10, 30, 31, 32).

The main emphasis of studies with Saccharomyces has been either enhancement or repression of fermentation due to alterations of substrate, or loss of respiratory activity during fermentation. In either event, the Crabtree effect was intimately involved. The data presented in this study have demonstrated the response of an obligately psychrophilic, Crabtree negative, yeast, Leucosporidium stokesii, to alterations in substrate, temperature, and atmospheric oxygen during the induction of fermentative enzymes.

Batch cultivation of S. cerevisiae with glucose as the carbon source is a biphasic process. During early stages of growth at high glucose concentration, the cells are subject to glucose repression and have a highly fermentative pattern of metabolism. Exhaustion of glucose is accompanied by derepression of respiratory enzymes and oxidation of accumulated ethanol (3, 5, 25).

Batch cultivation of L. stokesii, on the other hand, at high glucose concentration does not result in a biphasic growth curve. Throughout the early exponential phase of growth the metabolic pattern of the cells is oxidative. Fermentation does not occur until oxygen becomes limiting due to high respiration rates. As fermentation begins the rate of growth decreases and there is a smooth transition into the stationary phase. After the glucose is consumed, the accumulated ethanol is respired, but this also does not drastically alter the shape of the growth curve.

Although certain aspects of the growth of S. cerevisiae and L. stokesii are similar, i.e., alcoholic fermentation and oxidation of accumulated ethanol, the process of initial glucose utilization is different. Within the permissive growth temperature range, L. stokesii requires both a fermentable sugar and oxygen limiting conditions in order to initiate fermentation. If either of these two conditions is lacking, the yeast will not produce fermentative activity. S. cerevisiae, on the other hand, will ferment even under aerobic conditions if a suitable sugar is available in sufficient quantity (greater than 0.1 percent) (32).

The data discussed thus far indicate that L. stokesii does not undergo the Crabtree effect, but instead produces a negative Crabtree effect (an inhibition of fermentation by respiration).

In addition to differences in aerobic growth, anaerobic growth of L. stokesii differs from that of fermentative yeasts. S. cerevisiae has been shown to require ergosterol and Tween 80 for anaerobic growth (1). Addition of these compounds to anaerobically growing L. stokesii does not significantly alter the growth pattern. Moreover, once L. stokesii assumes a fermentative pattern of metabolism, it does not lose its ability to respire. After 12 hours of anaerobic growth, the QO_2 of L. stokesii was essentially unchanged (Table 6). Chapman and Bartley (10) have shown that aerobically grown S. cerevisiae cells containing high levels of activity of tricarboxylic acid cycle, glyoxylate cycle and electron transport enzymes lose up to 45 percent of their activity within 30 minutes of fermentative growth. They suggested a process of active removal of these enzymes to explain this loss of activity.

Fell et al. (17) have reported that L. stokesii is capable of fermenting glucose, galactose, sucrose, and maltose, although maltose and galactose fermentation are weak and sometimes latent. My data confirm and extend these observations. It appears that maltose fermentation requires the presence of maltose during the induction period. Glucose-induced cells are unable to ferment maltose and maltose-induced cells are unable to ferment glucose. Glucose-induced cells are also

unable to ferment two pentoses, xylose and arabinose, but cells induced in xylose contribute a factor which can induce fermentation of glucose.

The use of inhibitors has helped to ascertain the intracellular location of fermentative enzymes in L. stokesii. Cyclohexamide has been shown to inhibit cytoplasmic protein synthesis in yeast (36). Addition of this antibiotic during anaerobic induction of L. stokesii cultures completely inhibited the cells ability to ferment. Moreover, it provided evidence that the induction of fermentation requires the synthesis of protein. This observation supports and extends the finding of Ruiz-Amil et al. (36). These investigators showed that pyruvate decarboxylase is localized in the cytoplasm of Candida pulcherrima and S. cheresiensis. The fact that iodoacetic acid decreased the fermentation of glucose in L. stokesii by one-half but did not affect pyruvate fermentation, indicates that in addition to the Embden-Meyerhof pathway, L. stokesii also possibly utilizes the pentose phosphate pathway for glucose metabolism.

Studies involving the kinetics of induction clearly indicated that during the three hour lag period at 15 C before fermentation reached a maximum, fermentative enzymes were being either synthesized or activated. This conclusion is supported by observations using cell free extracts. Assays performed during the growth cycle show no PDC activity in early exponential phase cells. PDC activity becomes apparent only when the culture approaches stationary phase. Moreover, growth studies done in a fermentor under conditions of controlled aeration indicated increased PDC and ADH activity only during anaerobic

incubation. An increase in the QCO_2 reflected the increase in fermentative enzymes.

It is also apparent that the induction of ADH is regulated by different controls than PDC. The induction of PDC appears to respond to oxygen limitation only in the presence of a fermentable sugar. ADH induction also responds to these conditions, but in addition it responds to aerobic but not anaerobic incubation in ethanol, conditions to which PDC induction is insensitive. Anaerobic stimulation of ADH activity during incubation in ethanol is not unreasonable expected since oxidation of accumulated ethanol would require an enzyme which could produce acetaldehyde for processing into acetyl-CoA. ADH is a reversible enzyme which functions in the oxidation of ethanol to acetaldehyde. In addition, studies with S. cerevisiae have shown that ADH is repressed by glucose. According to Witt, Kronau and Holzer (44) glucose repression may indicate that this enzyme functions in the gluconeogenesis pathway from ethanol. Chapman and Bartley (10) observed that the activity of ADH increases on transfer towards anaerobic metabolism caused by the addition of sugar. These differing results are probably explained by the observation that yeast has several ADH isoenzymes, a fermentative ADH concerned with maintenance of glycolysis, and an oxidative ADH involved in the utilization of ethyl alcohol (37). Evidence that anaerobic incubation in ethanol does not enhance ADH levels is substantiated by the fact that during strict anaerobic growth, accumulated ethanol is not assimilated (Figs. 3 and 4).

There is considerable evidence that the ability of psychrophilic organisms to grow at low temperatures but not at moderate temperatures is related to the effect of temperature on enzyme synthesis and activity. Psychrophile enzymes are formed and function better at low temperatures and also are more heat sensitive than corresponding enzymes from mesophilic microorganisms (18, 33, 39, 40, 42). Substrate transport may also be more efficient at lower temperatures in psychrophiles (4, 11, 39).

Induction experiments performed at various temperatures with L. stokesii showed that the temperature range for induction of fermentation was 0 C to 20 C (Fig. 9). This is the same as the cardinal growth temperature range. In repeated experiments at 21 C no induction of fermentation occurred. Evidence supporting this finding was that PDC activity was evident in cell free extracts of cultures anaerobically incubated at 20 C but not at 21 C. This phenomenon also corresponds in part to results obtained by Silver (38). He found that L. stokesii undergoes an atypical morphological change when aerobically incubated at 21 C to 23 C. In contrast, morphology was not affected when cells were incubated under anaerobic conditions. He predicted that the failure to undergo morphological change anaerobically was due to reduced levels of adenosine tri-phosphate (ATP). Failure to produce an active PDC would accomplish this result since the glycolytic pathway would stop at pyruvate.

In addition to a temperature sensitive inductive process Fig. 9 also showed that decreasing temperature affects the rate of induction.

Once a maximum rate of fermentation was attained at the induction temperature, the water bath was increased to 25 C, the optimum temperature for fermentation, and the QCO_2 was calculated at that temperature. This provided an estimate of the fermentative enzymes present. Although the rate of fermentation at the induction temperatures varied, the QCO_2 at 25 C was approximately the same for each induction temperature tested. This indicated that once maximum fermentation was reached, the relative amounts of fermentative enzymes were equal irrespective of induction temperature. If the time required to reach maximum fermentative activity at a particular induction temperature was plotted against the induction temperature, an inverse proportion was seen. This suggests that fermentative enzymes in L. stokesii are produced faster as the temperature is decreased within the range of 0 C to 20 C. This differs from the experiments reported by Quist and Stokes (33) on formic hydrogenlyase induction in a psychrophilic Gram-negative bacterium designated Strain 82. They found that the range of induction was between 0 C and 20 C, but the rate of induction increased with temperature within this range.

Temperature-sensitive glucose fermentation in L. stokesii was first reported by Sinclair and Stokes (39). They found that glucose fermentation was abolished by heating resting cells for 30 minutes at 35 C. Grant et al. (18) subsequently reported that a survey of the enzymes of the yeast alcoholic fermentation pathway revealed that PDC was the only temperature sensitive enzyme of 12 assayed. Since these data suggest an inactivation of preformed enzyme rather than an

inhibition of enzyme synthesis, an examination of the thermodynamic stability of PDC was undertaken. The activity of PDC was checked after heating whole cells, crude cell free extracts, and partially purified cell-free extracts. The results showed that as the purity of the enzyme preparation increased, the thermal stability of PDC decreased. PDC activity was abolished within 15 minutes and 45 minutes with partially preparations and crude cell-free extracts respectively. In contrast, whole cells retained 100 percent PDC activity after 60 minutes heating at 35 C.

It is known that substrates stabilize enzymes against thermal denaturation (16). Therefore the protection of PDC in heated whole cell preparations and crude cell-free extracts may have resulted from enzyme stabilization due to substrate interaction. This was substantiated when partially purified PDC preparations, the most sensitive to heat, were heated in the presence of 0.1 M sodium pyruvate. Ninety-three percent of the substrate charged enzyme was stable to 30 C heat for as long as 45 minutes. At 35 C 66 percent of the substrate charged enzyme was stable for 30 minutes. In the latter case the substrate-charged enzyme was completely destroyed within 45 minutes.

The protection of PDC by pyruvate would account in part for thermal stability in whole cells exposed to 35 C heat. Whole cells nevertheless are unable to ferment glucose when exposed to 35 C heat for extended periods of time. Sinclair and Stokes (39) noted that almost all of the glucose added to heated cell suspensions was still present at the end of their experiment. To compensate for this they

added 400 micro-moles of glucose--an amount which they expected to permit penetration of the substrate by diffusion. Since fermentation still did not occur, they assumed that heating destroyed one or more enzymes in the fermentative pathway rather than impairing the permeability of glucose. Grant et al. (18), continuing this study, described heat inactivation of fermentation of both whole cells and cell free extracts, but they noted that substrate permeation was not examined and thus did not eliminate the possible involvement of heat-induced impairment of glucose permeation. They proposed that an unusually heat-labile PDC was at least partially responsible for the observed temperature sensitive glucose fermentation by whole cells.

My data not only confirm, but also extend the above observations. The data in Figs. 13 and 14 show that impairment of fermentative enzymes was not the primary cause of the decreased activity. Rather, heating at 35 C results in inability of the cells to assimilate glucose from the medium. Consequently, the reduced fermentation rates resulting from heat treatment previously described were probably due to alterations in the permeability of glucose rather than inactivation of temperature sensitive enzymes of the fermentative pathway.

In conclusion, L. stokesii, an aerobic, Crabtree negative, obligately psychrophilic yeast, is capable of shifting to a fermentative metabolism under conditions of excess sugar and limiting oxygen. Under these conditions pyruvate decarboxylase is induced. The induction of this enzyme occurs only over the cardinal growth temperature range, i.e., 0 C to 20 C and involves the synthesis of one or more

cytoplasmic enzymes. It is of interest to note that the rate of induction of PDC is inversely proportional to temperature and may in part be related to the obligately psychrophilic nature of this yeast.

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