GERMINATION

OF ASCOSPORES OF

SACCHAROMYCES CEREVISIAE HANSEN

CHANGES IN RESPIRATORY CAPACITY AND THE LOSS OF ACID-FASTNESS DURING YEAST ASCOSPORE GERMINATION

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SCOPE AND CONTENTS:

Experiments are described which investigate the effect of some nutritional and environmental factors on the germination of yeast ascospores. The factors stressed are the carbon and nitrogen sources and the gaseous environment. Loss of acid-fastness and changes in respiratory activity are investigated for their suitability as germination criteria.

In the discussion, the respiratory features of the three major phases in the life cycle of yeast, that is, growth, sporulation, and germination, are compared.

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INTRODUCTION

LIFE CYCLE OF YEASTS

The characteristic method of vegetative cell multiplication in Saccharomyces yeasts is budding. In this process the mother cell develops a bud which swells and then is released as a daughter cell. Cagniard-Latour (1838) observed the presence of " bud and birth scars " and described precise dimensions of the cells and their mode of reproduction; however, little attention was given these structures until the detailed study by Barton in 1950. Barton demonstrated the presence of both " bud scars " and " birth scars " on the walls of the mother cell and of the newly formed daughter cell. When these cells themselves produce more buds, upon detachment, each of the daughter cells leaves a bud scar upon the mother cell. The bud scar produced is situated diametrically opposite to the birth scar. Calculations based on an average size yeast cell by Bartholomew and Mittwer (1950) revealed that the maximum number of bud scars on a cell would be on the order of 100. Barton (1950) stated that buds never formed at the same site as an old scar and so the reproductive life of a yeast cell is definitely limited. More than 20 bud scars on any one cell was not found out of the several hundred cells that were photographed by Bartholomew and Mittwer. Bowers and McClary (1964) studied

thin sections of cultures of Saccharomyces cerevisiae in various phases of the budding cycle using the electron microscope. Budding began with an ever increasing protuberance of the cell wall from the mother cell. The nuclear division was mitotic and one of the resulting diploid nuclei remained in the parent cell. With increase in size of the bud separation occurred by an annular growth of cell wall material.

The first acceptable description of yeast ascospores was published by DeSeynes (1868). Sexual reproduction in yeast involves the production of ascospores, the " sporogenic " process. Nuclear reduction division occurs in the " ascus ", the last cell of the diploid stage, where meiosis takes place, and results in the production of four haploid nuclei of sexual spores in Saccharomyces cerevisiae. Unlike vegetative reproduction which occurs in common yeast nutrient media quite readily, sporulation of yeast cells requires the restriction of nutrients. Stantial (1935) reported that some strains of yeast would sporulate readily in dilute solutions of certain sugars, or acetates, or both together, and this has been abundantly confirmed by workers such as Elder, (1937); Adams, (1949); Fowell, (1952); Adams and Miller, (1954); and many others. Miller and Halpern (1956) found that optimum sporulation occurred in a hexose concentration that was much below the optimum for vegetative growth. Also, a supply of assimilable nitrogen and of minerals other than that contained in the cells was not required. Thus, yeast cells when

transferred to a restrictive medium from a nutritive medium will proceed to sporulate and produce ascospores. If the nutrient supply in a vegetative growth culture becomes exhausted, the vegetative cells become " starved " and may proceed to sporulate. It is emphasized here that the yeast ascospore is produced under conditions where vegetative reproduction is not possible.

Yeast ascospores are produced as a reaction to unfavorable nutritive conditions, but, unlike spores of bacteria, yeast spores are not markedly more resistant than vegetative cells to influences such as heat and disinfectants, though there are slight differences according to Ingram (1955). Using the data obtained by Lund (1951) and others, Ingram concluded that " wet " ascospores, that is, in suspension, usually could withstand temperatures which were 5-10 degrees higher than vegetative cells. However, when the ascospores have been dried the resistance to heat was much greater. Alcohol, which kills vegetative cells, was resisted by mature ascospores for a long time and viable ascospores can be separated from vegetative cells better in this way than by heating. Once ascospores are formed they remain in a dormant state until sufficient nutrient and/or favorable conditions are provided which will allow their germination to occur. Thus the significance of the sporogenic process in yeast included the provision of a mechanism which allows for survival when yeast cells are exposed to unfavorable growth conditions.

The provision of a mechanism which allows for genetic variation to occur is a second and equally important function of this sporogenic process in yeast. Comprehensive studies by Winge and Laustsen (1937) presented conclusive evidence that genetic segregations occurred during spore formation which was preceded by reduction division. These workers observed this phenomenon regularly, each time all the four spores of an ascus were isolated. By the formation of giant colonies from each of the isolated spores they found that the differences between the four haploid clones of yeast originating from the same ascus was often great. These workers established that heterozygosity in more than one gene was common in the segregations and that crossing-over may occur frequently during reduction division.

The mature ascospore of the Saccharomyces yeast germinates to produce haploid vegetative cells which proceed to reproduce vegetatively by budding. Matings or copulations occur between these haploid spores (or the budding haploid cells) to produce zygotes which bud off diploid vegetative cells. Ingram (1955) noted that it was unusual to find much vegetative proliferation in both haploid and diploid generations in any one yeast species. Saccharomyces cerevisiae for example, is normally diploid, that is, vegetative proliferation occurs mainly in the diploid phase. Winge (1935) showed that conjugations between the haploid cells occurred some generations subsequent to the germination of ascospores of Saccharomyces which did not conjugate pairwise.

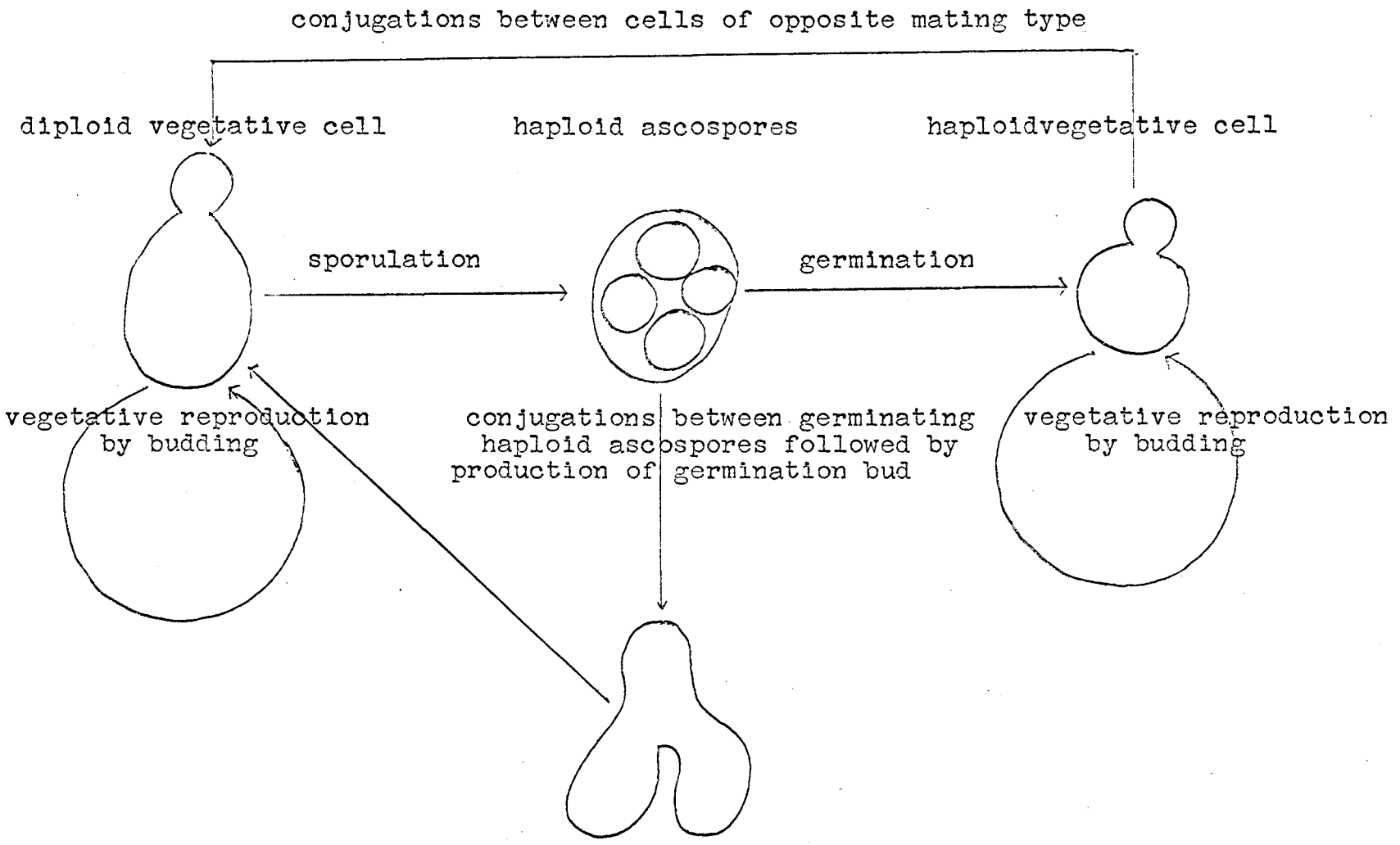


Figure 1. LIFE CYCLE OF SACCHAROMYCES YEAST

The main stages of the life cycle of Saccharomyces yeasts can be summarized as follows:

(1) The characteristic method of vegetative reproduction is by budding. A protuberance or bud is developed by the mother cell which swells and then is released as a daughter cell. As each bud develops, the nucleus undergoes mitotic division and one of the two daughter nuclei enters the bud before it detaches from the mother cell. This process continues indefinitely as long as favorable conditions for growth are maintained.

(2) Sporulation provides the mechanism for sexual reproduction of diploid vegetative cells. In this process, the nucleus in any such cell may undergo meiotic (reductional) division producing four haploid nuclei. A spore wall forms around each haploid nucleus to produce the ascospore. The vegetative cell has now become the ascus. The yeast is now in the resting or dormant phase of its life cycle.

(3) Germination occurs when the dormant spore is placed in a favorable environment, generally a medium suitable for growth. In this process spores swell, bud, and then continue to reproduce vegetatively by budding.

(4) Conjugations between pairs of haploid cells of opposite mating type result in the production of the diploid state once again. This may occur early in the germination process between spores or later between haploid vegetative cells produced from germinated ascospores.

SPORES OF BACTERIA AND FUNGI

Halvorson and Sussman (1966) employed the term " spore " to describe " any reproductive structure found in microbes ". A prefix to this term denoted the origin of the spore or the name of its container. For example, fungal ascospores are spores formed within the ascus; basidiospores are exospores formed on basidia; teliospores are spores within which nuclear fusion in the rusts occur; and bacterial endospores are spores formed inside bacterial cells.

Bacterial endospores occur most frequently in the gram-positive rods of the genera Bacillus and Clostridium, and much less often in some species of Vibrio, Spirillum, and Sarcina (Lamanna and Mallette, 1953). Bacterial spores and to a lesser degree fungal spores possess the ability to resist extremes in temperature and other physical stresses. VanNiel (1955) suggested that the property of spore resistance was an important factor in survival. For example, no spore-formers are known which thrive in media unsuitable for growth of nonspore-formers. Apparently, spore-formers have found competitive advantage in nature via their resistance to adverse conditions in the environment.

Spores can be considered to be timing devices which ensure that active growth occurs when conditions are most favorable, in addition to their roles of survivability and disseminability. This ecological adaptation would confer selective advantage upon organisms which live in fluctuating environmental conditions (Halvorson and Sussman, 1966).

Knaysi (1948) postulated that autogamic sexual processes were involved in the formation of endospores of bacteria. The claim was that at the time of sporulation the nucleus, consisting of a single chromosome, divided lengthwise equally, the two chromosomes subsequently uniting in an autogamous sexual process accompanied by chromatin reduction (Robinow, The Bacteria, 1960). However, recent electron microscopy of spore formation in bacteria has eliminated this possibility as fusion preceding spore formation was not observed (Young and Fitz-James, 1959). On the other hand, many dormant fungal spores are produced by the haploid portion of the life cycle (Halvorson and Sussman, 1966).

BACTERIAL SPORE FORMATION

Lamanna and Mallette (1965) state that (i) the optimum conditions for bacterial sporulation are like those for growth of the vegetative form, the permissible variation being within narrower limits than those for growth and (ii) the sporulation process commences after the period of most rapid vegetative growth. These two generalizations probably indicate that physiologically vigorous parent cells are required and that a trigger mechanism for sporulation is operative, as the sporulation in a culture is not a gradual process but one in which within a relatively short period of time sporulation is initiated and completed.

The sporulation process requires an extensive de novo synthesis based on low molecular weight presursors derived

from the breakdown of vegetative cytoplasmic components. Hölzmuller (1909) found that with five species of Bacillus endospores were formed only by well-nourished bacterial cells. Foster and Heligman (1949) demonstrated that Bacillus cereus grew well but sporulated poorly in a glutamate-salts medium. The addition of glucose increased growth by a factor of only 23% but increased sporulation almost 100 fold in comparison to the increase in growth factor. In this case it appeared that glutamate alone was adequate as a carbon source for vegetative growth but that cells produced in such a medium were lacking in energy reserves sufficient to cause a high degree of sporulation. Hardwick and Foster (1952) also found that several Bacilli grown on glutamic acid-salts medium sporulated poorly when transferred to distilled water; but, sporulation was increased by addition of glucose to the growth medium. They also found that cells of Bacillus mycoides produced in a medium low in nitrogenous material failed to sporulate when shaken in distilled water, whereas cells produced in a nitrogen-rich medium sporulated readily. Thus the ability of sporangia (cells) to form spores depends on the adequacy of the intracellular reserves. They concluded that a cell impoverished in regard to its protein content loses its ability to sporulate.

Many of the physiological features characteristic of bacterial sporulation are also found in the sporulation process of many fungi (Miller, 1959).

Sporulation or the conversion of a vegetative cell to the dormant state, is accompanied by extensive changes in the structure of the cell. The electron microscope has provided the means for the elucidation of the fine structure of the bacterial endospore: Bacillus subtilis, Takagi et al., (1956); Bacillus cereus, Young and Fitz-James, (1959); Bacillus polymyxa, Holbert, (1960); and many others. The role of internal membranes in the sporulation process has been clarified by the elegant studies of Fitz-James (1960, 1962a,b) in Bacillus and Clostridium and by Ohye and Murrell (1962) in Bacillus. Early in the sporulation process infolding of the cytoplasmic membrane occurs and a differentiated area termed the " forespore " is produced, a double membrane which surrounds the spore nuclear material. This process seems to be under the control of membranous organelles termed " mesosomes ".

Although the fine structure of endospores differs somewhat from one species to another, generally, the central core is surrounded by a delicate membrane termed the " spore wall ". This eventually transforms into the " cell wall " of the new vegetative cell produced upon germination (Mayall and Robinow, 1957; Hashimoto and Naylor, 1958; Robinow, 1960; Takagi et al., 1960). Surrounding the spore wall is a second layer, the " cortex ", which is thicker and of relatively low density. Enclosing the cortex is the " spore coat ", which is reported to consist of 1-4 layers depending on the

species (Robinow, 1953; Mayall and Robinow, 1957; Hashimoto and Naylor, 1958). The spore coat may be smooth, grooved, or raised into ridges. Finally, the " exosporium " may surround the entity fitting snugly at the sides but protruding beyond the end of the spore.

The remarkable resistance of the endospore implies that their chemical composition and physical structure differ radically from the parent vegetative cells. Sporulation is a process which includes chemical changes as well as physical changes which are visibly detected. One of the most striking features of endospores is that dipicolinic acid is present in all bacterial spores but is absent from the vegetative cells. This compound makes up 5-15% of the dry weight of the spore. Among others, this compound is released from the germinating spore and simultaneously to this the heat resistance property of the spore is lost. Thus it appears that dipicolinic acid is involved in the phenomenon of spore resistance (Powell, 1953).

FUNGAL SPORE FORMATION

Although most frequently filamentous in terms of microscopic structure, fungi also exist in single-celled forms, for example, many of the yeasts. Carpenter (1961) states that there are five classes of fungi belonging to a subgroup of the Thallophytes, which comprise the second division of the Plant Kingdom: Myxomycetes, Phycomycetes, Ascomycetes, Basidiomycetes, and Fungi Imperfecti. The terms

" molds " and " yeasts " have no taxonomic significance as they are colloquial designations for forms that cannot be accurately defined. Molds are usually described as filamentous, multicellular fungi in which the filaments or " hyphae " branch and sometimes rejoin to form a tangled mass which is referred to as " mycelium ". Molds reproduce by spore formation and two types of spores may be produced: sexual and asexual. Both of these types of spores are formed by the Phycomycetes and Ascomycetes, while only asexual spores are formed in the Fungi Imperfecti.

Asexual spores are produced by the mycelium without nuclear fusion. There are several kinds of asexual spores and the type of spore is more or less characteristic of the species. For example, sporangiospores are produced within a swollen structure, the " sporangium ", on the end of fertile hypha of a nonseptate mold or Phycomycete. The Ascomycetes and many Fungi Imperfecti bear exposed or unprotected spores, " conidia ", upon fertile hyphae called " conidiophores ".

The ascus is the one structure which is common to all fungi belonging to the Class Ascomyceteae. Sexual spores are produced following nuclear fusion. The young ascus is a binucleate cell which is well supplied with nutrients. The two nuclei which are usually considerably larger than those of the vegetative mycelium fuse forming a diploid nucleus. This nucleus enlarges quite considerably and divides meiotically to form four nuclei which in the great majority of cases

divides again. The portion of the ascus cytoplasm which surrounds each nucleus is separated from the remaining cytoplasm (= epiplasm) by the formation of the cell wall, thus forming the ascospore. The cytoplasm of the ascospore builds the endospore, the inner layer of the spore wall (Bessey, 1950).

Lowry and Sussman (1958, 1965) have studied the surface of ascospores of the fungus Neurospora tetrasperma in considerable detail. The " endospore wall " is the most internal surface structure of the spore and it surrounds the protoplast. The layer immediately surrounding the endospore is the " epispore wall ". The epispore and the endospore walls provide support and rigidity to the spore and maintain its shape. The " perisporic " structures include all the layers and all other elements of the spore which surround the spore proper and which can be absent without interfering with the functioning of the spore. The perisporic structures include the " subperisporic layer ", which is often very thin and on whose surface is a membranous pellicle, the " perispore ".

For the study of the physiology of endospore formation in " true fungi " the yeasts are the ideal representatives according to Miller (1959) in view of their predominately unicellular nature and the fact that the process of endospore formation can be accomplished by individual cells of sporogenic strains uncomplicated by a necessity for preliminary development of mycelium and fruiting bodies.

The ascospore of yeast was discovered in 1868 by DeSeynes and since that time intermittent attention has been given to the sporogenic process in yeast (Miller and Hoffmann-Ostenhof, 1964). Not as much is known concerning yeast sporulation as compared to bacterial sporulation, however (Palleroni, 1961).

Pontefract and Miller (1962) observed that yeast cells appeared to increase in size after transfer to a sporulation medium. It was discovered that small granules become visible in sporulating cells after five hours in sporulation medium; and these granules become more conspicuous as aporulation progresses. Nagel (1946) and Kleyn (1954) have noted the appearance of granular cytoplasm in sporulating yeast cells. Mundkur (1961b) using electron microscopy upon Saccharomyces yeast cells transferred from growth culture to acetate sporulation medium, observed that after six hours in this sporulation medium the dense heavy concentration of ribosomes typical of actively budding cells decreased greatly. Accompanying this decrease was a progressive vacuolization of the cell and the numerous small vacuoles which were formed increased in size and confluency with continued exposure to this sporulation medium.

Nagel (1946) and Pontefract and Miller (1962) observed that the nucleus appeared to become larger and less dense when the cell had been only a few hours in the sporulation medium. The diploid nucleus of the sporulating cell

divides reductionally (= meiotically) to form four haploid daughter nuclei. This was established by the work of Winge (1935) and Winge and Laustsen (1937) whose cytological and genetical evidence showed that in sporulation the diploid nucleus of the vegetative cell underwent reductional division to form a tetrad of haploid nuclei.

The deposition of the spore walls is the next stage in the sporogenic process. Mundkur (1961b) gave figures showing that each haploid nucleus along with a small quantity of cytoplasmic particles were enclosed within a vacuolar cavity and the spore wall was laid down along the outer margin of the clear zone surrounding each nucleus. Outer and inner spore walls form and as the spore matures the volume of the cytoplasm they enclose increases in size (McClary et al., 1957b; Hashimoto et al., 1958, 1960; Marquardt, 1963).

Nagel (1946), Magni (1958), and Pontefract and Miller (1962) observed unenclosed nuclei in the cytoplasm outside the spores. Thus even though a single nucleus is contained in each spore (Hashimoto et al., 1958) each nucleus of the tetrad does not necessarily become surrounded by a spore wall and so 1-, 2-, and 3-spored asci occur frequently.

A considerable amount of cytoplasm or epiplasm is located in the space between the immature spore and the ascus wall when the spore membrane first appears (Miller and Hoffmann-Ostenhof, 1964). Hashimoto et al. (1960) and Marquardt (1963) showed that this epiplasm contained lipid, glycogen, and mitochondria. The spores also contained mito-

During spore maturation the epiplasm diminishes in amount greatly and mostly disappears (Miller and Hoffmann-Ostenhof, 1964). Epiplasmic material was observed to add to the outer wall of the ascospore making it thicker and more electron dense by Marquardt (1963). Miller and Hoffmann-Ostenhof (1964) inferred that some of the disappearing epiplasm was transferred into the spores since the cytoplasm within the spore increased in volume during the maturation process. Marquardt (1963) showed that the spore cytoplasm became denser than the epiplasm so that the volume increase was not due to water absorption.

SPORE GERMINATION

CRITERIA

Germination has been described as a process which leads to an irreversible stage that is distinctly different from the dormant organism (Halvorson and Sussman, 1966). Thus the emergence of a germ tube from the spores of fungi or the unilateral swelling and/or release from the spore wall are examples of morphological alterations occurring which represent this irreversible stage. DeBary (1887) considered bacterial spores as having germinated when the spore had acquired all the characteristics of a vegetative cell. Physiological criteria such as loss of acid-fastness, or loss of resistance to environmental stresses, or respiratory rate increases, have been applied to the study of germination of spores in an attempt to detect the irreversible stage. The workers in the germination field are divided in their choice of criteria for germination- each selects the criterion which

Because the study of spore germination necessitates a criterion of germination, Halvorson and Sussman (1966) have presented " guide lines " useful for the evaluation of criteria of germination. They specify that the criterion chosen should adhere to the following principles. The technique should be accurate and convenient to use. Different criteria of germination will have to be used with different organisms as spores of various types germinate in a wide variety of patterns. The criterion should point out the stage which is irreversible or commits the spore to further development.

The subject of bacterial spore germination has been extensively studied for the last two decades. The bacterial spore is an excellent entity for the study of germination for many reasons. Sporulation of all of the cells in a culture can be obtained and many criteria of germination can be applied to follow the course of germination, such as differences in the morphological properties of bacterial spores and cells, or physiological and biochemical changes which occur during germination. No complications arise as there are only few vegetative cells, if any, present in the spore suspension and there is only one spore per cell. Thus optical density measurements and other physical-morphological techniques can be used, as there is little contribution to growth by vegetative cells present in the suspension to interfere with measurements.

According to Lamanna and Mallette (1965) cells soaked in carbol fuchsin take up phenol and dye preferentially from the staining solution. Organisms stained with carbol fuchsin generally appear more intensely coloured than the staining reagents applied; in other words, sharing of the dye and phenol occurs between the carbol fuchsin and cell according to a distribution coefficient favoring a higher concentration within the cell. This is expected as acid-fast organisms usually contain materials favoring the greater solubility of phenol and dye within the cell than in carbol fuchsin. Cellular lipids which are soluble in organic solvents (but insoluble in water) play a most important role in determining the distribution of dye and phenol between the cell and decolourizer. The phenol-dye in the cell acts as a part of the liquid phase. The cell wall of acid-fast organisms and bacterial endospores retards the penetration of the staining reagents due to permeability characteristics, and so, the temperature usually must be raised in order to drive the stain into the cells or spores within a reasonable length of time. However, this step was omitted in this study as it was found to be unnecessary for the staining of yeast ascospores, as well as eliminating a source of variability due to unequal heating times and strengths of application. A longer period of time of exposure to the stain at room temperature in a staining jar of the yeast ascospores allowed sufficient dye uptake. Upon addition of a decolorizer to nonacid-fast cells, the dye leaves the cell because of the greater solubility in the

inorganic acid. However, in the acid-fast entities the solubility of the dye-phenol is postulated to be relatively greater in the cells than in the decolorizer and as a result only small quantities of colour is lost. Acid-fast organisms must possess a cell wall which resists the rapid movement out of the cell of any of the dye and phenol that dissolved in the decolorizer within the cell.

Spores of yeast were freed from the asci by Emeis (1958) as a result of homogenization of sporulated cells with glass powder, a procedure which destroyed the ascus walls. Upon addition of paraffin oil to the mixture an emulsion or partitioning resulted as spores collected in the paraffin layer. As lipid molecules are more soluble in organic solvents it was assumed that the outer surface of the yeast spores must be lipid in nature. This and other evidence (Schumacher, 1926; Miller and Eelnurme, unpublished) indicates that a superficial lipid layer surrounds the spore wall in contrast to the vegetative cells. This hydrophobic lipid layer has been assumed to account for the failure of spores to stain readily with aqueous methylene blue (Lindegren, 1947) and ruthenium red (Miller and Kingsley, 1961) which easily stain vegetative cells. Electron micrograph study by Marquardt (1963) of yeast spores clearly showed that cytoplasmic material which was more electron dense that the inner spore coat or ascus wall was deposited on the surface of the spore forming an outer covering. The nature of the lipid substance surrounding the yeast spore has not been determined.

Vegetative cells of yeast and germinated ascospores do not retain the original dye colour as they are decolorized with acid-alcohol. Hashimoto et al., in 1958, used as their criterion of germination of yeast ascospores the complete loss of resistance of the yeast ascospores to staining by basic dyes after alcohol fixation.

Measurement of respiratory activity can serve as an indicator of germination using the Warburg respirometer and standard manometric techniques. This technique has been used with great success with both fungal and bacterial spores, as the germination of spores usually proceeds with increased respiratory activity. For example, Goddard and Smith (1937) studied the germination of Neurospora tetrasperma ascospores and observed increased respiration in the germinating ascospores. Also, Halvorson and Church (1957) used the Warburg respirometer to measure the oxygen uptake by dormant, activated, and germinating endospores of Bacillus cereus in the presence of glucose. It was found that dormant spores had an extremely low oxygen quotient whereas the value increased greatly during activation and germination processes.

BACTERIAL SPORE GERMINATION

The process of germination comprises several distinct stages and there is great variation in the process itself as there are a great variety of spore types which may germinate in different ways. Upon the transfer of bacterial endospores to a favorable environment the endospore proceeds to germinate

or revert back to the vegetative state once again. Various criteria of germination, such as dye uptake, manometric measurements, and direct microscopy, reveal that germination of the bacterial endospore occurs in stages.

The first stage consists of an increase in stainability of the endospore, a decrease in optical density, and a loss in the property of heat resistance. Also, aerobic species of bacteria show a rapid increase in respiratory activity. The spore volume undergoes its greatest increase in the second stage of germination. The linear increase to some final rate characteristic of the vegetative state in this second stage, as the germ cell having broken through the spore coat elongates and arrives at the point of fission. Almost all of the dipicolinic acid present in the spore is eliminated into the medium probably as a mixture of free acid and calcium chelate with a small quantity released as the monoethyl ester (Lamanna and Mallette, 1965).

FUNGAL SPORE GERMINATION

When fungus spores are placed in a suitable germination medium there occurs swelling of the spore followed by germ tube formation. Mandels and Darby (1953) observed that the spores of the fungus Myrothecium verrucaria swelled rapidly and germinated when in the presence of sucrose and yeast extract, a suitable germination medium for this organism. They found that the swelling of the spores was not a simple osmotic process but was accompanied by an increase in dry weight of the spores. Ohmori and Gottlieb (1965) also

observed swelling, the degree of which increased gradually with time when the following spores were placed in a germination medium: Trichoderma viride, Aspergillus niger, Penicillium atroventum, and Penicillium oxalicum. The morphology of fungus spore germination in Neurospora has been studied in great detail. One end of the ascospore develops a bulge and there is formation of a hyphal wall upon germination which is continuous with that of the ascospore (Halvorson and Sussman, 1966). Yanagita (1957) studied the germination of conidia of Aspergillus niger in great detail and distinguished two stages of swelling. The primary stage, endogenous swelling, occurred in the absence of carbon dioxide whereas the secondary or exogenous swelling stage required the presence of carbon dioxide for its completion. It was found by Wood-Baker (1955) working with Mucor that oxygen was required in the second phase instead of carbon dioxide and not in the first.

In addition to these morphological changes which occur early in the germination process, other physiological and biochemical changes occur as well. Spores of fungi like those of bacteria have been found to show marked increases in respiratory activity during the germination process. Sussman (1961) reported that dormant ascospores of Neurospora remain quiescent and metabolize at slow rates until they are activated by heat or chemical treatment. After the activation treatment, there is initiated in the spore a series of changes which result in increased respiratory rates

as well as increases in the respiratory quotient and loss in thermal resistance. The respiratory rate as a result of the " induction " to germinate or activation treatment, increases 10-40 times. However, the high rate has to continue for 2-3 hours if germination is to occur. A second increase in the respiratory rate is found upon germination, nearly doubling that of the activation period. Thus, three distinct phases in the rate of the respiration of the ascospores are detected: (1) dormancy, (2) activation, and (3) germination. Activation and germination in Neurospora occur readily in distilled water so that external substrates are not required. Lingappa and Sussman (1959) performed more work in this area of metabolism of germination of spores. As judged by their gradual disappearance from dormant ascospores of Neurospora tetrasperma, lipids are the substrates which are utilized during the dormant period. Carbohydrates do not appear to be used during the dormant period but begin to disappear almost immediately after the spores are activated. Lipids are also used during this activation period but in lesser amounts than carbohydrates. The proposed hypothesis by Sussman (1961) was that activation consisted of induction of an enzyme system through which trehalose was metabolized, whereas the dormant stage was restricted to a diet of endogenous lipids.

In contrast to Neurospora a great number of other fungus spores require exogenous substrates for germination. Mandels and Norton (1948), Terui and Mochizuki (1955), and Yanagita (1957) have all found this to be the case in

fungi such as Myrothecium viride and Aspergillus niger. Ekundayo and Carlile (1964) found that the initiation of germination in sporangiospores of Rhizopus arrhizus required the presence of glucose or fructose. Maximal spore swelling required in addition the presence of a nitrogen source. When germinating spores were transferred to a medium lacking glucose from glucose medium the swelling of the spores soon ceased.

Excretion of cell materials is another common physiological occurrence in germinating spores. It is well known that dipicolinic acid is excreted in large amounts from bacterial spores during germination (Powell and Strange, 1953). However, fungus spores, unlike bacterial spores are not known to accumulate dipicolinic acid in sporulated cells or excrete it into the medium. Pontefract and Miller (1962) made attempts to detect dipicolinic acid in ascospores of Saccharomyces cerevisiae but the results were negative. They concluded that if dipicolinic acid occurred in yeast spores at all, it was far less abundant than in bacterial spores. Sussman et al. (1956) showed that immediately after the start of germination in ascospores of Neurospora, ethanol and acetaldehyde accumulated in the medium. These substances were present in minimal amounts only in the dormant ascospores but were produced in large amounts immediately after activation. However, 2-3 hours later, the activated cells no longer produced these fermentation products. They concluded that enzymes and intermediates of the citric acid cycle have probably been synthesized

by this time and so more complete or further oxidation of the fuel molecules occurred. Nishi (1961) observed the exchange of labelled phosphate in conidia of Aspergillus niger with unlabelled phosphate in the medium during germination, and Yanagita et al. (1961) observed the release of polyphosphate. During the germination of these spores that was induced by L-alanine, it was found that carbon dioxide, pyruvate, and ammonia accumulated in the medium (Hoshino et al., 1962).

Sussman (1954) found that inorganic substances accumulated in the medium during germination of ascospores of Neurospora tetrasperma including calcium and sodium ions. Fermentation products and ions were not the only materials excreted from germinating spores. Mandels (1956) for example, detected that invertase was released from Myrothecium verrucaria spores. Thus enzymes can be released as well.

The sequence of events in the germination of conidia of Aspergillus niger is quite similar to the events observed in bacteria in regard to cellular composition and metabolism (Halvorson and Sussman, 1966). Yanagita et al. (1961) reported that in germination of Aspergillus niger, loss of heat resistance followed decreases in polyphosphate and subsequent synthesis of macromolecules. RNA synthesis began at approximately three hours after the start of incubation of conidia of Aspergillus niger whereas DNA and protein synthesis began later according to Yanagita (1957). Shepherd

(1957) found that protein nitrogen rose from 26% dry weight in the spore to 36% dry weight in the mycelium of Aspergillus nidulans.

Henney and Storck (1963a,b) have performed much work concerning RNA and ribosomes in the morphological states of Neurospora. They found that the dormant spores contained no polysomes but that these appeared in the early phase of germination. They concluded that germination was accompanied by mRNA synthesis and subsequent binding of ribosomes to form polysomes.

YEAST SPORE GERMINATION

Only very little systematic work on the germination of ascospores of yeast has been performed outside of the purely observational or descriptive early work. However, this is not the case for other fungi where spore germination studies have been a major area of research for a long period of time. Descriptive reports on the mode of germination are well represented in the earlier literature on reports such as Hansen (1894), Guillermond (1920), and many others. Most recently in 1958, Hashimoto et al. performed electron microscopy upon resting and germinating ascospores of Saccharomyces cerevisiae. However, very little work has been performed on the physiological basis of spore germination.

Hashimoto et al. (1958) placed yeast ascospores in a germination medium and observed the ascospores to swell, the result of which was breakage of the outer spore coat at

more than one point. The inner spore coat was more resistant to disruption as no breakage occurred in it. Simultaneously with the swelling process, there was formed a large vacuole near the nucleus in addition to several smaller ones, prior to the shedding of the outer spore coat. The resistant inner spore coat gave rise to the cell wall of the newly formed vegetative cell produced upon liberation. The ductile nature of the ascus wall made it possible for the bud to carry part of the ascus wall in the direction of bud formation. No alteration in internal or external structure of the nucleus was observed during germination.

For these purely observational studies upon yeast germination undefined media were used since there was no chemical study intended. An early worker, Hansen (1894), germinated Saccharomyces spores on a medium of variable composition- moût de bière or beer wort. Tomato juice was the medium used by Winge and Laustsen (1937) for their genetic work. Hashimoto et al. (1958) used a semi-defined germination medium consisting of glucose, yeast extract, peptone, KH_2PO_4, and $MgSO_4$ for their electron microscope work.

Nagashima (1959) must be regarded as the pioneer in the study of the nutritional and environmental aspects of spore germination in yeast. Purified agar with added substances was adjusted with phosphate buffer to the required pH. Loopfuls of spore suspension were placed upon sterile slides, covered with a cover glass, and sealed with vaseline. However, the purely observational criterion along with his

very incomplete series of experiments provide little information concerning the nutritional requirements for spore germination outside of a few basic facts. Nagashima found that the ascospores of the wine yeast employed did not germinate on plain agar even in the course of 50 hours. It was found that glucose alone, or tomato juice, was sufficient to allow germination to occur. Though ammonium sulphate alone did not permit germination to occur its admixture to glucose in an appropriate concentration promoted germination. Various glucose concentrations (0, 0.1%, 1.0%, 10%) were tested for their effect and Nagashima determined that 1% glucose sufficed for abundant germination. Too much ammonium sulphate, such as 5% or more, exerted an inhibitory effect upon germination when added to 1% glucose. The combination of 0.05% ammonium sulphate and 1% glucose proved to be the most effective germination medium. Germination appeared to be indifferent to the presence of vitamins and salts.

The optimum pH for germination on this agar medium was determined to be pH 6.5 and it was noted that the germination rate sank abruptly above pH 7. The spores were capable of vigorous germination on this medium at a temperature of 27-30° C.. Attempts were made to substitute sodium salts of pyruvic, citric, and acetic acids for glucose in the presence of ammonium sulphate; however, none of them allowed germination to occur. Some amino acids (number and type not revealed) were tested in 0.1% concentrations as substi-

tutes for ammonium sulphate for their availability as a nitrogen source. Without the concurrent presence of glucose these amino acids caused no germination of spores. L-glutamic and L-aspartic acids were found to show slight stimulatory effects when added to 1% glucose, but not to such a degree as ammonium sulphate, while DL-alanine had no effect.

Palleroni in 1961 was the only other worker to deal with the nutritional aspect of yeast ascospore germination. No reference to Nagashima was made by Palleroni so that it is unlikely that he was aware of his predecessor's work. His observations upon the germination of a strain of Saccharomyces cerevisiae, 20SJ, were similar to those of Nagashima's who employed a wine yeast (unrevealed strain and species). However, Palleroni's experiments were performed more systematically. Again a purely observational or morphological criterion of germination was used as Palleroni isolated single ascospores by the use of a Leitz micromanipulator and germinated them on washed agar containing various additions. Palleroni arbitrarily considered a spore as having germinated when it reached a specific morphological size and shape. " The observations reported in this paper constituted only a preliminary attempt to elucidate the nutritional factors involved in the process of germination of the yeast spore". (Palleroni, 1961).

It was found by Palleroni that maximum spore germination of Saccharomyces cerevisiae ascospores occurred in Wickerham's yeast nitrogen base-glucose medium (1946) which

is described under materials and methods. When this medium was diluted the rhythm of the morphological stages described by Palleroni was considerably altered. For example, with a 1:6 dilution the arbitrarily chosen stage which indicated that germination had occurred was delayed 3-5 hours. The ascospores were found to be able to germinate under " anaerobic " conditions ", that is, under cover slips, but the onset of germination was considerably delayed under this condition. Palleroni assumed that conditions would be anaerobic under this condition, that is, under cover slips. The influence of the various components of Wickerham's medium were tested alone and in combinations for their effect upon germination of the ascospores. The result was that glucose alone jproved as effective as the complete medium in promoting germination as germination only occurred when glucose was present. Some sugars other than glucose in a concentration of 1% were also tested with no other additions. Only fructose and mannose gave results comparable to those of glucose. Sucrose and galactose permitted the germination of a limited number of spores only, although Palleroni indicated that this germination could be due to impurities. Ascospores of the strain used did not germinate in the presence of acetate, ethanol, or pyruvate, as the sole source of carbon in germination media. The ascospores were unable to germinate in Wickerham's YNB medium where acetate was substituted for glucose. Optimum environmental conditions of temperature and pH were found to be similar to those obtained by Nagashima.

Yeast nitrogen base is a chemically defined medium developed by Wickerham (1946) for use in yeast taxonomy. This medium contains all the essential nutrients required for yeast growth except that carbon sources suitable for vegetative reproduction are totally absent. Included in this YNB medium is 0.5% ammonium sulphate which serves as a nitrogen source, as well as mineral salts, compounds containing trace elements and vitamins, all present in minute amounts. Three amino acids, L-histidine, DL-methionine, and DL-tryptophan, are also included in trace amounts because a few yeasts require these growth factors. This medium is marketed commercially by the Difco company. There is a description in greater detail of this medium to be found in Appendix I.

Subsequently, Difco has supplied a YNB medium modified by omission of these amino acids, as well as another YNB medium modified by omission of ammonium sulphate and the three amino acids. These media are of value for physiological studies as well in allowing the growth of yeast in a chemically-defined medium containing carbon and nitrogen sources in desired combinations and concentrations.

Because Miller (1959) has shown that " purified " agar may contain sufficient nutritives to support limited growth of yeast cells, it was not added to the YNB media in this study.

PURPOSE OF INVESTIGATION

The purpose of this investigation was to study the requirements for germination of ascospores of Saccharomyces cerevisiae using physiological criteria of germination (changes in acid-fastness and respiratory activity) in addition to the purely optical one used by the two previous workers. A more systematic study of the effect of both carbon and nitrogen sources upon germination was undertaken. In an attempt to explain the effect of these nutrients, germination was compared in aerobic and anaerobic environments. The final aim was to compare the responses of the three main phases of the life cycle of Saccharomyces cerevisiae, strain F493, that is , growth, sporulation, and germination, to the factors studied.

MATERIALS AND METHODS

YEAST CULTURES

The primary yeast culture employed during the course of this investigation was Saccharomyces cerevisiae Hansen, strain F493. This culture was originally isolated from a package of Fleischmann's yeast and has been used for many years at this institution by Dr. J. J. Miller and his associates in research upon sporulation. Preliminary experiments were performed with the F493 strain of yeast as well as with two other yeast strains:

(i) NCYC 79- a Fleischmann strain of Saccharomyces cerevisiae Hansen, obtained from the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, England.

(ii) Bobadilla 18- a strain of wine yeast of Saccharomyces chevalieri Guillermond, which was obtained from the Estación de Viticultura y Enologiá, Jerez de la Frontera, Spain.

Stock cultures of these organisms were maintained in the refrigerator at 6° C. on agar slants of a nutrient medium of the following composition:

1% glucose

2% Bacto-Agar

0.67% yeast nitrogen base

Yeast nitrogen base is a chemically defined medium developed by Wickerham (1946) containing 0.5% ammonium sulphate,

trace amounts of the amino acids L-histidine, DL-methionine, and DL-tryptophan, as well as minerals and growth factors in trace amounts. The detailed description of the yeast nitrogen base medium can be found in Appendix I. These stock cultures were transferred to fresh agar slants regularly at weekly intervals and allowed to grow 2 days at 27° C before being placed in the refrigerator. Inocula were removed from these slants when required.

Spores of the above mentioned 3 strains were produced in the usual manner, to be described below; however, in some instances, once the spores were produced they were air-dried following filtration through Millipore filters (pore size 0.8μ) and then stored in tightly capped jars containing anhydrous silica gel in the refrigerator. Thus a source of dried spores of the 3 types was available when required.

It was considered to be of considerable advantage to use dried ascospore preparations in addition to freshly prepared ascospores. This had the advantage of eliminating the variability which might be induced by slight differences in the physiological conditions among similarly prepared fresh ascospores, because dried samples were always taken from the same identical source. A second advantage is that the drying process should kill most, if not all, of the unsporulated cells: therefore, in respiratory experiments the contribution from these unsporulated cells would be

expected to be small. In comparison, however, approximately 30% of the cells in the fresh spore suspensions are viable-unsporulated and so would contribute to respiratory measurements. Since the production of 100% sporulation was not found possible in the F493 strain, the dried ascospore preparations provided an excellent basis for comparison with the fresh spore suspensions in regard to respiratory activity measurements. Ideally, total separation of unsporulated cells from asci would be desirable for this work; however, efforts by Snider and Miller (1954) to achieve this were not successful.

PRESPORULATION AND SPORULATION MEDIA

Vegetative cells were harvested by inoculation from yeast stock cultures into 250 ml. Erlenmyer flasks containing 50 ml. of " high nutrient medium ". This medium was devised by Wickerham (1951) and is of the following composition:

2% glucose
0.5% peptone
0.3% yeast extract
0.3% malt extract

The medium was modified by the inclusion of 0.1% KH_2PO_4 which reduced the pH of the medium to pH 5. This ensured an ample phosphate supply for growth and the low pH would discourage the development of any chance bacterial contaminations.

The flasks were placed on a water bath shaker, Warner-

Chilcott Laboratory Model, set at 27° C and were allowed to shake for 2 days at a speed of 60 shakes per minute, each with a horizontal displacement of 5 cm.. This water bath shaker was used for all the required incubations except for the early preliminary experiments where cultures were grown on an Eberbach shaker with a horizontal displacement of 5 cm., at room temperature, with a shaking speed of 60 per minute. Thus, conditions with respect to temperature and oscillation frequency were kept constant in this investigation.

After the growth period the cells were separated by centrifugation, washed 3 times with sterile distilled water, and placed in 250 ml. Erlenmyer flasks containing 1% potassium acetate. The cell density was adjusted to 50 million per ml. by the use of a haemacytometer counting chamber. These " sporulation flasks " were then placed on the water bath shaker again at 27° C for 2 or 3 days, depending upon the sporulation time desired. Pontefract and Miller (1962) observed that the first nuclear divisions occurred in some F493 cells after 15 hours in a similar sporulation medium. After 20 hours or longer in this acetate medium about 50% of the cells had developed into spore-containing asci and the spores were in varying stages of maturation. After 3-4 days 75-80% of the cells contained spores.

GERMINATION STUDIES

After incubation in the acetate sporulation medium the cells were again collected by centrifugation, washed 3

times with sterile distilled water, and then suspended in M/30 pH 6 potassium hydrogen phthalate buffer at a concentration of 15 million cells per ml. (adjusted with a haemacytometer counting chamber).

RESPIRATION

The Warburg respirometer employed to measure the gaseous exchanges was a Bronwill Series 5UV. For these experiments cells that had been 2 days in the sporulation medium were used. Although the percentage sporulation was not extremely high, approximately 65%, it was fairly constant from one experiment to the other so that there was nearly the same proportion of nonsporulated to sporulated cells in each suspension used. For convenience, suspensions of cells from sporulation cultures are termed ascospore suspensions although these are not suspensions of 100% asci. Two ml. of a spore suspension containing 15 million cells per ml. was placed in Warburg flasks and when carbon dioxide adsorption was required 0:4 ml. of 10% KOH was added to the center well. Anaerobic conditions were produced by the flushing of nitrogen gas through alkaline pyrogallate solution contained in a Fisher-Milligan gas washer (to remove trace amounts of oxygen from the cylinder nitrogen gas) through the Warburg flasks for a period of 10 minutes. The flasks were allowed to equilibrate to the 27° C temperature of the water bath before the contents of the side arm were tipped in.

The final conditions of the germination period in the Warburg vessels were a temperature of 27° C and a 10 mil-

lion per ml. spore suspension with an oscillation frequency of 90 per minute. The measurement of the respiratory activity of the spores was performed in yeast nitrogen base-germination medium containing individual carbon sources, each at a concentration of 1% by weight. The composition of the yeast nitrogen base medium is given in Appendix I. Readings were taken for a 4 hour period and the oxygen uptake, aerobic carbon dioxide production, and anaerobic carbon dioxide production were measured using standard manometric techniques as outlined by Umbreit, Burris, and Stauffer (1949). Some similar germination media were tested for their ability to support growth with 2 day vegetative growth cultures so as to obtain comparisons as to the effect of the carbon source upon both germination and growth.

ACID-FASTNESS

The second procedure used to follow the course of germination of the yeast ascospores was the utilization of the acid-fast staining technique. Yeast ascospores have a high lipoidal content and this is presumably why they are acid-fast organisms while vegetative cells and germinated cells are not. Thus staining samples of cells from the germination media at various time intervals can give an estimate of the proportion of acid-fast/nonacid-fast cells in the suspension.

The Ziehl-Neelsen acid-fast staining procedure (Society of the American Bacteriologists Manual of Methods, 1957) was modified slightly in this investigation by eliminating the heating step which was found to be unnecessary

and the method was standardized before use in this study. Smears were made of loopfuls of spore suspension on clean glass slides and these were immersed in Ziehl-Neelsen carbol fuchsin stain (0.3g. basic fuchsin in 10 ml. 95% ethanol mixed with 5g. phenol dissolved in 95 ml. distilled water) for 5 minutes in a staining jar. The slides were removed, rinsed by dipping thrice in beakers containing distilled water, and then placed in a staining jar containing acid-alcohol (9 ml. 37% HCl mixed with 300 ml. 95% ethanol) for 10 seconds for decoloration. Three dippings in distilled water to rinse the slides was followed by counterstaining in a staining jar containing methylene blue stain (0.3 g. methylene blue dissoved in 30 ml. of 95% ethanol and diluted with 100 ml. of distilled water) for 75 seconds. After this time the slides were again rinsed in distilled water, air-dried, and examined under oil-immersion.

The result was that spores stained red whereas non-sporulated cells and germinated spores stained blue. Each ascospore was counted individually as was each vegetative cell in random microscopic fields. All spores in an ascus were included in the enumeration as well as nonsporulated cells; for example, an ascus containing 4 ascospores, 3 of which remained red following staining, is counted as 3 acid-fast staining entities/ one nonacid-fast. In this strain of yeast, F493, most asci contain more than one ascospore and so the value of the percentage acid-fastness exceeds the per-. percentage sporulation. As the spores germinate a change in

the colour retained after staining results due to the differential staining properties of spores and germinated spores. The extent of the decrease in the value of acid-fastness in a given time period was used to express the relative amount of germination which had occurred. These experiments were performed in duplicate and at least 200 randomly selected cells were counted for each determination.

Other modifications or changes in the procedures used in this investigation will be made explicit when results obtained from these are presented.

RESULTS

LOSS OF ACID-FASTNESS DURING GERMINATION OF ASCOSPORES

FRESHLY PREPARED ASCOSPORES

Suspensions of ascospores of Saccharomyces cerevisiae, strain F493, from 2 day sporulation cultures were prepared in M/30 phthalate buffer. These ascospores were placed in Warburg flasks and the germination substrate, at final concentration 1% glucose plus 0.67% yeast nitrogen base, was tipped in from the sidearms at the beginning of each experiment. The ascospore suspensions at final concentrations of 10 million per ml. were allowed to germinate in these flasks at a constant temperature of 27^{0} C with shaking. At various time intervals, samples were removed from these " germination flasks " via platinum wire inoculating needles ending in 2 mm. loops and placed on clean glass slides. These slides were then subjected to the acid-fast staining procedure described above: the samples were then examined under oil-immersion to determine the percentage of red entities. The result of this procedure is provided in the following graph, Figure 2, which represents the average of 3 separate trials, each performed in duplicate.

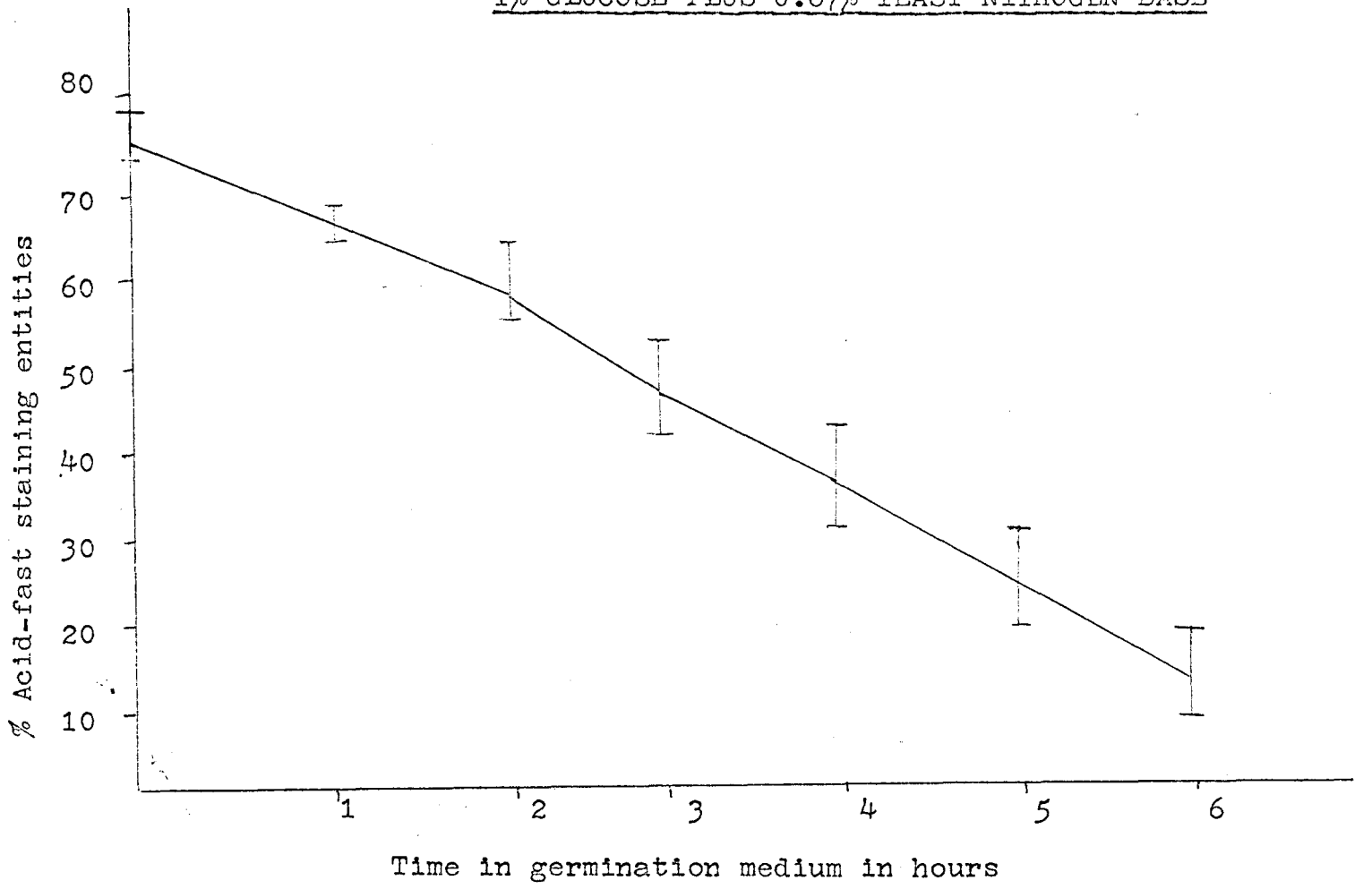
It was observed that the yeast ascospores had swelled or enlarged slightly after 1-2 hours in incubation in this germination medium under the conditions of the experiment. The initial sign of ascospore germination occurred as early

as one hour as the proportion of nonacid-fast staining entities had increased; however, it was not until after 2-3 hours of incubation that abundant asci were observed to contain enlarged ascospores having both red and blue colour in the same ascus. Conjugations between germinating ascospores were first observed at approximately 4 hours and became more frequent after 5 hours in the germination medium. Germination buds were also visible in the yeast ascospore samples around the same time that abundant conjugations were found, that is, after 5 hours in 1% glucose plus 0.67% yeast nitrogen base.

Many asci were found that contained both germinated and ungerminated ascospores during the germination period. The progressive increase in the proportion of nonacid-fast ascospores, shown in Figure 2, accompanied some other aspects of yeast ascospore germination, that is, the swelling of the spores, conjugations between spores, and the production of germination buds. No spores were observed which after having produced a bud remained acid-fast. Almost invariably, a sharp distinction was evident between the acid-fast and the nonacid-fast entities, and so as a result of this sharp " endpoint " the ascospores could be categorized without any doubts. Thus this staining procedure was found to be very useful for following the progress of yeast ascospore germination and gave a measure of the extent of ascospore germination at any given time.

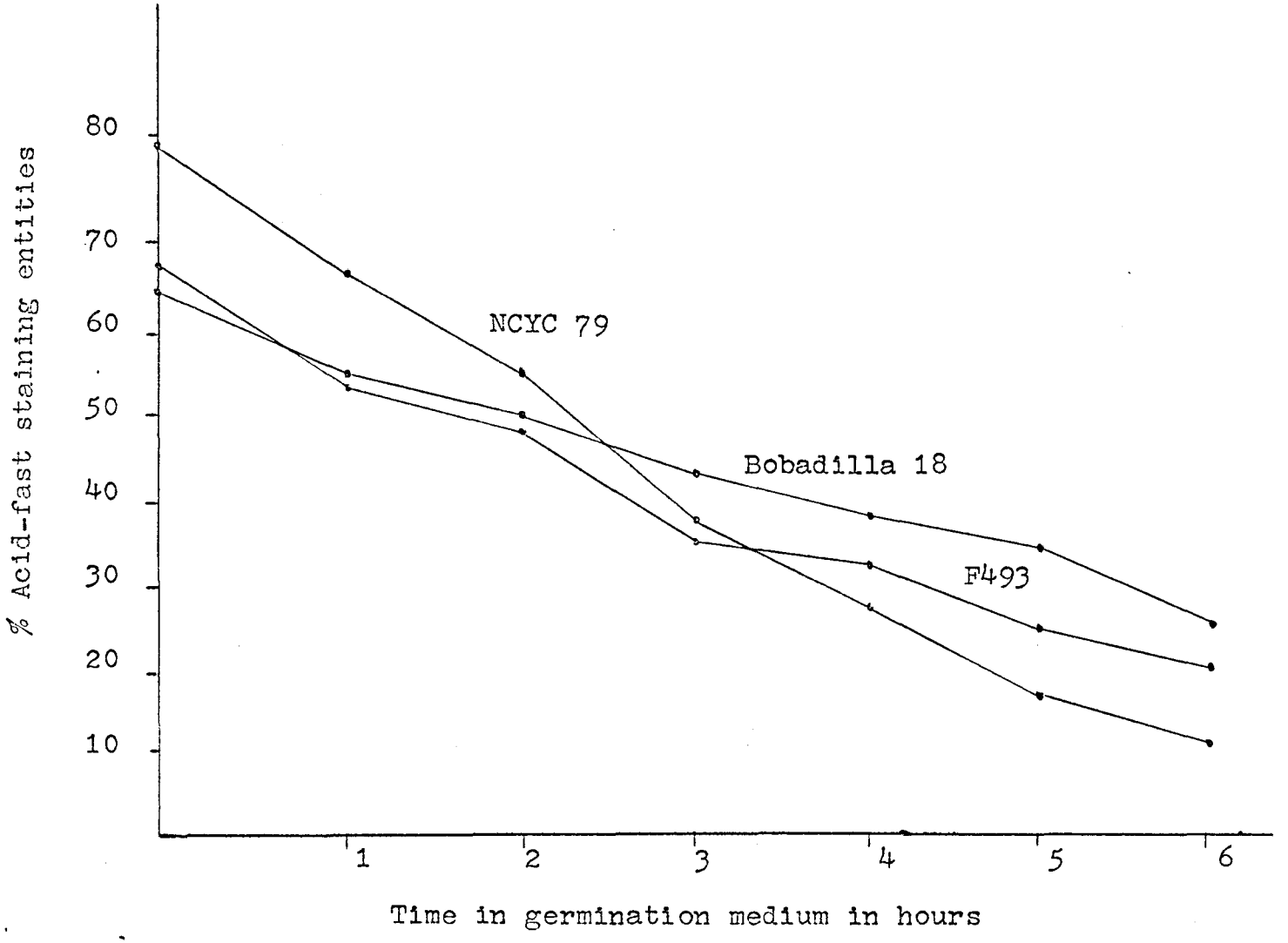
Note I. Final conditions of these experiments: temperature, 27° C.; yeast density, 10 million per ml.; total volume per flask, 3.0 ml.; pH, 6.0.

Figure 2. GERMINATION OF FRESH 2 DAY ASCOSPORES IN 1% GLUCOSE PLUS 0.67% YEAST NITROGEN BASE



Note I. Final conditions of these experiments: temperature, 27° C.; yeast density, 10 million per ml.; total volume per flask, 3.0 ml.; pH, 6.0.

Figure 3. GERMINATION OF DRIED F493, NCYC 79, AND BOBADILLA 18 ASCOSPORES IN 1% GLUCOSE PLUS 0.67% YEAST NITROGEN BASE



DRIED ASCOSPORES

Spore suspensions were prepared in M/30 pH 6 phthalate buffer of dried ascospores of F493, NCYC 79, and Bobadilla 18, which had been stored 2 months in the refrigerator in tightly capped jars containing anhydrous silica gel. The ascospores were allowed to germinate for a period of 24 hours in a germination medium consisting of 1% glucose plus 0.67% yeast nitrogen base. Loopfuls of samples of ascospores were removed at various time intervals and a similar procedure was followed as used for the freshly prepared F493 ascospores and the results obtained are provided in Figure 3, where each curve represents the average of 2 determinations for each strain.

It was observed that the proportion of acid-fast staining entities decreased with the time of incubation in the glucose plus yeast nitrogen base medium for all 3 strains. NCYC 79 germinated at a faster rate than either F493 or Bobadilla 18, whose rates of germination, that is, loss of acid-fastness were similar. After 6-8 hours in the germination medium the process of spore germination had been essentially completed with all 3 strains of yeast, and after 24 hours, no acid-fast entities were detected. By this time, a great deal of vegetative budding had occurred. The stages of germination similar to those observed with the fresh F493 ascospores were obtained with the dried F493 ascospores. It is observed in Figures 2 and 3 that initially both the dried and fresh ascospores lost their acid-fastness at about the same rate. However, in the final 3 hour period it appeared that the fresh ascospores lost their acid-fast staining capacity

at a slightly faster rate than the corresponding dried ascospores.

RESPIRATORY ACITIVITY OF ASCOSPORES IN 1% GLUCOSE PLUS 0.67% YEAST NITROGEN BASE

FRESH F493 ASCOSPORES

Ascospores of F493 from 2 and 3 day sporulation cultures were suspended in M/30 pH 6 phthalate buffer. These ascospore suspensions were placed in Warburg flasks at a final concentration of 10 million per ml. and their respiratory activity was measured using the respirometer after the germination substrate, 1% glucose plus 0.67% yeast nitrogen base, was tipped in from the sidearms. The results obtained are presented in Table 1 and Figure 4.

It is shown that the respiratory activity of the ascospores which had been the longest in the sporulation medium is less than those which were in the sporulation culture for a shorter period of time. The high respiratory quotient values and the large volume of carbon dioxide production under anaerobic conditions indicate that fermentation is more active than respiration in the early stages of ascospore germination.

DRIED F493 ASCOSPORES

Ascospore suspensions of dried 2 day spores of F493 which had been stored in the refrigerator in tightly capped jars containing anhydrous silica gel for 2 months were prepared and added to Warburg flasks. Glucose and yeast nitrogen base were added from the sidearms of the flasks and the

TABLE 1. Respiratory activity of freshly prepared F493 ascospores in glucose plus yeast nitrogen base.

% Sporulation	Age	Respiration in µl/ 4 hours/ flask: Anaerobic CO_2 production	Aerobic CO_2 production	Oxygen uptake	R.Q.
87%	2 days	311.9	281.5	63.6	4.4
		301.0	297.0	77.4	4.0
75	"	286.6	320.6	67.2	4.8
		272.7	285.2	70.1	4.0
74	"	301.0	296.0	77.4	3.8
		242.3	256.4	56.6	4.5
68	3 days	179.5	167.6	41.8	4.0
		172.6	153.6	38.5	4.0

TABLE 2. Respiratory activity of dried F493 ascospores in glucose plus yeast nitrogen base.

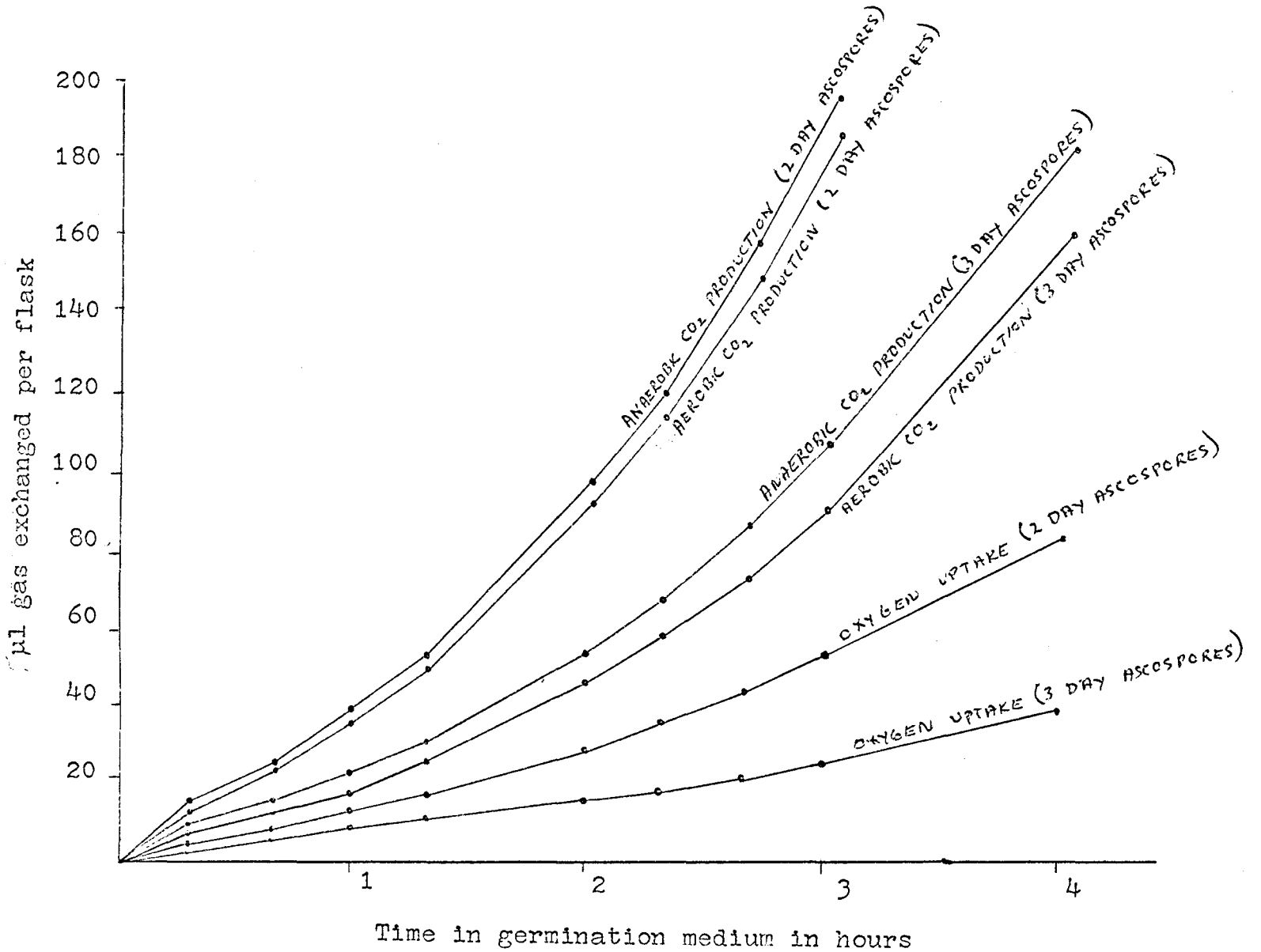
% Sporulation	Age	Respiration in µl/ 4 hours/ flask: Anaerobic CO_2 production	Aerobic CO_2 production	Oxygen uptake	R.Q.
67%	2 months	162.1	143.2	13.9	10.3
		160.5	146.4	15.2	9.6
		170.2	158.0	16.4	9.6

Note I. Each flask contained 10 million cells per ml. of which approximately 2/3 are sporulated. The corrsponding dry weight of suspension in each flask equivalent to 10 million cells per ml. is 1.6 mg..

Note II. Final conditions of these experiments: temperature 27°C., yeast density 10 million per ml., total volume per flask 3.0ml., pH 6.0.

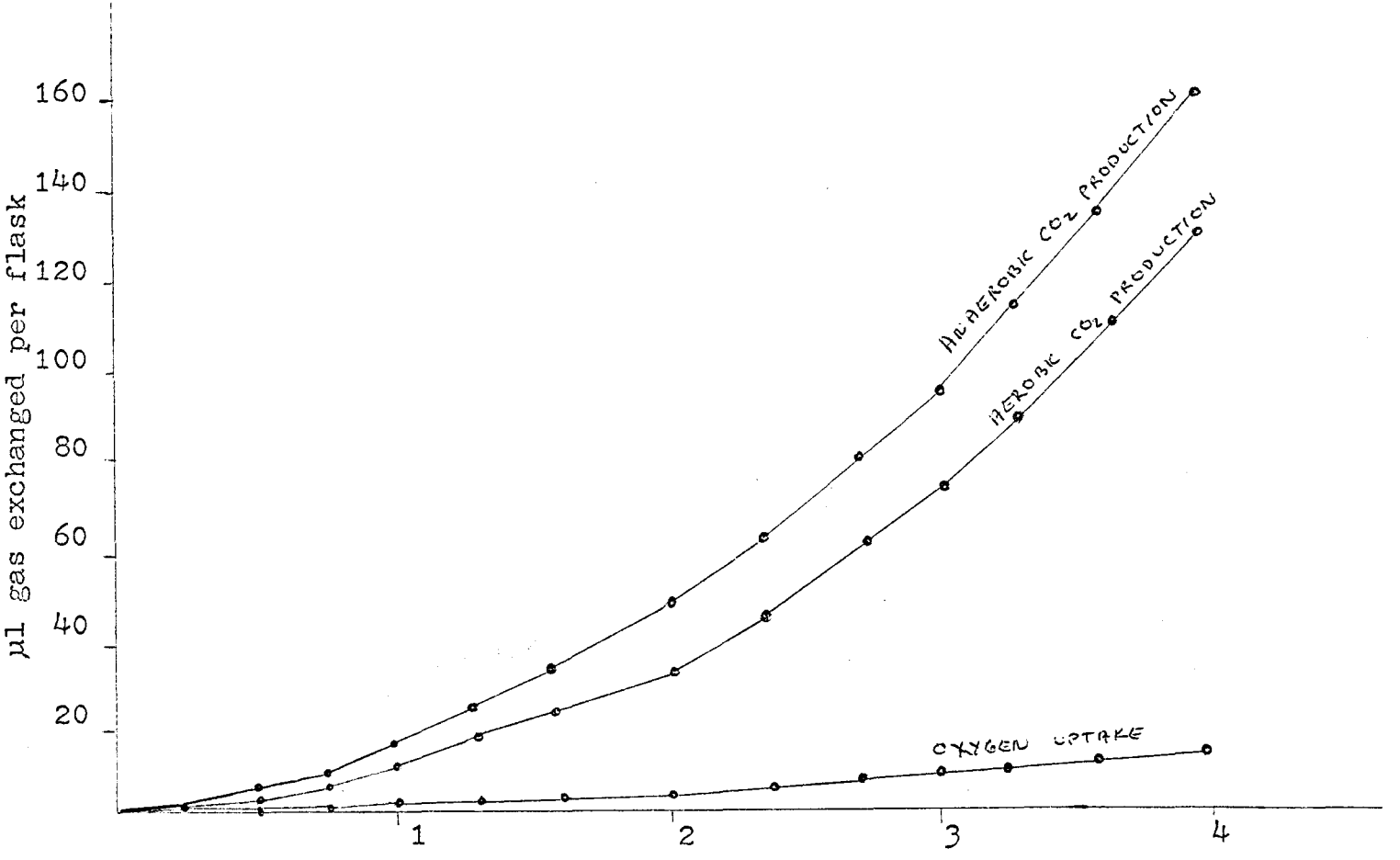
Note I. Final conditions of these experiments: temperature, 27°C.; yeast density, 10 million per ml.; total volume per flask, 3.0 ml.; pH, 6.0.

Figure 4. RESPIRATORY ACTIVITY OF FRESH F493 ASCOSPORES IN GLUCOSE PLUS YEAST NITROGEN BASE



Note I. Final conditions of these experiments: temperature 27°C.; yeast density, 10 million per ml.; total volume per flask, 3.0 ml.; pH, 6.0.

Figure 5. RESPIRATORY ACTIVITY OF DRIED F493 ASCOSPORES IN 1% GLUCOSE PLUS 0.67% YEAST NITROGEN BASE.



respiratory activity of the dried ascospores at a final concentration of 10 million per ml. was measured using standard manometric techniques as used above. The results obtained are given in Table 2 and Figure 5.

As shown, the amount of carbon dioxide produced both anaerobically and aerobically was fairly similar in both total volume and rate of production, but it was liberated in an amount which was far greater than the oxygen uptake. Although the amount of oxygen used was low in total volume in the 4 hour hour incubation period, in the glucose plus yeast nitrogen base medium, oxygen uptake became detectable after a long initial lag of approximately 90 minutes. Carbon dioxide production began after only a short lag of approximately 5 minutes. The great volume difference in the carbon dioxide production and oxygen uptake indicates that essentially only fermentation was occurring when the dried F493 ascospores germinated.

RESPIRATORY ACTIVITY OF DRIED ASCOSPORES IN VARIOUS CONCENTRATIONS OF GLUCOSE

Miller and Halpern (1956) performed a series of experiments comparing sporulation and growth of Saccharomyces cerevisiae, strain F49, in varying concentrations of glucose. They found that glucose in low concentrations (0.02-0.1%) stimulated sporulation whereas glucose in higher or moderate concentrations (0.33-1.0%) inhibited sporulation. On the other hand, growth was stimulated by the higher concentrations.

The oxygen uptake of suspensions of dried ascospores of F493 was measured using the Warburg apparatus. The germination medium consisted of 0.67% yeast nitrogen base to which 0, 0.05, 0.1, 0.5, and 1.0% glucose was added. The results obtained from this experiment are shown in Table 3.

It was observed that with low concentrations of glucose, or in the absence of glucose, the oxygen uptake by the dried ascospores was low in amount after 4 hours in the germination media. The greatest oxygen uptake occurred at the highest concentration of glucose used (1%). Thus the oxygen uptake increased as the concentration of glucose was increased up to the 1% concentration. The initial lag preceding oxygen uptake by the dried ascospores lasted approximately 90 minutes. The data presented in Table 3 indicate that 1% glucose plus 0.67% yeast nitrogen base is preferable to more dilute amounts of glucose in germination medium. This was the same concentration of glucose and ammonium sulphate (yeast nitrogen base contains 0.5% ammonium sulphate) found to allow maximum ascospore germination of wine yeast by Nagashima (1959). As a result of this evidence, 1% glucose plus o.67% yeast nitrogen base was chosen as the standard germination medium for this study.

TABLE 3. Oxygen uptake of dried F493 ascospores in various concentrations of glucose.

Germination medium	Percentage sporulation	Age	µl oxygen uptake/ 4 hours/ flask
0.05% glucose + 0.67% YNB	67%	2 months	4.7 3.9
0.1% glucose + 0.67% YNB			7.5 4.7
0.5% glucose + 0.67% YNB			11.1 10.6
1.0% glucose + 0.67% YNB			13.9 13.6
0.67% YNB			1.6 0

Note I. Final conditions of these experiments: temperature 27 C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

EFFECT OF VARIOUS COMPONENTS OF WICKERHAM'S MEDIUM UPON GERMINATION OF YEAST ASCOSPORES

A series of experiments was performed using both ascospores from 2 day sporulation cultures and dried ascospores of strain F493 in order to evaluate the effect of various components present in Wickerham's medium (1946). Measurements were made of the oxygen uptake of freshly prepared F493 suspensions from 2 day sporulation cultures containing 65% ascospores in phthalate buffer to which various additions were made. It can be concluded from the examination of the results presented in Table 4 that 1% glucose alone stimulates the uptake of oxygen by the freshly prepared ascospores. However, it is observed that the addition of 0.67% yeast nitrogen base to 1% glucose increased the uptake of oxygen. Addition of 0.1% ammonium sulphate to 1% glucose also increased the uptake of oxygen. Much less oxygen uptake was detected in germination media which lacked 1% glucose. The presence of the nitrogen source (ammonium sulphate) and vitamins and minerals without the concurrent presence of 1% glucose resulted in very little oxygen uptake.

Various components of Wickerham's yeast nitrogen base medium were tested further, both individually and in combination with 1% glucose, to determine their effect upon germination of ascospores of F493 as indicated by change (decline) in acid-fastness. The results presented in Table 5 again indicate that ammonium sulphate, either alone or in yeast

nitrogen base or in yeast nitrogen base minus amino acids, stimulates the germination of the ascospores when added to 1% glucose as detected by the decline in the percentage acid-fastness. Vitamins and salts appear to have no stimulatory effect either in the presence or absence of ammonium sulphate in glucose solution. Vitamin-free yeast base allowed germination to occur without further addition of glucose as it already contains 1% glucose along with 0.5% ammonium sulphate, and so stimulation of the yeast ascospore germination would be expected to occur and does so in this germination medium. No germination of ascospores occurs when the carbon source (glucose) is absent with both dried and freshly prepared F493 ascospores.

EFFECT OF CARBON SOURCES UPON GERMINATION OF ASCOSPORES

EFFECT UPON RESPIRATORY ACTIVITY OF FRESH ASCOSPORES

Suspensions of ascospores from 3 day sporulation cultures were allowed to germinate in Warburg flasks containing germination media consisting of 1% carbon sources plus 0.67% yeast nitrogen base. The results obtained from the measurement of the respiratory activities of the ascospores in the various media are given in Table 6.

TABLE 4. Effect of various components of Wickerham's medium upon oxygen uptake of freshly prepared F493 ascospores.

Germination medium	µl oxygen uptake/ 4 hours/ flask
1% glucose	31.5 34.1
1% glucose + 0.67% YNB	56.7 59.2
1% glucose + 0.1% ammonium sulphate	46.7 44.3
0.67% YNB	25.2 22.7
0.67% YNB minus amino acids	21.3 23.4
0.67% YNB minus amino acids and ammonium sulphate	19.6 25.4
buffer alone	24.2 23.8

Note I. Final conditions of these experiments: temperature 27 C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

TABLE 5. Effect of components of Wickerham's medium upon germination of dried and freshly prepared F493 ascospores.

Germination medium	Decrease in percentage acid-fast staining entities after 5 hours	
	Dried ascospores	Fresh ascospores
1% glucose	12, 14, 14	14, 12½, 14
1% glucose+0.67% yeast nitrogen base	41, 36½, 41	50, 52½
1% glucose+0.67% yeast nitrogen base minus amino acids	43½, 41	52, 54
1% glucose+0.67% yeast nitrogen base minus amino acids and ammonium sulphate	10, 12	12½, 13½, 11
1% glucose+0.67% vitamin-free yeast base	41, 44	50, 52
1% glucose+0.1% ammonium sulphate	31, 34	36, 38
0.67% yeast nitrogen base	0, 0	0, 2
0.67% yeast nitrogen base minus amino acids	0, 0	1, 3
0.67% yeast nitrogen base minus amino acids and ammonium sulphate	2, 0	0, 1
0.67% vitamin-free yeast base	18, 17	18, 20
0.1% ammonium sulphate	0, 1	0, 1
buffer alone	0, 2	0, 1½

Note 1. Final conditions of these experiments: temperature 27 C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

TABLE 6. Respiratory activity of freshly prepared F493 ascospores in various carbon sources.

Germination medium (+ 0.67% YNB)	% Sporulation	Respiration/ 4 hours/ flask: Anaerobic CO_2 production	Aerobic CO_2 production	Oxygen uptake	R.Q.
1% glucose	68	179.5 µl	167.6 µl	41.8 µl	4.0
		172.6	153.6	40.2	3.8
1% fructose	63	221.2	188.0	38.4	4.8
		246.1	205.0	44.0	4.6
1% mannose	64	112.1	98.3	23.5	4.2
		126.4	113.4	25.3	4.5
1% sucrose	67	135.0	126.1	31.1	4.0
		129.4	115.2	25.0	4.6
1% trehalose	67	4.2	19.6	20.4	0.9
		3.9	18.6	19.8	0.9
1% maltose	68	57.2	70.6	21.3	3.3
		61.4	71.7	24.7	2.9
1% galactose	69	56.0	68.7	20.9	3.3
		60.2	70.1	24.2	2.9
1% pyruvate	64	4.2	14.4	22.3	0.6
		4.2	15.8	23.1	0.7
1% potassium acetate	64	4.4	11.9	14.0	0.8
		4.4	8.6	13.9	0.6
1% dihydroxy-acetone	66	3.9	14.2	16.5	0.8
		2.8	12.2	13.4	0.9
1% ethanol	71	4.4	11.8	14.8	0.9
		4.5	14.2	15.0	0.8
buffer alone	68	5.2	29.5	28.2	1.0
		5.2	27.3	26.0	1.1
		6.0	29.0	23.7	1.2
		4.9	30.0	30.0	1.0

Note I. Final conditions of these experiments: temperature 27 C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

Some endogenous aerobic respiratory activity was observed from freshly prepared F493 ascospores, producing an R.Q. value of unity, whereas anaerobic endogenous respiration was slight. In germination media containing glucose, fructose, mannose, or sucrose, as the sole carbon source, active respiratory activity greater than the endogenous was measured. Large volumes of carbon dioxide production occurred in these germination media, both aerobically and anaerobically, and high values for the R.Q. of over 4 were obtained. With maltose or galactose as the sole carbon source in germination media, intermediate active respiratory activity was produced during germination of the F493 ascospores. Fairly large volumes of carbon dioxide production, both aerobically and anaerobically, were measured resulting in R.Q. values of approximately 3. Adaptation to the use of these 2 sugars occurred as the rate of respiration exhibited by the yeast ascospores in their presence increased more rapidly after 1-1½ hours in the germination medium. High R.Q. values and active anaerobic carbon dioxide production were characteristics of the germination of F493 ascospores in carbon sources supporting the germination process. Slight respiratory activity, both anaerobic and aerobic, were exhibited by the ascospores in trehalose, pyruvate, acetate, ethanol, and dihydroxyacetone germination media. R.Q. values of less than unity were produced in germination media containing these carbon compounds as sole carbon sources. By the use of the

increase in respiratory activity as the criterion of germination it was indicated that trehalose, acetate, ethanol, pyruvate, and dihydroxyacetone, did not support the germination of F493 ascospores.

EFFECT OF VARIOUS CARBON SOURCES UPON THE LOSS OF ACID-FASTNESS OF FRESH F493 ASCOSPORES

Suspensions were prepared using ascospores from 2 day sporulation cultures. Various carbon sources each at 1% concentration , plus 0.67% yeast nitrogen base, were added to the ascospore suspensions contained in Warburg flasks. These ascospores were allowed to germinate at the conditions of the study. Both initially and after 4 hours incubation in these germination media samples of the ascospores were removed and stained using the acid-fast staining procedure. The results obtained showing the decrease in percentage acid-fast staining entities are as shown in Table 7.

Maximum ascospore germination as evidenced by greatest loss in percentage acid-fast staining entities was obtained in germination media consisting of 0.67% yeast nitrogen base and 1% glucose, fructose, sucrose, and mannose. One percent maltose and 1% galactose allowed a smaller loss in the number of acid-fast staining entities in the presence of 0.67% yeast nitrogen base indicating that these sugars are either utilized at a slower rate or that adaptation to the utilization of these two sugars had to occur. The remaining carbon sources listed in Table 7 did not allow significant germination to occur. Thus, raffinose, melibiose, inositol, malic acid, xylose, ribose, glycolic acid, trehalose, and lactose, did

TABLE 7. Effect of carbon sources upon F493 ascospore germination as determined by loss in acid-fast staining entities.

Germination medium (+ 0.67% YNB)	Decreases in percentage acid-fast staining entities after 4 hours
1% glucose	53½, 58, 55½, 54½, 56
1% fructose	53½, 57
1% mannose	41½, 48
1% sucrose	50½, 49½
1% maltose	31, 34
1% galactose	27½, 38
1% trehalose	0, ½
1% lactose	½, ½
1% raffinose	1, 1
1% melibiose	4½, 2
1% D-xylose	1, 2
1% D-ribose	½, ½
1% malic acid	0, 2
1% i-inositol	3, 6
1% glycollic acid	4, 3
1% pyruvate	0, 1½, 1, 2½
1% potassium acetate	½, 1½, 3, 3½
1% dihydroxyacetone	0, 1½, 3, 3
1% ethanol	1, ½, 2½
buffer alone	2, 0, 1, 1

Note I. Final conditions of these experiments: temperature 27° C., yeast density 10 million per ml., total volume per flask 3.0ml., pH 6.0.

not allow significant germination to occur as evidenced by the slightness in the decline of acid-fast staining entities in these carbon sources. Ethanol, pyruvate, acetate, and dihydroxyacetone, did not allow any significant germination of the yeast ascospores. No germination occurred in buffer solution alone.

GERMINATION OF DRIED F493 ASCOSPORES IN VARIOUS CARBON SOURCES

Ascospore suspensions of dried ascospores of F493 from the same supply of dried spores as used in the previous experiments were prepared in M/30 pH 6 phthalate buffer and utilized in this study to test the respiratory activity of dried ascospores in various carbon sources. Conditions of the experiments remained constant except that in the germination media the carbon source to be tested was substituted for 1% glucose in the identical concentration. The results are provided in Table 8.

Highest respiratory activity was observed with the germination medium consisting of 1% glucose plus 0.67% yeast nitrogen base. There was low respiratory activity with acetate, ethanol, and dihydroxyacetone, each at 1% concentrations as the sole source of carbon in the germination medium. Pyruvate seemed intermediate between these three carbon sources and glucose in respiratory activity in allowing some carbon dioxide production to occur both anaerobically and aerobically. Endogenous respiration, that is, respiration in a medium lacking a carbon source was very slight. Oxygen uptake by the dried ascospores in media other than that containing glucose was very low and the characteristic long

TABLE 8. Respiratory activity of dried F493 ascospores in various carbon sources.

Germination medium (plus 0.67% YNB)	Respiratory activity/ 4 hours/ flask		
	Anaerobic CO_2 production	Aerobic CO_2 production	Oxygen uptake
1% glucose	162.1µl 160.5	143.2µl 146.4	13.9µl 15.2
1% potassium acetate	2.8 3.4	7.1 6.0	4.4 4.4
1% pyruvate	8.9 10.2	8.1 10.4	7.5 6.2
1% dihydroxyacetone	1.4 2.8	4.1 4.7	5.2 4.4
1% ethanol	3.3 3.0	2.8 3.8	4.4 3.3
buffer alone	1.3 2.7	1.4 2.8	1.2 1.6

Note I. Final conditions of these experiments: temperature 27°C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

initial lag before commencing was also observed. With glucose as the carbon source in the germination medium the initial lag period was shortest as at approximately 90 minutes a slow oxygen uptake was detected and the rate increased until the end of the 4 hour period.

The respiratory activity obtained when 1% pyruvate was the carbon source was slightly greater than the respiratory activity obtained with acetate, ethanol, or dihydroxyacetone. However, with glucose as the carbon source in the germination medium, a much greater respiratory activity was displayed by the dried ascospores as seen in Figure 5.

TABLE 9. Respiratory activity of dried F493 ascospores in various sugars as the sole carbon source in germination medium.

Germination medium (plus 0.67% YNB)	µl oxygen uptake/ 4 hours/ flask
1% glucose	15.9 14.2
1% fructose	14.8 13/9
1% sucrose	9.1 8.9
1% lactose	0 1.2
1% galactose	1.3 0

Note I. Final conditions of these experiments: temperature 27°C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

In another set of experiments, oxygen uptake by dried F493 ascospores in glucose was compared to oxygen uptake in germination media containing various sugars as the sole carbon source, each at 1% concentration, with the addition of 0.67% yeast nitrogen base. Results obtained are provided in Table 9 which follows. Fructose at a concentration of 1% was shown to be equally effective as 1% glucose in its utility as a carbon source for respiration as comparable volumes of oxygen were taken up by the dried ascospores in germination media containing each of these two sugars. Respiration also occurred in 1% sucrose as indicated by uptake of oxygen, but the volume of oxygen taken up was less than with either glucose or fructose. No respiration occurred in 1% lactose or 1% galactose. Freshly prepared ascospores of F493 have been shown previously to not germinate in lactose but to germinate in galactose to a moderate degree. Similarly, freshly prepared ascospores of F493 utilized glucose, fructose, and sucrose as sole sources of carbon for their germination.

EFFECT OF CARBON SOURCES UPON THE RESPIRATORY ACTIVITY OF VEGETATIVE CELLS OF F493

Vegetative cells from 2 day growth cultures were placed in buffer solution at a final concentration of 10 million per ml.. Various carbon sources at a final concentration of 1% were added to the cells contained in Warburg flasks in addition to 0.67% yeast nitrogen base. The respiratory activity resulting are provided in Table 10.

High respiratory activity of the vegetative cells was observed in the medium containing 1% glucose as the sole carbon source. A large volume of carbon dioxide, anaerobic and aerobic,

production, but which, nevertheless, was large. A high value of the R.Q. of approximately 5 was obtained indicating that fermentation was the main respiratory process functioning in the actively budding vegetative cells. With 1% sucrose a similar active respiratory activity was measured (although slightly less than 1% glucose) giving an R.Q. value of slightly less than 5. Intermediate values for the R.Q. were obtained with 1% maltose and 1% galactose (approximately 3 and 2 respectively) as the sole carbon sources in growth medium containing yeast nitrogen base. An adaptation period for the utilization of these sugars by the cells was required however, before the active respiratory activity observed began. R.Q. values less than unity were obtained with 1% acetate and pyruvate as carbon sources. Here aerobic processes predominated whereas anaerobic carbon dioxide production was slight. With trehalose, an important reserve carbohydrate, no respiratory activity was detected as it was not different from the controls. The features of the respiratory activity observed with trehalose closely resembled that obtained for the endogenous respiration of the vegetative cells as low anaerobic carbon dioxide production and a slightly active aerobic respiration producing an R.Q. approximating unity was obtained. Dihydroxyacetone as the carbon source inhibited the endogenous respiration of the vegetative cells as respiratory activity less than the endogenous values were obtained. Ethanol also seemed to inhibit the endogenous respiration of the yeast cells; however, the oxygen uptake was slightly greater than the endogenous value.

TABLE 10. Respiratory activity of F493 vegetative cells in various carbon sources.

Growth medium (+ 0.67% YNB)	Respiratory activity/ 4 hours/ flask			R.Q.
	Anaerobic CO_2 production	Aerobic CO_2 production	Oxygen uptake	
1% glucose	427.0 µl	533.4µl	105.6 µl	5.0
	470.4	559.3	107.8	5.2
1% sucrose	462.0	476.7	108.9	4.4
	443.0	461.6	92.4	5.0
1% maltose	127.4	200.8	63.8	3.1
	117.6	180.0	59.4	3.0
1% galactose	160.2	225.7	101.6	2.2
	154.0	208.3	98.3	2.1
1% trehalose	4.2	29.4	30.8	1.0
	4.2	28.0	29.7	1.0
1% acetate	13.7	122.0	143.3	0.9
	12.7	114.7	127.4	0.9
1% dihydroxyacetone	1.3	17.6	20.9	0.8
	0	16.5	19.8	0.8
1% ethanol	2.8	28.6	66.0	0.4
	2.8	22.0	56.1	0.4
1% pyruvate	15.0	69.0	91.0	0.8
	11.2	45.7	59.1	0.8
buffer alone	4.2	37.4	45.1	0.8
	4.2	30.8	35.2	0.9
	3.9	35.5	39.1	0.9
	5.2	31.4	35.0	0.9

Note I. Final conditions of these experiments: temperature 27°C., yeast density 10 million per ml., total volume per flask 3.0 ml., pH 6.0.

EFFECT OF NITROGEN SOURCES UPON SPORE GERMINATION

As indicated in the Introduction very little work has been devoted to the effect of nitrogen compounds upon ascospore germination. Palleroni (1961) tested only ammonium sulphate at 0.05% concentration. Nagashima (1959) found optimum germination in 1% glucose with 0.05-0.5% ammonium sulphate. Three amino acids only were tested by Nagashima for their effect upon ascospore germination. L-glutamic acid and L-aspartic acid, each at 0.1% concentrations, stimulated ascospore germination in the presence of 1% glucose, whereas DL-alanine (0.1 and 0.2%) was without effect. In order to compare Nagashima's results using a different strain of yeast and also to increase the information available on the subject of the effect of nitrogen compounds upon ascospore germination some further experiments were performed in this study.

Twenty-one individual nitrogen compounds were tested in this regard of which 15 were amino acids. L-glutamic acid, L-aspartic acid, L-proline, L-alanine, L-leucine, and L-phenylalanine, have been shown to be of value as nitrogen sources for growth of the F493 strain of Saccharomyces cerevisae by Miller (1963). L-proline has been found to be the only amino acid which accumulated during the sporulation process in this yeast as well (Ramirez and Miller, 1962). Also included were 2 amino acids which were found to support little, if any, growth of this strain of yeast, β-alanine and L-lysine. D-alanine was also included as DL-alanine was tested by Nagashima. A number of other common amino acids were tested as

well for comparison: L-leucine, hydroxy-L-proline, L-glutamic acid, L-asparagine hydrate, DL-aspartic acid, L-glutamine, and L-tryptophan. Some others, inorganic nitrogen sources, were also tested. Ammonium sulphate is one of the most commonly used nitrogen sources in yeast media. In addition, ammonium nitrate was also found by Miller (1963) to be of value as a nitrogen source for growth. Potassium nitrate was included in order to test the effect of nitrate-nitrogen upon germination in the absence of the ammonium ion, although Saccharomyces yeasts are not able to assimilate nitrate. Urea was included as it has been shown to be valuable as a nitrogen source for growth of this yeast strain as well as being a frequent inclusion in yeast nutrient media for the provision of a nitrogen source in yeast industry. Allantoin (a urea derivative) is known to be assimilated by yeasts (Ingram, 1955). A similar compound to allantoin, alloxan, was available in the laboratory and so was tested as well. Peptone and casein hydrolysate, two common mixtures of amino acids, were also included.

The effect upon germination of these nitrogen sources on both fresh and dried spores of F493 was measured using the loss in percentage acid-fast staining entities as the criterion of germination. The Warburg apparatus was employed so that the conditions of the experiments would remain constant. The ascospores were allowed to germinate for a 5 hour period after which time samples were removed and stained in the usual manner. The dried ascospores used were

from the same supply of refrigerated dried ascospores of F493 that were used earlier. Fresh ascospores which were used were obtained from 3 day sporulation cultures. The results obtained are presented in Table 11.

The nitrogenous substances were employed in 0.1% concentrations both in the presence and absence of 1% glucose, the same concentration of nitrogen used by Nagashima (1959). In addition, this concentration is of the same order (0.01M) that was used by Miller (1963) who tested the effect of nitrogen compounds on the growth and sporulation phases of this same yeast strain. Wickerham (1951) also used this approximate concentration of nitrate for nitrogen assimilation tests in yeast taxonomy.

Little germination occurred in media containing nitrogen sources where glucose was absent with the exception of peptone and casein hydrolysate, both of which promoted germination. Slight germination in the absence of glucose may have occurred possibly with L-alanine, L-leucine, L-aspartic acid, L-glutamic acid, and L-proline. Negligible decline of acid-fastness of both fresh and dried F493 ascospores occurred in buffer solution alone. When 1% glucose was added to the phthalate buffer solution declines resulted in both these types of ascospores.

The decline in the percentage acid-fastness in germination medium consisting of glucose and yeast nitrogen base minus ammonium sulphate and amino acids was similar to the decline produced in glucose alone. The other 2 yeast nitrogen

base media (which contain ammonium sulphate) allowed 3-4 times the decline in percentage acid-fastness as compared to 1% glucose alone. Thus it is evident that the ammonium sulphate contained in yeast nitrogen base is responsible for the stimulation of of germination in the presence of glucose. This confirms the conclusions drawn earlier from the results shown in Table 5.

Clear increases in the amount of germination was produced in the presence of 1% glucose by ammonium sulphate, urea, L-leucine, peptone, casein hydrolysate, L-glutamine, L-aspartic acid, and L-asparagine hydrate. Possibly smaller increases were caused by ammonium nitrate, allantoin, L-glutamic acid, and DL-aspartic acid. No nitrogen source was found to be more effective than ammonium sulphate although peptone and casein hydrolysate were equally effective. L-lysine seemed to have no effect upon germination although a slight reduction could also be possible. Very little germination in comparison to glucose alone was observed with D-alanine, β-alanine, potassium nitrate, alloxan, L-phenylalanine, and L-tryptophan, in glucose solution. L-isoleucine allowed somewhat less ascospore germination to occur than did glucose alone.

The results obtained for the effect of nitrogen sources upon freshly prepared ascospores in glucose were similar to those obtained with dried ascospores except that L-leucine, urea, L-glutamine, and L-aspartic acid, resulted in less decline in acid-fastness of dried ascospores.

TABLE 11. Effect of nitrogen sources upon F493 ascospore germination.

Nitrogen source	Loss in percentage acid-fast ataining entities			
	Fresh ascospores		Dried ascospores	
	plus glucose	no glucose	plus glucose	no glucose
D-alanine	0, 0, 0, 0	0, 1	7, 2	5, 2
B-alanine	2, 1, 2, 2	2, 0	2, 1	0, 1
L-alanine	10, 9, 10, 10	5, 7	15, 11	6, 4
L-leucine	31, 33, 36, 37	6, 2	13, 15	5, 3
L-isoleucine	5, 6, 8, 8	3, 6	8, 7	6, 3
L-proline	26, 17	5, 6	14, 15	6, 4
OH-L-proline	10, 9	2, 4	0, 4	2, 3
L-glutamic	20, 17	8, 5	18, 15	2, 0
L-glutamine	30, 33	0, 2	12, 16	1, 2
L-aspartic	36, 32	4, 6	15, 22	8, 9
DL-aspartic	17, 24	3, 7	15, 17	5, 2
L-asparagine hydrate	33, 36	1, 0	20, 23	2, 0
L-phenylalanine	1, 2	4, 2	0, 1	2, 3
L-trytophan	0, 2	3, 2	0, 0	0, 0
L-lysine	14, 11	0, 1	10, 13	2, 3
urea	28, 32, 33, 34	1, 8	19, 12	8, 3
NH_4NO_3	15, 14, 19, 18	0, 2	13, 15	2, 3
KNO_3	2, 4, 3, 2	3, 0	3, 1	0, 1
$(NH_4)_2SO_4$	36, 38, 51, 49	0, 1	31, 34	3, 4
peptone	40, 38	19, 17	28, 31	14, 16
casein hyd.	42, 47	12, 12	35, 37	10, 12

TABLE 11-continued

alloxan	4, 6	1, 1	4, 5	1, 0
allantoin	16, 18	3, 6	18, 15	3, 6
yeast nitrogen base	50, 55	0, 0	44, 41	2, 0
yeast nitrogen base minus amino acids	52, 54	1, 3	42, 45	3, 2
yeast nitrogen base minus amino acids and ammonium sulphate	15, 12	0, 1	10, 13	2, 0
buffer alone	15, 13, 12, 13, 14, 14, 12, 13	1, 1 1, 2	12, 13, 13, 12	0, 0 0, 0

Note I. Final conditions of these experiments: temperature, 27°C.; yeast density, 10 million per ml.; total volume per flask, 3.0 ml.; pH, 6.0.

EFFECT OF ANAEROBIC CONDITIONS UPON YEAST GERMINATION AND GROWTH

The results obtained in this study regarding the effect of various carbon sources upon yeast ascospore germination as measured by loss in acid-fast staining entities and respiratory activity have indicated that the process of ascospore germination is anaerobic. The achievement of strict anaerobic conditions is technically difficult (Wood-Baker, 1955). As a check upon whether or not yeast ascospores germinate anaerobically, further experiments were performed using the Brewer anaerobic dish.

In this method anaerobic conditions are obtained by adding reducing agents such as sodium thioglycollate to agar media and covering the agar with a Brewer's anaerobic lid. This lid touches the agar at all points at its periphery and so a perfect seal is obtained. The oxygen in the center area is removed by the reducing agent and so completely anaerobic conditions are achieved.

In this series of experiments both vegetative cells and ascospores of F493 were placed on agar media contained in Brewer anaerobic dishes. Two types of agar medium were used: (1) Wickerham's medium (1951) to which 5% S. P. agar and 0.2% sodium thioglycollate were added in pH 6 M/30 phthalate buffer. S. P. agar is a special purity agar of exceptional clarity and minimum metabolizable and inhibiting impurities obtained from Fisher Scientific. (2) N. I. H. thioglycollate broth which is of the following composition:

Bacto-yeast extract	5g.
Bacto-casitone	15g.
Bacto-dextrose	1g.
sodium chloride	2.5g.
L-cystine	0.05g.
thioglycollic acid	0.3ml.
distilled water	1000ml.

KH_2PO_4 was added to reduce the pH to pH 6 and then the medium was diluted with an equal amount of phthalate buffer and 5% S. P. agar was added. Once the agar had set in the petri dishes, 0.1ml. of both vegetative cells and ascospores, at a cell density of 10 million per ml., were placed in the center of each dish and the lids were set on, forming anaerobic conditions. These plates were incubated for a 5 hour period in the 27°C incubator after which they were removed and the yeast was examined under the microscope. The control plates consisted of 5% S. P. agar without addition.

Initially, the vegetative cells were well-separated on the control plates and 23% of them possessed a bud. After 5 hours no growth or vegetative budding was observed. As this is a highly purified agar containing no nutritives, no growth of vegetative cells would be expected.

After 5 hours incubation of the vegetative cells upon the agar plates where nutritives were added, a definite growth was observed as indicated by the large increase in the number of cells possessing a bud. Upon Wickerham's medium, plentiful growth occurred and the usual observation

was a group of 4-5 cells originating from an original single cell. Of the vegetative cells present after 5 hours incubation, 85% possessed a bud. Similar growth was obtained in the N. I. H. thioglycollate medium where again 85% of the cells possessed a bud after 5 hours. However, most of the cell groups comprised only 2 cells in comparison with cells grown upon Wickerham's medium where groups containing larger numbers of cells were common.

Ascospores of F493 did not germinate on plain 5% S. P. agar after 5 hours incubation. However, the ascospores did germinate on both types of nutritive agar plates under anaerobic conditions. Upon Wickerham's medium plentiful germination buds had developed as well as budding of the vegetative cells which were present. Similarly, germination was very frequent on the N. I. H. thioglycollate medium. Conjugations were seen in these dishes as well and so it is interesting to note that this phase of the life cycle can occur anaerobically also. This is strong evidence that ascospore germination occurs under strict anaerobic conditions as produced via the Brewer anaerobic dish.

DISCUSSION

RESPIRATORY CHANGES DURING SPORE GERMINATION

Yeast ascospores of strain F493, Saccharomyces cerevisiae, possess the capability for aerobic endogenous respiration. The endogenous respiration of freshly prepared ascus suspensions was determined in this study in phthalate buffer and the result was that some aerobic respiratory activity was observed producing an R.Q. of approximately unity. Anaerobic endogenous respiration was very slight as low volumes of carbon dioxide produced under anaerobic conditions were measured in comparison to the larger volumes of carbon dioxide produced aerobically. Upon measurement of the endogenous respiration of dried F493 ascospores, however, it was observed that this feature was almost nonexistent, both aerobically and anaerobically, as very low (probably not significant) values of respiratory activity were measured. It can be assumed as a result of these observations that the drying and storage process to which the F493 ascus suspensions were exposed in some manner destroyed the ability of the yeast ascospores to respire endogenously.

When freshly prepared F493 ascospores were placed in germination medium consisting of 1% glucose and 0.67% yeast nitrogen base, it was observed that after 4 hours very active respiration, both aerobic and anaerobic, had occurred which

resulted in an R.Q. value of approximately 4. It was also observed that the respiratory activity of ascospores which had been the longest in the sporulation culture was less than those in sporulation culture for a shorter period of time. The high R.Q. values and the great volumes of carbon dioxide production under anaerobic conditions indicate that fermentation is more active than respiration in the early stages of F493 ascospore germination, that is, germination of the F493 ascospore is an anaerobic fermentation process.

Similar observations were obtained when dried F493 ascospores were allowed to germinate in a similar germination medium as active anaerobic respiration was measured. These dried ascospores displayed active carbon dioxide production, both anaerobically and aerobically, whereas oxygen uptake was very low in comparison. The total volume of oxygen used by the dried ascospores was low in the 4 hour incubation period, and oxygen uptake became detectable only after a long initial lag of approximately 90 minutes. Carbon dioxide production and oxygen uptake resulting in an R.Q. of approximately 10 for the dried ascospores indicates that essentially only fermentation was occurring when the dried ascospores germinated.

In the case of the freshly prepared ascus suspensions it is inevitable that a portion of the respiratory activity measured is due to the presence of vegetative cells in the suspensions. However, in the suspensions of dried as-

cospores it is highly probable that vegetative cells have not survived the drying and storage process and so have been eliminated.

Although both the freshly prepared ascospore suspensions and dried ascospore suspensions exhibited anaerobic respiratory activity, it was much less in the dried ascospore suspensions. Also, the volume of oxygen taken up by the freshly prepared ascospores was much greater than the corresponding uptake by the dried ascospores and so it seems that the drying process employed in the storage of the F493 ascospores has damaged the oxidative mechanisms in the dried ascospore, possibly the mitochondria. However, it is reasonable to assume that more active respiratory activity would be exhibited by the freshly prepared " wet " ascospore in comparison to the dried ascospore as rehydration must occur in the dormant dried ascospore as well as activation and synthesis of enzyme systems, which either need not occur in the " wet " ascospore or can be performed much more quickly as the whole cellular metabolism has probably not been stopped completely as in the dried ascospore.

Germination of spores of fungi resembles germination of bacterial endospores in that increased respiratory activity usually accompanies the germination process. Gottlieb (1950) stated in his review upon fungal germination that germination was characterized by the physiological transformation to a state of high metabolic activity from low metabolic states in Sclerotinia fructicola, Neurospora sitophila, Neurospora tetrasperma, and Myrothecium verrucaria. Goddard (1935)

and Gottlieb and Smith (1937) used manometric techniques to measure the respiratory activity of ascospores of Neurospora tetrasperma: dormant ascospores when activated by heat or chemical treatment began to germinate using their endogenous substrates as energy sources (Sussman, 1961). Simultaneous to activation of the ascospore was increased respiration of the ascospores as oxygen uptake and carbon dioxide production was stimulated.

LOSS OF ACID-FASTNESS DURING SPORE GERMINATION

In this study, the modified Ziehl-Neelsen acid-fast staining procedure proved to be of great value not only in allowing the detection of ungerminated and germinated forms by the distinct colour differences, but also in allowing morphological changes in the process of germination to be clearly visible upon microscopic examination. Before germination occurs, there is a swelling of the yeast ascospores after 1-2 hours in a germination medium consisting of 1% glucose plus 0.67% yeast nitrogen base. Loss in percentage of acid-fast staining entities was measurable after 1-2 hours, but is was not until 2-3 hours in this germination medium that enlarged ascospores were more abundantly observed being red and blue in colour in the same ascus. The rate of loss of acid-fast staining entities increased slightly at this period in the germination process. Conjugations between adjacent ascospores were clearly visible on the stained slides

at the 4th hour, and these phenomena increased in frequency after this time. At approximately this time, buds became visible indicating that vegetative reproduction is beginning in the germination of F493 ascospores at the 5th hour in the incubation.

Since asci were detected frequently which contained both germinated and ungerminated ascospores, it can be assumed that asynchrony is a characteristic of ascospore germination in this yeast strain. A plausible explanation is that the meiotic segregations preceding sporulation produce spores of different capability for speed of germination.

The morphological aspects of yeast ascospore germination, that is, swelling, conjugations, appearance of germination buds, as well as the physiological (increased anaerobic respiratory activity) have been found to accompany the progressive loss of acid-fast staining entities in the germination medium.

Oxygen uptake, especially with the dried ascospores, was observed after approximately 90 minutes. By this time approximately $\frac{1}{4}$ of the spores had germinated as indicated by the decline in the acid-fastness of the ascospore suspensions. This indicates that oxygen uptake is not a good criterion of germination for the detection of the early phase of yeast germination. However, carbon dioxide production began almost immediately, both anaerobically and aerobically, and was found to progressively increase in rate during the period

of the experiment. This would suggest that carbon dioxide production is more suitable than oxygen uptake as a germination criterion for the early phase of spore germination. Possibly, oxygen uptake can be employed as a criterion of germination for the latter phases in spore germination.

In contrast, the decline of acid-fastness as a criterion of ascospore germination appears more suitable. The change was always sharp between acid-fastness and nonacid-fastness, that is, no intermediate colour between red and blue was observed. Unlike respiration, this criterion of germination is not affected by the presence of nonsporulated cells.

CARBON SOURCES AND SPORE GERMINATION

Before discussion can be made into the aspects of carbon metabolism in germinating spores it is necessary to distinguish between those fungus spores that require an exogenous (extracellular) supply of energy and those which contain sufficient endogenous reserves in order that spore germination can occur. It is generally accepted that germination of spores is an energy requiring process: the source of this energy is believed to be the oxidation of the endogenous reserves of the spore, and if sufficient carbon reserves are not present, then the organism requires an exogenous source of carbon. Conidia of Fusarium solani despite the fact that they contain approximately 20% lipid still require the addition of a carbohydrate in order to germinate.

Similarly, Myrothecium conidia also have sufficient endogenous reserves to germinate but still require exogenous substrates. In contrast to these spores is Neurospora, the ascospores of which can germinate even in distilled water. Uredospores of Puccinia utilize lipids as endogenous fuel for respiration and do not require the addition of exogenous nutrients (Gottlieb, 1950).

According to Colrider and Gottlieb (1963) the germination of fungus spores involves the oxidation of carbon compounds to produce the energy and the carbon skeleton needed for synthetic activities. These workers stated that the dissimilation of carbon compounds for both the production of energy and the synthesis of the carbon skeleton of organic molecules must be accomplished before a germ tube is developed. Enzymes required to mediate these reactions should either have been incorporated in the spore at the time it was formed or be synthesized de novo from endogenous or exogenous materials during the germination period.

It was found that ascospores of F493 do not germinate in phthalate buffer alone as indicated by the lack of loss of acid-fast staining entities. Thus the yeast ascospore requires an exogenous nutrient supply in order that it may proceed to germinate. Nagashima (1959) found that ascospores of the wine yeast employed did not germinate on plain agar which had been thoroughly washed with distilled water even in the course of 50 hours. Palleroni (1961) in working with strain 20SJ of Saccharomyces cerevisiae observed

that the ascospores did not germinate upon purified agar with no additions as no morphological changes were seen to have occurred.

The oxygen uptake of the dried F493 ascospores was measured in the presence of 0.67% yeast nitrogen base with varying concentrations of glucose added. In the absence of glucose, negligible oxygen uptake occurred after 4 hours incubation in phthalate buffer containing yeast nitrogen base. However, addition of increasing concentrations of glucose to the dried F493 ascospores resulted in oxygen uptake which was proportional to the concentration of glucose used. Maximum oxygen uptake was found at the highest concentration used, that is, 1%. One percent glucose alone allowed a fair amount of oxygen uptake to occur although the addition of 0.67% yeast nitrogen base or 0.1% ammonium sulphate stimulated oxygen uptake. In other experiments using acid-fast staining as the criterion of germination with both freshly prepared F493 ascospores and dried F493 ascospores, it was observed that without the addition of a carbon source to the germination medium very little, if any, decline in the proportion of acid-fast staining entities occurred in the course of 5 hours. One percent glucose alone allowed ascospore germination to occur as decline in acid-fastness was detected as well as morphological signs of yeast ascospore germination under microscopic examination. Addition of ammonium sulphate or yeast nitrogen base resulted in maximum germination as

greatest decline in acid-fastness occurred as well as plentiful germination buds and conjugations. Nagashima (1959) observed that glucose alone, or tomato juice (diluted), was sufficient to allow germination to occur. Although ammonium sulphate alone did not permit any germination to occur, its admixture to 1% glucose in a 0.05% concentration stimulated ascospore germination. Thus the stimulative effect of ammonium sulphate upon yeast ascospore germination in this study confirmed what was earlier found by Nagashima although the concentrations used varied slightly.

In this study evidence has been obtained that yeast ascospore germination is an anaerobic fermentative process, where energy for the germination is obtained through the breakdown of utilizable sugars. Respiratory measurements of the ascospores in the sugars supporting F493 germination indicate that very high R.Q. values and active anaerobic carbon dioxide production are characteristics of the germination process. Fructose and mannose as sole carbon sources in germination media containing yeast nitrogen base yielded respiratory activity similar to that produced in glucose, as active anaerobic respiratory activity and high R.Q. values of over 4 were obtained. When sucrose was utilized as the sole source of carbon in Wickerham's medium, respiratory activity slightly lower than that obtained with the hexoses was obtained which, nevertheless, was active anaerobically and produced a similar value of over 4 for the R.Q.. The

decline in acid-fast staining entities was greatest when the following sugars were used as sole sources of carbon in Wickerham's medium: glucose, fructose, mannose, and sucrose. Thus, maximum ascospore germination was found to occur in these sugars as evidenced by two distinct criteria for germination. Palleroni (1961) germinated strain 20SJ ascospores in Wickerham's medium containing individual sugars replacing glucose at the 1% concentration and it was observed that fructose and mannose gave results comparable to glucose. Sucrose allowed the germination of a limited number of ascospores only, and so it seems that quicker adaptation to/or faster production of the enzyme invertase occurred in the F493 ascospore in comparison to the ascospore of strain 20SJ.

Maltose and galactose gave a fair amount of germination as evidenced by the intermediate values for the loss of acid-fastness. These F493 ascospores also showed the phenomenon of adaptation to the utilization of a sugar as increased respiratory activity was measured after approximately 1½ hours incubation in germination media containing these sugars as the sole carbon sources. As a result of this adaptation, active respiratory activity was produced which although being less than that of the hexoses gave R.Q. values of approximately 3, as well as active carbon dioxide production under anaerobic conditions. Vegetative cells of the F493 strain of Saccharomyces cerevisiae have been shown to adapt to the utilization of these 2 sugars as carbon sources for vegetative

growth; thus, it would be expected that the ascospores of the same strain would adapt to these sugars. Palleroni (1961) obtained the germination of a limited number of ascospores of strain 20SJ upon galactose as a carbon source in germination medium; however, no germination was obtained with maltose. It seems that the F493 strain of yeast can adapt to the utilization of both these sugars while this did not happen in the yeast strain employed by Palleroni.

No germination of F493 ascospores was observed in germination medium containing trehalose as the carbon source as there was no loss of acid-fastness of ascospores in this sugar. In respiration experiments using this sugar as the carbon source, it was observed that the respiratory activity displayed by the ascospores was similar to the endogenous values, although being slightly lower in total volume of gases exchanged. This is not surprising as it was found that this important reserve substance in yeast inhibited the endogenous respiration of vegetative cells of F493. Thus it seems that this carbohydrate is metabolized only very slowly and is, in fact, a true storage or reserve carbohydrate in this yeast. Thus this strain of yeast does not germinate endogenously as occurs in Neurospora which utilizes its trehalose reserves in germination of its ascospores.

No germination of F493 ascospores was observed to have occurred as evidenced by lack of loss of acid-fastness when the following carbon sources were employed solely in germination media: raffinose, melibiose, malic acid, xylose,

ribose, glycolic acid, and lactic acid. These extraneously supplied carbon sources are not growth-supporting to the vegetative form to F493 cells and so it would be expected that no germination would occur upon them.

No germination or loss in acid-fastness of F493 ascospores was observed in germination media containing ethanol, acetate, pyruvate, or dihydroxyacetone, as the sole carbon source in Wickerham's medium. Very slight aerobic respiratory activity of the spore preparations was detected with these substances using the Warburg apparatus, less than endogenous, while the carbon dioxide production under anaerobic conditions was even less. R.Q. values of less than unity were obtained. Thus the end product of fermentation (ethanol) as well as acetate and pyruvate are not used as substrates for germination as they have to be broken down oxidatively via the TCA cycle. F493 vegetative cells can utilize these substances via terminal oxidation and as a result aerobic activity was detected. However, under anaerobic conditions terminal respiration is prevented due to the lack of oxygen. Dihydroxyacetone is an unusual compound in regard to this strain of yeast in that is supports sporulation in the presence of ammonium sulphate but does not allow vegetative growth. Nagashima (1959) obtained no germination of ascospores of a strain of wine yeast upon acetate; similarly, Palleroni (1961) observed no germination with either acetate or ethanol as carbon sources in germination media..

However, no respiratory activity measurements were made by these workers and it is quite conceivable that spores could respire acetate and not germinate on it. Here it is shown that failure to support germination is associated with failure to be respired as respiratory activity in the absence of germination was not observed.

NITROGEN SOURCES AND SPORE GERMINATION

From the examination of the results obtained in this study regarding the effect of various nitrogen sources upon germination of ascospores of Saccharomyces cerevisiae, strain F493, both in the presence and absence of 1% glucose as the sole source of carbon in germination media, the prominent feature is that maximum germination of the yeast ascospores does not occur in the absence of the carbon source. Furthermore, the concurrent presence of a nitrogen source may or may not result in stimulation of ascospore germination as measured by loss in acid-fastness during the incubation period. Because an incubation period of only 5 hours was used in this study, the results obtained are indicative of only the comparative rates of germination of the yeast ascospores in the various germination media and not the ultimate possibility of germination. Further germination could possibly occur if the arbitrarily chosen time period of 5 hours was entended.

This series of experiments was performed on freshly prepared ascospores as well as dried ascospores of the same strain. Similar results were obtained with both types of ascospore suspensions in germination media of similar composition although there were a few exceptions. The amount of germination of dried ascospores

in general, appaared to be slightly less in glucose medium containing the various nitrogen sources than the corresponding freshly prepared ascospores. Those nitrogen sources that stimulated germination of fresh spores had less effect upon the dried spores in glucose medium. This effect could possibly be due to the greater time required for " activation " of the dried spores in comparison to the freshly prepared ascospores.

In germination media lacking the carbon source, peptone and casein hydrolysate promoted the germination of a fair number of yeast ascospores of both fresh and dried types. It might be expected that all amino acids together, as in peptone and casein hydrolysate, should promote considerable germination as Thorne (1950) observed that mixtures of amino acids such as in casein hydrolysate required less sugar for assimilation into yeast protein by vegetative cells. Some of the individual amino acids, L-alanine, L-leucine, L-proline, L-aspartic acid, L-glutamic acid, resulted in slight declines in the percentage of acid-fast staining entities.

In germination media containing D-alanine, L-alanine, and β-alanine, in the presence of 1% glucose, germination of F493 ascospores occurred in L-alanine only, and to approximately the aame extent as in 1% glucose alone. With D- and β-alanine little occurred

in the presence of glucose. The inhibitory role of D-alanine to bacterial germination has been studied (Hills, 1949,1950; Powell, 1950,1957; Church et al., 1954). Wolf and Mahmoud (1957) observed that in bacterial strains responsive to L-alanine the inhibitory effect of D-alanine was only temporary in those strains where alanine racemase was present. Alanine racemase is not present in yeast (Hoffmann-Ostenhof, 1954). D-alanine is not included in protein synthesis. L-alanine has been shown by Miller (1963) to support excellent growth of this strain of yeast while β-alanine did not.

It is noteworthy that although L-leucine is highly stimulatory to ascospore germination, with L-isoleucine germination is less than in the controls, both in the presence of glucose. Both of these amino acids have been found to support excellent growth of vegetative cells when used as the sole source of nitrogen in growth media (Thorne, 1941; Miller, 1963). Differences may exist between the nutritional factors involved in the germination of bacterial and yeast spores as Hachisuka et al. (1955) reported that isoleucine was an effective germination stimulant for endospores of Bacillus subtilis when in the presence of glucose.

A similar effect of closely related compounds was obtained with L-proline and hydroxy-L-proline. L-proline was highly stimulatory to yeast ascospore germination in the presence of glucose while hydroxy-L-proline did not stimulate germination in glucose solution and may have reduced the germination of the dried ascospores. L-proline has been

found to accumulate in the yeast ascospore during the sporulation process. Thus it is interesting to note that although the spore contains a supply of L-proline, the exogenous addition of this amino acid in glucose medium results in stimulation of germination. Ramirez and Miller (1962) speculated that the endogenous supply of L-proline would function as a nitrogen source for ascospore germination and this is supported by the present study.

Great stimulation of F493 ascospore germination by ammonium sulphate resulted when the ascospores were placed in germination medium containing 1% glucose. Yeast have been shown to be able to derive an extracellular supply of nitrogen from simple substances in the course of vegetative growth (Ingram, 1955; Tremaine and Miller, 1956; Cook, 1957; Miller, 1963). Ammonium nitrate may have been slightly stimulating to ascospore germination in 1% glucose but not to the same extent as ammonium sulphate. Potassium nitrate was found to be unacceptable as a nitrogen source in Saccharomyces for vegetative reproduction (Miller, 1963) and so it is not expected to stimulate germination; but, its surpressing action was unexpected. Urea was also found to be a nitrogen source highly stimulating to ascospore germination when in the presence of glucose. This compound is known to serve well as a nitrogen source for vegetative growth as it has readily available amide groups.

Two aromatic amino acids, L-phenylalanine and L-tryptophan, were found to strongly reduce the germination of yeast ascospores in glucose medium as evidenced by the lack of decline of acid-fastness. Toxic aftereffects may be implied in the catabolism of amino acids. For example, Harris (1958) found that tryptophol produced from tryptophan inhibited yeast growth; this toxic alcohol could be produced here and prevent germination. The action of L-phenylalanine is unexpected as it has been shown to be useful as a nitrogen source for vegetative growth of this yeast (Miller, 1963). L-lysine had no effect upon germination in this study as an equal amount of loss of acid-fastness occurred in its presence as in glucose alone. This is not unexpected as L-lysine does not support vegetative growth of this yeast strain (Miller, 1963). Thus, there seems to be no close correlation between the utilization of nitrogen sources for growth and germination in this strain of yeast.

Aspartic acid, asparagine, glutamic acid, and glutamine, were all found to promote germination in the presence of glucose. These amino acids have all been found to be readily assimilated by yeast (Thorne, 1950; Ingram, 1955; Miller, 1963). Thus their stimulatory effect upon germination in glucose is expected. This also confirms the findings of Nagashima (1959) with aspartic acid and glutamic acid.

With respect to the reduction of germination in glucose with β-alanine, D-alanine, L-isoleucine, potassium nitrate, alloxan, L-phenylalanine, and L-tryptophan, this effect cannot be definitely attributed to inhibitory action at this time. Further experiments in this area are desirable involving the addition of ammonium sulphate along with these nitrogen sources in glucose solution so as to ensure an adequate nitrogen nutrition at the time of addition of these nitrogen compounds. The results presented here comprise only a preliminary study of the nitrogen nutrition in ascospore germination but are an advance on the contributions from the two previous workers.

GASEOUS ENVIRONMENT AND SPORE GERMINATION

The process of F493 yeast ascospore germination is characterized by increased respiratory activity and loss of acid-fastness. An end product of the glycolytic pathway in yeast (ethanol) and acetate were shown in respiration and acid-fast staining experiments not to allow significant germination of these ascospores to occur. But other carbon sources which are metabolized by this strain of yeast anaerobically, for example glucose, did support ascospore germination.

The two earlier workers in the area of yeast ascospore germination have also hinted that yeast ascospore germination is an anaerobic fermentation process. Nagashima (1959) germinated wine yeast ascospores on agar media under coverslips sealed with vaseline. Palleroni (1961)

approximated anaerobic conditions by also germinating ascospores of strain 20SJ Saccharomyces cerevisae under cover slips. In this study, the flushing of cylinder nitrogen gas through the Warburg flasks in order to create purely anaerobic conditions was performed in respiration experiments with the Warburg respirometer. Good ascospore germination occurred in these flasks in the presence of a fermentable sugar. However, the technical achievement of anaerobiosis is very difficult according to Wood-Baker (1955). Therefore, in order to check under more exacting conditions the capability of F493 ascospores to germinate anaerobically, use was made of the Brewer anaerobic dish.

In this method anaerobic conditions are obtained by the addition of a reducing agent, sodium thioglycollate, to agar media and covering the agar with a Brewer's anaerobic lid which creates a perfect seal by touching the agar at all points at its periphery. The reducing agent contained in the agar is oxidized at the expense of the oxygen is the center area, and so, completely anaerobic conditions are achieved.

Ascospores of F493 were observed to germinate on both types of nutritive (germination) media used under the anaerobic conditions obtained. Conjugations were observed to occur in these dishes after 5 hours incubation as well as the production of germination buds. Thus, conju-

gation , an important phenomenon in the yeast life cycle, can occur under anaerobic conditions as well. These observations are further strong evidence for the germination of yeast ascospores by the process of anaerobic fermentation.

Oxygen markedly affects the germination of spores, either by its presence or absence in the germination atmosphere, according to Halvorson and Sussman (1966). For example, oxygen has been found to be an absolute requirement for the germination of many fungus spores since most fungi are aerobic; for example, ascospores of Neurospora tetrasperma (Goddard, 1935). The amount of oxygen required for germination, however, may vary greatly depending upon the type and species of spore (Lopriore, 1885; Brown, 1922; Platz, 1928; Child, 1929; Wood-Baker, 1955).

Germination of some fungus spores may also occur under anaerobic conditions, as was found in spores of Tilletia contraversa (Boing et al., 1953), conidia of Sclerotina fructicola (Lin, 1940), and oospores of Phytophthora infestans (Uppal, 1926). The first stage in the germination of spores of Mucor has been found to be able to occur anaerobically (Goddard, 1935) but the spores were deactivated if oxygen was not admitted after 1 hour.

UNIQUE FEATURES OF THE THREE MAJOR PHASES OF THE LIFE CYCLE

Actively growing F493 cells have a strong endogenous respiration under aerobic conditions and the R.Q. approximates unity. Slight anaerobic carbon dioxide production

was detected only and according to Ingram (1955) these features are usually observed in the endogenous respiration of yeast in general.

Upon transfer of vegetative cells from growth culture to Warburg flasks containing 1% glucose and 0.67% yeast nitrogen base uptake much greater than the endogenous was measured. The carbon dioxide production under aerobic conditions was much greater than the oxygen uptake indicating the occurrence of a strong aerobic fermentation. The carbon dioxide production under anaerobic conditions was great and so active anaerobic respiration and a high R.Q. value were characteristic of the growing F493 cells.

Aerobically grown vegetative cells of Saccharomyces cerevisiae were found to possess numerous typical mitochondria (Agar and Douglas, 1957; Vitols et al., 1961) and an active cyanide-sensitive respiratory chain involving the cytochromes (Ephrussi and Slonimski, 1950; Slonimski, 1953). However, anaerobically grown cells were devoid of cyanide-sensitive respiratory chain and the classical cytochrome complement, that is, functional mitochondria, but, these cells regain these characteristics (mitochondrial function) upon aeration (Ephrussi and Slonimski, 1950; Ephrussi, 1953; Lindenmayer and Estabrook, 1958; Chaix, 1961; Lindenmayer and Smith, 1964). The results obtained from experiments by Criddle and Schatz (1969) indicate that anaerobic growth of Saccharomyces cerevisiae cells does not arrest the synthesis of mitochondrial inner membranes but merely modifies their composition. Thus the mitochondrial membranes present in anaerobic cells lack an

integrated electron transfer chain and presumably a functional oxidative phosphorylation system.

Upon transfer of vegetative cells of F493 to sporulation medium by Miller et al. (1959) a distinct decline in the value of the R.Q. became apparent within 4-6 hours. As the length of time in the sporulation medium increased, accompanying the great decline in the value of the R.Q. was the production of ascospores. Most responsibility for the decrease in the R.Q. lay in the great decrease in the carbon dioxide production although the ability to consume oxygen decreased as well.

Biochemical changes must be involved in the sporulation of yeast cells when the nuclei cease to divide mitotically and instead divide meiotically (Miller and Halpern, 1956). The decline in the value of the R.Q. is the most marked physiological change observed in sporulating cells and indicates a change over from the predominently fermentative mechanism occurring in growing cells to the oxidative mechanisms in sporulating cells (Miller et al., 1959). Vegetative cells did not sporulate in buffer and no decline in the value of the R.Q. appeared when the cells were placed in glucose solution. They speculated that the low R.Q. value was associated with the sporulation process as the decline

preceded the first appaarance of the ascospores and also that no ascospores were formed under anaerobic conditions.

The endogenous respiration of freshly prepared ascus suspensions in phthalate showed some aerobic respiratory activity producing an R.Q. of unity. Anaerobic activity was very slight as little anaerobic carbon dioxide production resulted. However, upon measurement of the endogenous respiration of dried F493 ascospores it was observed that this feature was almost entirely absent. Thus the drying and storage process to which the ascospores were exposed in some manner destroyed the ability of the ascospores to respire endogenously and to consume oxygen in the early portion of spore germination.

Upon transfer of ascospores of F493 to a germination medium consisting of 1% glucose and 0.67% yeast nitrogen base, it was observed that after 4 hours in this medium very active respiratory activity, both aerobic and anaerobic, had occurred producing an R.Q. value of approximately 4. This high R.Q. value and the great volume of anaerobic carbon dioxide production indicates that the germination of these ascospores is predominently an anaerobic fermentation process. Respiratory measurements of freshly prepared F493 yeast ascospores in a germination medium consisting of 1% acetate plus 0.67% yeast nitrogen base showed that slight aerobic respiration only was exhibited by the ascospores. Little germination, if any, was observed in this same medium using loss of acid-fastness as the criterion for germination. However,

vegetative cells of this yeast strain show active aerobic respiratory activity in acetate. As it would be expected that acetate would be oxidized via the TCA cycle, it is reasonable to assume that either the mitochondria in the ascospores are not functional or that the glycolytic pathway is the only mechanism which can provide the energy and intermediates required for ascospore germination. Thus, germination is somewhat similar to the vegetative growth of F493 cells as fermentation is the preferred mechanism functioning there as well, as high R.Q. values for both stages in the life cycle of yeast were obtained in glucose medium. The process of yeast sporulation obviously involves a dramatic change in the metabolism of the yeast cell. The R.Q. decreases greatly indicating that the capacity for aerobic glycolysis is almost lost. By restoration of the cell to germination medium, the characteristic high R.Q. value of growing cells reappears in a short period of approximately 4 hours.

The process of sporulation in yeast requires energy producing mechanism(s) in order to produce the energy and intermediates needed for synthetic processes. Sporulation is characterized by highly oxidative processes indicative of the changeover from the more fermentative processes occurring in vegetative reproduction. The mitochondria are, perhaps, the most logical sources for the production of this required energy and intermediates as evidenced by the utilization of acetate and ethanol as carbon sources for sporulation of the F493 vegetative cells. However, once the ascospores have

been produced, this high production is not required further as the ascospores are in the resting phase of their life cycle and as a result the mitochondria may begin to degenerate. As the length of time in the sporulation medium increases more cells reach their resting phase and so increasing loss of function of the mitochondria may occur. When the ascus suspensions are transferred to a suitable germination medium energy is again required; however, for germination processes. Hashimoto et al. (1958) have reported the lack of distinct cristae in spore mitochondria in Saccharomyces cerevisiae. As a result, the energy required for germination of the yeast ascospore could be produced from the anaerobic fermentation of fuel molecules provided in the germination medium. Much less respiratory activity and higher R.Q. values were obtained during the germination of dried F493 ascospores. Here the mitochondria probably have been highly damaged by the drying and storage process and so anaerobic fermentation predominates for a longer period of time. The synthesizing or repairing of spore mitochondria in a much shorter period of time by the freshly prepared yeast ascospores could possibly be another explanation of the slighly faster germination of the freshly prepared F493 ascospore in comparison to its dried counterpart.

The process of reversal from sporulation is a more rapid process than the change from the vegetative state to the dormant state according to observational evidence by Miller and Hoffmann-Ostenhof (1964). These workers put forward a hypothesis concerning the regulatory mechanisms controlling sporulation and germination. Derepression of biosynthetic enzymes which the cell

is genetically capable of forming occurs upon sporulation and this derepression results from the lack of nutrients in the sporulation medium. Germination or the reversal to the vegetative condition as induced by application of exogenous nutrients is caused by the repression of the biosynthetic enzyme systems active in the sporulation process. Thus, no exogenous trigger is involved for the initiation of sporulation, but rather a deprivation of essential nutrients for growth lowers the concentrations of repressors below the threshold value and the metabolism of the cell is changed accordingly. The placing of the ascospores in a medium favoring growth allows germination to occur. A mechanism may operate here as the mutrients could act as repressors to the enzyme synthesizing systems formed during sporulation and the metabolism characteristic of vegetative cells reappears.

Further work in this area of yeast ascospore germination especially in the role of mitochondria and enzymes in the germination process is required and the results that would be obtained should be extremely interesting. This study represents one of the first contributions to the study of the physiological aspect (metabolism) of yeast ascospore germination and the author hopes that further interest in this area will be aroused.

CONCLUDING REMARKS

Some conclusions concerning the Biology of Saccharomyces were able to be drawn from the results obtained in this study.

The fact that growth of Saccharomyces could occur both aerobically and anaerobically has been known for a long time. Also well established is the aerobic sporulation of Saccharomyces (Miller and Hoffmann-Ostenhof, 1964). In this study, three types of evidence have been obtained which indicate that the process of ascospore germination in Saccharomyces is an anaerobic process. (1) Germination has been found to be promoted by those carbon sources that can be fermented such as glucose, mannose, and fructose, whereas acetate or ethanol which are not fermented, did not. These observations confirmed the earlier findings of Palleroni (1961). (2) Carbon dioxide evolution but not oxygen uptake was found to accompany the early stages of the germination of the dried F493 ascospores. (3) Germination was observed to occur in this study under anaerobic conditions. Although this was previously claimed by Palleroni (1961) doubts can be given to the conditions he employed to achieve them, that is, germinating ascospores on agar under cover slips. The conditions used in this study were much more rigorous as nitrogen atmospheres in Warburg flasks as well as the Brewer anaerobic dish with thioglycollate medium were employed.

Thus the respiratory characteristics of the three main phases of the yeast life cycle were found to differ. This relationship has never been pointed out previous to

this study.

This aspect of the germination of the yeast ascospore as being anaerobic is potentially of great importance in Nature regarding the survival of Saccharomyces. Germination is likely to be favored by contact with fermentable carbon sources, and thus the yeast spores produced late in the fall should tend to remain in the dormant state until ripe fruit is again encountered the next year. Amino acid mixtures have also been found to stimulate ascospore germination; however, it seems less likely that these would be encountered in quantity in the natural environment of the yeast than the fermentable sugars.

SUMMARY

(1) Acid-fast staining proved to be valuable as a criterion for germination of yeast ascospores. The progressive loss of acid-fastness during germination was correlated with increased anaerobic respiration and morphological signs of germination.

(2) Upon germination of ascospores, increased respiratory activity was measured which was greater from fresh ascospore suspensions than from suspensions of dried ascospores. If the sporulation period of fresh ascospores was legthened, the respiratory activity detected during the subsequent germination was less.

(3) Germination of yeast ascospores was found to be predominently an anaerobic fermentation process. High R.Q. values active anaerobic carbon dioxide production were features of

the germination process which only occurred upon carbon sources which were fermentable by the F493 strain of yeast. An end product of the glycolytic pathway (ethanol) and acetate did not allow germination to occur.

(4) Further evidence for the anaerobic germination of yeast ascospores was provided by the use of the Brewer anaerobic dish. Conjugations between ascospores were also observed to occur anaerobically.

(5) Nitrogen sources and amino acids alone, in general, did not promote germination unless the carbon source (glucose) was concurrently present. Peptone and casein hydrolysate were the primary exceptions. The ammonium sulphate in the yeast nitrogen base growth medium stimulated germination with glucose. Peptone, casein hydrolysate, and ammonium sulphate, were equally effective in stimulating germination in glucose. Vitamins and mineral salts appeared to have no effect upon yeast ascospore germination.

(6) Some unique features, mainly respiratory in nature, of the three main phases in the yeast life cycle were pointed out and discussed.

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APPENDIX I

WICKERHAM'S (1946) YEAST NITROGEN BASE MEDIUM

ammonium sulphate	5g.
L-histidine monohydrochloride	10mg.
DL-methionine	20mg.
DL-tryptophan	20mg.
biotin	2mcg.
calcium pantothenate	400mcg.
folic acid	2mcg.
inositol	2000mcg.
niacin	400 mcg.
p-aminobenzoic acid	200mcg.
pyridoxine hydrochloride	400mcg.
riboflavin	200mcg.
thiamine hydrochloride	400mcg.
boric acid	500mcg.
copper sulphate	40mcg.
potassium iodide	100mcg.
ferric chloride	200mcg.
manganese sulphate	400mcg.
sodium molybdate	200mcg.
zinc sulphate	400mcg.
potassium phosphate monobasic	1g.
magnesium sulphate	0.5g.
sodium chloride	0.1g.
calcium shloride	0.1g.
distilled water	1000ml.

YEAST NITROGEN BASE MINUS AMINO ACIDS

Same as above except that L-histidine monohydrochloride, DL-methionine, and DL-tryptophan have been excluded.

YEAST NITROGEN BASE MINUS AMINO ACIDS AND AMMONIUM SULPHATE

Same as above except that ammonium sulphate in addition to the three amino acids have been excluded.