THERMAL INJURY AND RECOVERY OF *SACCHAROMYCES CEREVISIAE* Y25

by

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ABSTRACT

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The purpose of this investigation was to determine the influence of thermal stress on Saccharomyces cerevisiae Y25. Viability and respiration were measured in resting cells of S. cerevisiae subjected to heating at 56 C for 0 to 5 minutes. Immediately after heating plate counts on potato dextrose agar (PDA) were up to 1.5 log cycles lower than those on plate count agar (PCA). The proportion of the population affected was related to the severity of heat-stress. Delayed plating after storage at 22 C in distilled water resulted in increased plate counts on both PCA and PDA. Cannibalistic growth studies and recovery in the presence of growth inhibitors revealed increased plate counts during the first 12 hours of storage were related to recovery from injury rather than cryptic or cannibalistic growth. Recovery was prevented by storage at 4 C or in the presence of 2,4-dinitrophenol but was not prevented by storage in the presence of cycloheximide, chloramphenicol, hydroxyurea, or actinomycin D. Reduced recovery of thermally injured

cells on PDA in comparison to PCA was related to glucose concentration. Recovery on a minimal medium (MM) was also related to glucose concentration, however, recovery on MM containing filter-sterilized glucose was considerably higher than recovery on MM containing steam-sterilized glucose.

Respiratory activity of thermally stressed cells reflected the severity of the heat-stress. The endogenous respiration was approximately 40 µl/mg/hr for cells heated for 2 minutes at 56 C as compared to 2 µl 0_2/ml/hr for nonheated cells. There was a distinct decrease in respiration after 1 to 3 hours, but after 20 hours the respiration rate of heated cells was less than that of nonheated cells. Along with the abnormal rates of endogenous respiration, respiratory quotients of cells were altered after heat stress. Addition of 2,4-dinitrophenol stimulated 0_2-uptake in nonheated cells but decreased 0_2-uptake of heated cells. Due to the high rate of endogenous respiration, addition of glucose resulted in no substantial change in the rate of respiration of heated cells. However, glucose caused a delay in the characteristic decrease in respiration observed in heated cells.

DEDICATION

To my parents.

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INTRODUCTION

Over the past decade food microbiologists have become increasingly aware of the implications of sublethal injury to microorganisms with respect to the interpretation of data from the microbiological examination of food (Ordal, 1971; Hobbs and Olson, 1971; Busta, 1976). With continued emphasis being placed on food safety and quality as illustrated by the adoption of microbiological standards for foods, methods of microbiological analyses must undergo further scrutiny to ensure the best possible and most representative evaluation of the microbial population.

Although bacterial injury and recovery has been, and continues to be, investigated extensively, injury and recovery of yeasts and molds has received less attention. Investigations concerned solely with injury and recovery of yeasts and molds are few; however, a number of other studies contain data pertaining to this area.

One example concerns the use of acidified potato dextrose agar (APDA) which is recommended for the enumeration of yeasts and molds in many food products (APHA, 1976). Acidified potato dextrose agar gave lower estimates of fungal populations in foods than media which were nearer to neutrality in pH and contained antibiotics or dyes to inhibit bacterial growth (Skidmore and Koburger, 1966; Mace and Koburger, 1967; Koburger, 1970,

1971, 1972, 1973; Ladiges et al., 1974). Undefined environmental stresses were assumed to result in a sublethally injured fungal population sensitive to low pH. However, Koburger and Farhat (1975) reported use of PDA plus 100 mg/l each of chloramphenicol and chlortetracycline gave estimates of fungal populations similar to several other media, including plate count agar, malt agar, and mycophil agar.

One specific factor often considered important in consideration of environmental sensitivity of microorganisms is heat-stress. Nelson (1972) reported maximum recovery on PDA of heat-stressed yeast required adjustment of PDA to pH 8. Recovery of injured yeast populations at pH values above and below pH 8 varied and was markedly reduced with some strains. Stevenson and Richards (1976) found that pH was not the major factor in differences observed in plate counts of thermally stressed yeasts obtained on APDA, PDA, and plate count agar (PCA). Plate counts on APDA and PDA were equivalent and those counts were substantially lower than plate counts on PCA. Obviously, several questions remain concerning the recovery of thermally injured yeasts. The purpose of this investigation was to further study thermal injury and recovery of Saccharomyces cerevisiae Y25.

LITERATURE REVIEW

Manifestations of thermal injury reported to occur in yeasts and molds may be divided into several categories (Stevenson and Graumlich, 1978). These categories include increased sensitivity to environmental conditions, alterations in growth and cell characteristics, and subcellular alterations.

Sensitivity to Environmental Conditions

As a result of thermal injury, some fungi exhibit increased sensitivities to NaCl, water activity, pH and temperature.

NaCl and Water Activity. Fries (1969) reported thermally stressed cells of Ophiostoma mutiannulatum and Rhodotorula glutinis were sensitive to NaCl and other inorganic salts containing Cl^- or Br^-. The sensitivity, expressed as decreased recoveries on media containing Cl^- or Br^-, was partially reversed by D- and L-histidine as well as some other imidazole compounds. Later, Fries (1972) found exposure to 2,4-dinitrophenol resulted in a similar sensitivity to halogens.

Tsuchido et al. (1972a) also found Candida utilis had an increased sensitivity to NaCl as a result of thermal stress. Candida utilis was normally able to grow on Czapek-Dox medium containing 7% NaCl. However,

sublethal thermal stress (45 C for 10 min) resulted in a 90% reduction in viability on Czapek-Dox medium + 7% NaCl as compared to Czapek-Dox medium. Delayed plating following storage in culture medium, phosphate buffer, or distilled water for 5 to 6 hours allowed restoration of salt tolerance. Inhibition of recovery by 8-azaadenine and cycloheximide suggested RNA synthesis and protein synthesis were necessary for recovery. Tsuchido et al. (1972b) and Shibasaki and Tsuchido (1973) reported that although sorbic acid did not prevent recovery of salt tolerance, thermal destruction was greater in the presence of sorbic acid. Inhibitions of protein synthesis and respiratory activity were noted when sorbic acid was present during storage.

Adams and Ordal (1976) investigated the effects of thermal stress on Aspergillus parasiticus and reported decreased viability of conidia on solid media containing 10% NaCl. Reduced viability was also noted on MPN enumerations in liquid media at lowered water activity (A_w). Storage at an A_w of 0.92 prevented recovery when NaCl, glycerol and sucrose were used as solutes to control A_w.

Gibson (1973) also reported an increased sensitivity to A_w after thermal injury. Thermal destruction of Saccharomyces rouxii and Torulopsis globosa was studied at high sucrose or sucrose + glucose concentrations. After heating, these osmophilic yeasts appeared to require less osmophilic growth conditions.

pH. The effect of pH on recovery of thermally stressed yeast was investigated by Nelson (1972). Ten species of yeast were subjected to sublethal temperatures and recovered on PDA adjusted to a wide range of pH values (2 to 10). Maximum recovery for all species was at pH 8, except C. utilis, which had maximal recovery at pH 10. Recoveries at pH 3.5 ranged from 1 to 100% of recoveries at pH 8 for the various species. The results would seem to correspond with several investigations concerning the effects of pH on the recovery of yeast and molds from foods. Skidmore and Koburger (1966, Mace and Koburger (1967), Koburger (1970, 1971, 1972, 1973), Jarvis (1973), and Ladiges et al. (1974) reported that acidified media gave lower estimates of fungal populations in foods than media which have pH values nearer to neutrality and which incorporate dyes or antibiotics to inhibit bacterial growth.

Temperature. In a series of articles Fries (1963, 1964, 1965, 1970, 1972; Fries and Soderstrom, 1963), reported thermosensitivity of fungal growth due to thermal injury. Exposure of a number of fungi, Ophiostoma, Rhodotorula, Dipodascus, Exobasidium, and Tilletiopsis, to sublethal temperatures resulted in inability to grow at temperatures slightly below their normal maximum temperature for growth. Cells exposed to 2,4-dinitrophenol or UV light demonstrated similar thermosensitivity.

Incubation at lower temperatures resulted in full recovery of injured populations.

Gibson (1973) reported similar findings in yeast populations exposed to supramaximal temperatures. Injured cells of S. rouxii and T. globosa were reported to have lowered optimal growth temperatures.

Several investigators have noted storage of injured populations at low temperatures prevented recovery from thermal injury. Schenberg-Frascino (1972) reported storage at 4 C prevented recovery of viability in thermally stressed yeast. Baldy et al. (1970) also reported recovery from thermal injury was prevented in conidia of Penicillium expansum by storage at 0 C, although recovery occurred during storage at 23 C.

Alterations in Growth and Cell Characteristics

In addition to greater sensitivity to extreme environments, thermally injured fungal populations were reported to have increased nutritional requirements, as well as altered growth and morphology.

Nutritional Requirements. Yeast growth at supraoptimal temperatures was dependent on nutrient concentration. Sherman (1959a) reported growth of S. cerevisiae was limited by the concentration of yeast extract in the medium. When cultured in yeast extract plus 4% glucose broth, the yeast was unable to grow at 40 C in the broth containing 0.5% yeast extract, but grew in broth

containing 1.0% yeast extract. Van Uden and Madeira-Lopes (1975) reported growth of S. cerevisiae was dependent on glucose concentration and approached the optimum temperature of growth with decreasing glucose concentrations.

Thermally stressed yeast also have increased nutritional requirements. Exposure of Candida nivalis, an obligate psychrophile, to temperatures above its maximum temperature of growth of 20 C resulted in losses in viability as well as in injury to surviving cells (Nash and Sinclair, 1968). Injury was demonstrated by reduced viability on a minimal medium as compared to growth on a complex medium and recovery of cells was enhanced by the presence of glutathione or thioglycollate. Supplementation of the minimal medium with yeast extract reduced the ability of glutatione or thioglycollate to enhance recovery. Stevenson and Richards (1976) reported differences in recovery on PCA and PDA of thermally stressed S. cerevisiae which apparently resulted from differences in nutrient composition rather than pH. Acidification of PCA to the pH of PDA (5.6), did not resolve differences in recovery.

Growth and Morphology. Longer lag periods before resumption of growth were noted in many fungal populations after thermal stress. Organisms in which extended lag periods were observed include: Typhula idahoensis, T. incarnata, and T. trifoli (Dejardin and Ward, 1971);

Sclerotinia borealis (Ward, 1966a and 1968b); Aspergillus parasiticus (Adams and Ordal, 1976); T. globosa and S. rouxii (Gibson, 1973); Cryptococcus sp. (Hagen and Rose, 1961); and Candida spp. (Evison and Rose, 1965; Meyer, 1975). The extended lag period was generally proportional to the time and temperature of exposure (Hagen and Rose, 1961; Evison and Rose, 1965; Ward, 1966a and 1968b; Dejardin and Ward, 1971). The increased lag periods were attributed to time necessary for repair of thermosensitive components or systems within the cells.

Exposure to supramaximal temperatures also caused a change in morphology. A thickening of older hyphae and loss of normal coordination of hyphae within colonies occurred when Sclerotinia borealis was exposed to temperatures above its maximum for growth (Ward, 1968b). Thermal destruction of the more sensitive hyphal tips apparently resulted in loss of apical dominance. Dejardin and Ward (1971) noted atypical growth with development of fan-shaped sectors after exposure of Typhula spp. to sublethal temperatures. The obligately psychrophilic yeast Leucosporidium stokesii formed enlarged cells and buds when exposed to temperatures above the maximum for growth (Silver et al., 1977).

Subcellular Alterations

Indications of the effect of thermal injury at the subcellular level have also been obtained. Changes in

metabolic activity, disruption of cell membranes and organization, and thermosensitivity of nucleic acids were reported.

Metabolic Activity. Decreased respiratory activity associated with thermal injury was reported in a number of investigations on fungi (Bacter and Gibbons, 1962; Evison and Rose, 1965; Sinclair and Stokes, 1965; Ward, 1968b; Dejardin and Ward, 1971; Baldy et al., 1970; Spencer, 1972; Shibasaki and Tsuchido, 1973; and Meyer, 1975). Decreased fermentative activity was also found in fungi exposed to supramaximal temperatures (Sinclair and Stokes, 1965; Sinclair and Grant, 1967; Grant et al., 1968).

One of the more intensive studies of thermal injury and recovery of fungi was conducted by Meyer (1975). An association between active metabolism and thermal injury was demonstrated in Candida P25, an obligate psychrophile, after exposure to 30 C. Cells respiring exogenous materials were less resistant to thermal injury than those respiring endogenous materials, regardless of their physiological state. Heat injury affecting endogenous respiration was irreversible when cells were heated in the presence of glucose but was reversible when heated in the absence of glucose. The presence of glucose during storage did not affect recovery.

In contrast, Baldy et al. (1970) reported glucose inhibited recovery from thermal injury during storage

of heat-stressed conidia of P. expansum. Conidia heat stressed in water at 54 C for up to one hour were observed to recover viability up to 20-fold during storage in water at 23 C for three days. The presence of glucose, potassium phosphate, ammonium or sodium acetate, sodium azide, 2,4-dinitrophenol, and sodium or potassium salts of pyruvate and acids from the tricarboxylic acid cycle prevented recovery. Malate, citrate, succinate, and acetate stimulated respiration in unheated condidia and inhibited respiration in heated conidia.

Other thermosensitive metabolic activities have been reported for fungi. Baxter and Gibbons (1962) observed decreased alcohol dehydrogenase activity and reduced uptake of glucosamine in a psychrophilic Candida sp. after exposure to supramaximal temperatures. Hagen and Rose (1961, 1962) reported synthesis and uptake of amino acids, and synthesis of α-oxoglutarate decreased after thermal stress. Protein synthesis was also sensitive to heat (Sinclair and Grant, 1967; Nash et al., 1969; Spencer, 1972). Thermosensitivity of protein synthesis in L. stokesii was correlated with thermolability of a number of aminoacyl-*t*RNA synthetases and soluble enzymes involved in formation of ribosomal bound polypeptide chains (Nash et al., 1969) as well as thermal instability of ribosomes (Nash and Grant, 1969). Heat-damaged ribosomes were deficient in binding of *t*RNA. Recently, Silver et al. (1977) reported that the maximum temperature of

growth for L. stokessi was due to temperature-sensitive inhibition of DNA synthesis. RNA synthesis was inhibited at a slightly higher temperature.

Disruption of Membranes and Cellular Organization. Thermal injury to fungal cell membranes was reported in a number of investigations. Leakage of intracellular constituents after thermal stress was reported in O. multiannulatum (Fries, 1972), A. parasiticus (Adams and Ordal, 1976), C. utilis (Tsuchido et al., 1972ab; Shibasaki and Tsuchido, 1973; Rudenok and Konev, 1973), S. cerevisiae (Rudenok and Konev, 1973; Hagler and Lewis, 1974), Candida sp. (Spencer, 1972; Meyer, 1975), C. navalis (Nash and Sinclair, 1968), Leucosporidium frigidum and L. stokessi (Spencer, 1972).

The environmental sensitivity described previously has been attributed to thermally induced membrane damage by some investigators. Nash and Sinclair (1968) considered damage to a "permeability barrier" responsible for altered nutritional requirements of thermally stressed C. nivalis. Likewise, Fries (1969, 1970) concluded sensitivity to Cl^- and Br^- in heat-shocked cells of O. multiannulatum and R. glutinus resulted from membrane damage. Rudenok and Konev (1973) observed what they termed "self-protection" of S. cerevisiae and C. utilis cells from thermal injury. High concentrations of cells released sufficient intracellular materials during heat treatment to provide increased thermal

resistance, presumably due to protection of cell membranes. Low concentrations (<0.01 mM) of nonpolar aromatic and heterocyclic amino acids also provided a similar protection from thermal injury. However, in contrast to the finding of Fries (1969, 1970), histidine did not provide significant protection during heating. Hagler and Lewis (1974) reported heat stress of S. cerevisiae in the presence of glucose resulted in membrane damage as demonstrated by extracellular ATPase activity and loss of maintenance of sorbose gradients. The effect was noted uniquely with utilizable sugars and was inhibited by Ca^{++} or inhibitors of sugar utilization.

Other indications of thermally disrupted cellular organization have been described. Meyer (1975) found thermally stressed Candida P25 had extensive ultrastructural changes including aggregation, alteration, and loss of mitochondria along with the appearance of numerous large vacuoles. Arnold and Lacy (1977) reported extensive membrane damage in heat-killed cells of S. cerevisiae.

Nucleic Acids. Evidence of nucleic acid sensitivity to thermal treatment was reported in several investigations. Sherman (1956, 1959b) found growth at supraoptimal temperatures or exposure to lethal temperatures resulted in increased proportions of respiratory-deficient cells (petite mutants) of S. cerevisiae. Respiratory-deficiency was attributed to inactivation of

cytochrome oxidase and the self-replicating cytoplasmic units responsible for its production. Other investigators have since demonstrated the cytoplasmic factor was actually mitochondrial DNA (Mounolou et al., 1966; Nagley and Linnane, 1970). Schenberg-Frascino and Moustacchi (1972) reported recovery from lethal and mutagenic effects resulting from heat treatment of a haploid strain of S. cerevisiae. Increased viability and repair of cytoplasmic mutations (petite mutants) and nuclear mutations (canavanine resistance) occurred in injured cells stored at 28 C in agitated distilled water. Repair was inhibited by storage at 4 C or by inhibitors of protein synthesis. The degree of injury was dependent on the physiological state of the cells; exponential cells were much more susceptible to injury than stationary phase cells.

Bullock and Coakley (1976) also found evidence relating thermal sensitivity of DNA to cell physiology. Heat sensitivity of synchronous cultures of Schizosaccharomyces pombe was highest during nuclear division. They concluded decreased viability resulted from thermal damage of DNA.

Additional evidence of thermal injury was provided by Parry and Zimmerman (1976). They found increased numbers of monosomic colonies after heat treatment of a diploid yeast which was capable of monitoring mitotic non-disjunction through phenotypic expression of a set of

coupled and recessive markers. Expression of the markers through monosomic colony formation required a post-treatment growth period in a non-selective medium suggesting repair of injury.

SUMMARY

Thermal injury in yeasts and molds was reported to result in increased sensitivity to environmental conditions, alterations in growth and cell characteristics, and subcellular alterations. Environmental sensitivity was apparent through altered sensitivity to NaCl and A_w, pH and temperature. Increased nutritional requirements and altered cellular and colony morphology were also noted after thermal stress. Subcellular manifestations of thermal injury were reported to include altered metabolic activity, membrane damage and damage to nucleic acids.

MATERIALS AND METHODS

Organism and Cultural Conditions

Saccharomyces cerevisiae Y25, a diploid yeast, from the culture collection of the Michigan State University Food Microbiology Laboratory, was utilized in all experiments. Stock cultures were maintained on YM (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1.0% glucose) agar slants at 4 C.

Growth from a YM agar slant incubated at 25 C for 24 hours was used to inoculate 500 ml of YM broth in a 1-liter erlenmeyer flask. After incubation for 4 days at 25 C and 200 rpm on a Model G-25 gyratory shaker (New Brunswick Scientific Co.; New Brunswick, N.J.), the cells were harvested by centrifugation for 10 minutes at 1500 x *g*, washed 3 times by centrifugation with 200 ml of distilled water, and suspended in 50 ml of distilled water. The resulting cell suspensions were held at 4 C until use, normally less than 1 hour later, at which time they were equilibrated to room temperature (22 C) by immersion in tap water.

Thermal Stress

Cells of *S. cerevisiae* were subjected to thermal stress utilizing the flask method described by the National Canners Association (1968). After preliminary

experiments indicated the destruction rates of S. cerevisiae Y25 at various temperatures in water, a temperature of 56 C was chosen for subsequent experiments. Five millimeters of cell suspension were added to 245 ml of water preheated to 56 C in a 500-ml screw-capped erlenmeyer flask. Temperature was maintained by immersing the flask in a water bath heated by a Bronwill Model 20 Constant Temperature Circulator (Bronwill Scientific; Rochester, N.Y.). The flask was stabilized by large metal washers and sufficient water was added to the bath to maintain a level which was within one inch of the screw cap. The contents of the flask were mixed with a magnetic stirring bar to help provide a uniform temperature and to maintain suspension of the yeast.

Heated cell suspensions were withdrawn at appropriate times from 0 to 5 minutes by pipetting. The samples were placed in 16 x 125-mm screw-capped test tubes and cooled by immersion of the tubes in cold tap water. The samples were plated immediately, or, for delayed plating, were held at room temperature in test tubes on a New Brunswick Model TC-6 rotating drum which provided aeration and maintained the yeast in suspension.

Enumeration of "Viable" Cells

Viability of the unheated and heated cell suspensions was determined from plate counts on plate count agar (PCA; Difco Laboratories; Detroit, Michigan),

potato dextrose agar (PDA; Difco), and a minimal medium (MM) composed of 0.67% yeast nitrogen base (Difco), 0.2-6.0% glucose, and 1.5% agar. The samples were serially diluted in water and duplicate pour plates were prepared of the appropriate dilutions. The media were tempered to 45 C before pouring the agar plates. Colonies were counted after incubation at 25 C for 5 to 6 days.

Factors Affecting Viability

A number of environmental variables and metabolic inhibitors were evaluated for their influence on recovery of heat-stressed cells. The following variables were tested: composition of media, temperature of storage, temperature of incubation, presence of heat-killed cells, and metabolic inhibitors.

Composition of media. Compositional differences between PCA and PDA were explored with regard to the recovery of heat-injured cells. PDA was supplemented with components of PCA such as 0.5% yeast extract or 0.25% tryptone. The pH of PDA was adjusted by addition of 0.1 M Na_2HPO_4 to a final pH of 6.6. The pH was measured on a Beckman Research pH Meter (Beckman Instruments Inc.; Fullerton, California) by immersion of a combination pH electrode into solidified media at room temperature. Laboratory-prepared potato dextrose agar (LPDA) was also utilized (Van der Walt, 1970). Glucose concentration of the laboratory-prepared medium was 2.0% as described for

PDA or 0.1% as in PCA. In addition, recovery of heat-stressed cells on PCA and PDA was compared to recovery on MM adjusted to several concentrations of glucose ranging from 0.2 to 6.0%. For MM, glucose and agar were normally steam sterilized; however, in some experiments glucose was filter-sterilized along with the yeast nitrogen base.

Temperature of Storage. Thermally stressed cells were diluted 1:10 in distilled water immediately after heating. The samples were divided with one portion held at room temperature and 150 excursions/minute on an Eberbach no. 6000 reciprocating shaker (Eberbach Corporation; Ann Arbor, Michigan) and the other at 4 C and 160 rpm on a New Brunswick Model V gyratory shaker. Unstressed cells were treated in a similar manner to serve as controls. The samples were plated at appropriate intervals from 0 to 24 hours.

Temperature of Incubation. Heat-stressed cells and unheated controls were plated immediately after heating and after storage for 24 hours at 22 C. Duplicate plates of PCA and PDA inoculated with appropriate dilutions from each sample were incubated at 20, 25, 30 and 35 C for 5 to 6 days.

Heat-killed Cells. A set of experiments was designed to determine the extent of cannibalistic growth in various suspensions. Cell suspensions containing approximately 3.5×10^7 cells/ml were heated for 25-30

minutes at 56 C to produce heat-killed cells. The heat-killed cell suspensions were inoculated with unheated cells, held in loosely-capped 16 x 125-mm test tubes at room temperature on a New Brunswick RT-6 rotating drum, and plated at intervals up to 21 days. Unheated cells were used as controls. For comparison, cell suspensions heated at 56 C for 3 minutes were held undiluted, or, diluted into various concentrations of heat-killed cells, and were plated at similar intervals.

Metabolic Inhibitors. Several metabolic inhibitors were utilized to determine their effect on the recovery of heat-stressed cells. Various concentrations of some of the inhibitors were added to a yeast nitrogen base (0.67%) plus glucose (0.5%) broth to determine the minimum inhibitory concentrations (MIC) of inhibitors as described by Spooner and Sykes (1972). The media were inoculated with S. cerevisiae and growth was measured for 24 hours by direct cell counting utilizing a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, Florida). Information concerning the metabolic inhibitors is given in table 1.

Heat-stressed cell suspensions and controls were divided so that a comparison of recoveries with and without inhibitors could be made. Appropriate amounts of the inhibitors were added immediately after heat stress to yield the desired final concentration. Serial dilution of samples before plating prevented inhibition

Table 1. Information concerning metabolic inhibitors utilized in this investigation.

Inhibitor	Source	Site of Inhibition	Concentration
2,4-dinitrophenol	U.S. Biochemical Corporation	(Uncouples) Oxidative phosphorylation	0.10 mM
Hydroxyurea	U.S. Biochemical Corporation	DNA synthesis	0.075 M[a]
Cycloheximide	Upjohn Co.	Protein synthesis	1.0µg/ml[a]
Chloramphenicol	Sigma Chemical Co.	Protein synthesis (mitochondrial)	4.0 mg/ml
Actinomycin D	Sigma Chemical Co.	RNA synthesis	0.10 mg/ml[a]

[a]Minimum inhibitory concentration

interference with growth in the various media.

Manometric Measurements

Respiration of heat-stressed and nonstressed cells suspended in water at 30 C was studied by conventional manometric techniques (Umbreit et al., 1972) using a Gilson Differential Respirometer, model GR-14 (Gilson Medical Electronics, Inc.; Middleton, Wisconsin). In order to provide cell concentrations sufficient for measurement of respiration, the thermal stress procedure was modified. Eleven milliliters of cell suspension were added to 99 ml of water preheated to 56 C in a 250-ml erlenmeyer flask and samples were withdrawn after 1 and 2 minutes. In some experiments heat-stressed and nonstressed cells were washed by filtration on a Nucleopore membrane filter (Nucleopore Corp.; Pleasanton, California) with 0.40-μm pores, and suspended in water. Alternately, heat-stressed and nonstressed cells were added directly to flasks after appropriate dilution. Each flask contained 2.0 ml of yeast suspension (2.0-4.5 mg/ml dry weight). The center will contained 0.2 ml of 20% KOH or water and a folded 2 x 2-cm strip of Whatman #1 filter paper. Glucose (11 μmoles or 2.2 μmoles in 0.2 ml) or DNP (0.2 ml of 10^{-3}M) was sometimes added from the side arm. In some experiments DNP was added immediately after heating. The gas phase was air. A dry weight-turbidity curve was utilized to determine the dry

weight of the yeast. Production of CO_2 was determined by the direct method (Umbreit et al., 1972).

Cell Leakage

Cell suspensions heated from 0 to 5 minutes at 56 C were measured for leakage of materials absorbing at 260 and 280-nm. The suspensions were centrifuged at 3000 x *g* for 10 minutes and the supernatant was decanted. Absorbance was measured on a Beckman DB-G Spectrophotometer.

Statistical Analyses

Student's t test, linear regression, and correlation coefficients were calculated as detailed by Snedecor and Cochran (1967).

RESULTS

Recovery of Thermally Stressed Cells of Saccharomyces cerevisiae Y25 on PCA and PDA

Sampling times of 0, 1.5, 3.0 and 4.5 minutes were normally employed for studying the thermal injury and destruction of S. cerevisiae Y25 at 56 C in water. Figure 1 summarizes plate counts immediately after heating on PCA and PDA compiled during the course of the study. Analysis of recovery data by linear regression indicated slopes of -0.62 and -0.93 for PCA and PDA, respectively. Comparison of the regression coefficients by the student t-test indicated the difference between them was highly significant ($P<0.001$); the correlation coefficients of the survivor curves were -0.88 and -0.90 for recovery on PCA and PDA, respectively.

Comparison of plate counts on PCA and PDA by student t-test revealed no significant difference ($P>0.10$) between the two media for recovery of unheated cells. However, plate counts of cells heated for 1.5, 3.0, and 4.5 minutes at 56 C were significantly different ($P<0.001$). A further comparison of plate counts on PCA and PDA of heat-stressed cells is presented in Figure 2. If plate counts on PCA and PDA were equal, one would expect a plot of $\log_{10}$ PCA counts vs. $\log_{10}$ PDA counts for recovery of heat-stressed cells to have a slope of 1.0 and

Figure 1. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA) and potato dextrose agar (PDA). Cells were heated at 56 C.

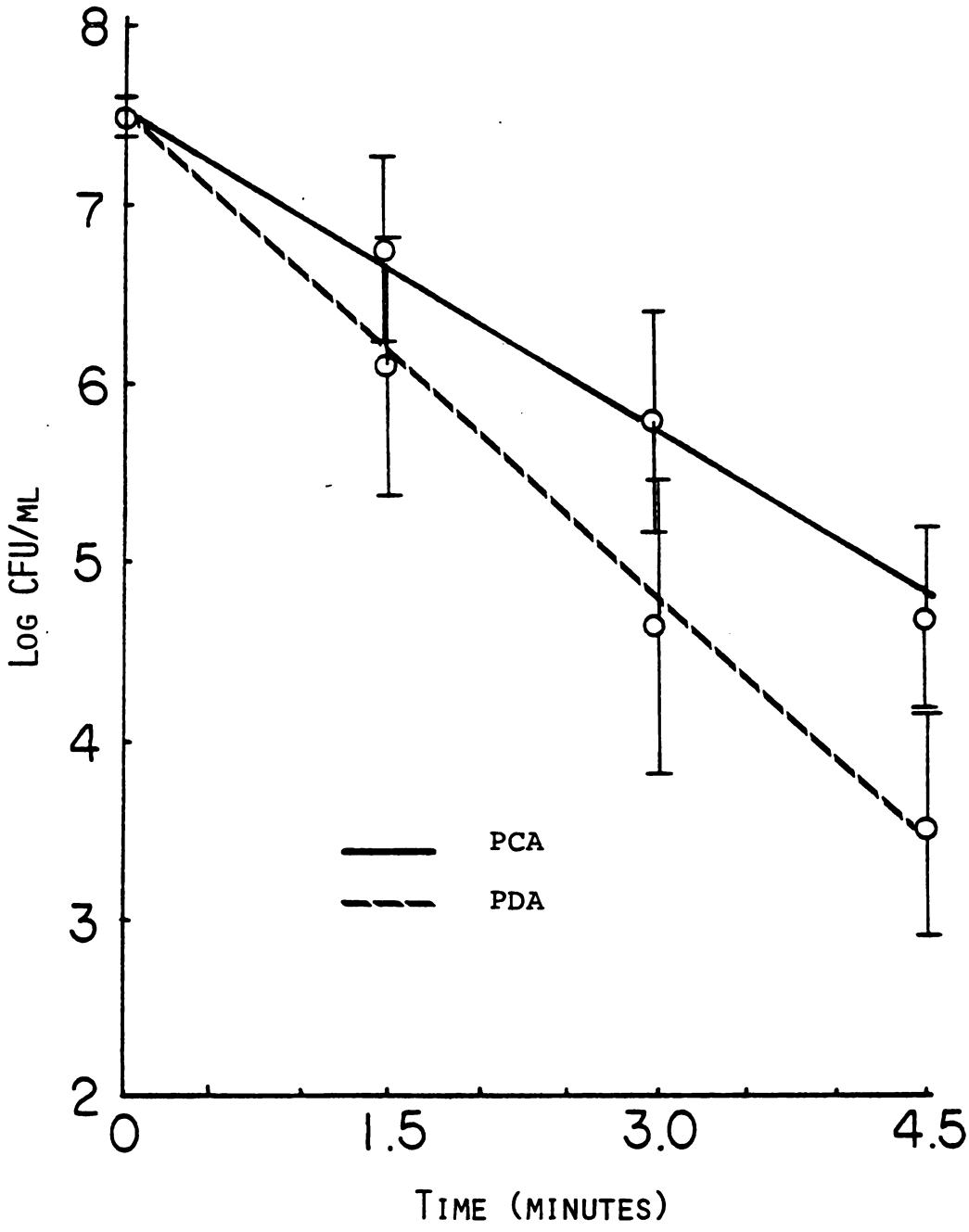
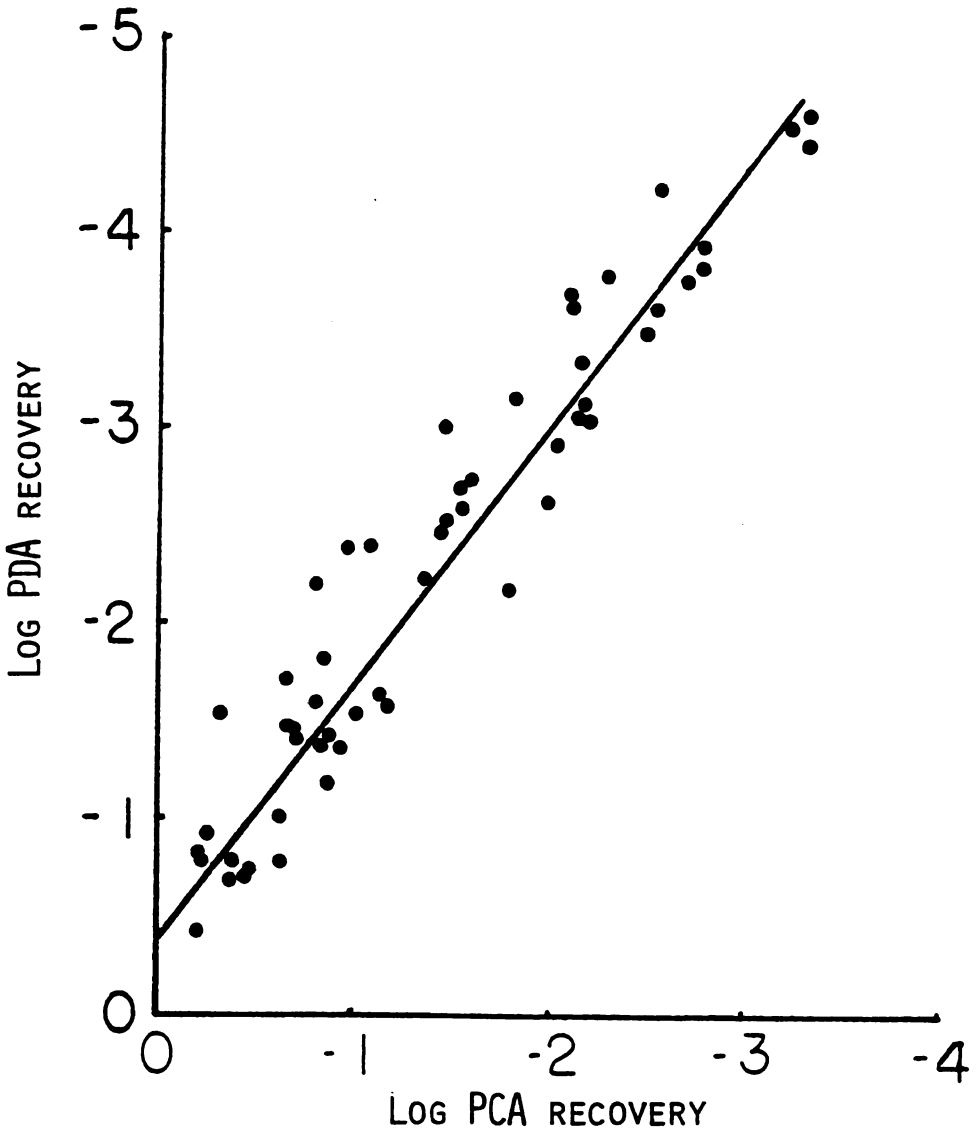


Figure 2. Comparison of recovery of heat-stressed Saccharomyces cerevisiea on plate count agar (PCA) and potato dextrose agar (PDA). Cells were heated at 56 C.



an intercept of 0. Analysis of the results by linear regression gave a slope of 1.3, an intercept of -0.45 and a correlation coefficient of 0.96. Plate counts of unheated (0 minute) samples were not included in the analysis of data in Figure 2.

Variations in recovery of heat-stressed cells on PCA and PDA were observed between experiments. Slight differences in temperature and sampling times, coupled with the rapid destruction rate of S. cerevisiae Y25 at 56 C in water probably contributed to this variation. For example, after heating for 4.5 minutes there would be a 110-fold difference in survivors on PDA between 56.5 C and 55.5 C using a z-value of 5 (Stevenson et al., 1975). Differences in culture sensitivity to heat between experiments also may have contributed to variations in recovery of heat-stressed cells. Despite varying rates of destruction, predictable differences were observed with different levels of destruction as shown in Figure 2. One of the primary reasons for choosing 56 C was the high rate of destruction which allowed rapid sampling times over a wide range of destruction. Although sufficient data were collected to compare average recoveries on PCA and PDA at the various sampling times, in other experiments such as inhibition studies where fewer replications were conducted, data from individual experiments are presented. All studies were conducted at least twice for verification of results.

Media Composition

Several aspects of media composition were investigated to determine their affect on the recovery of heat-injured cells. These included pH, variations in glucose concentrations, and supplementations of PDA with PCA components.

Neutralization of PDA with Na_2HPO_4 had no apparent effect on recovery of heat-stressed cells (Table 2). The final pH values of PCA, PDA, and PDA + 0.1 M Na_2HPO_4 were 6.9, 5.4, and 6.6, respectively.

One major difference in composition between PCA and PDA is the glucose concentration. Potato dextrose agar contains 2.0% glucose and PCA contains 0.1% glucose. Supplementation of PCA with glucose to a concentration of 2.0% resulted in decreased recovery of heat-stressed cells and the counts obtained were intermediate between those obtained on nonsupplemented PCA and PDA (Table 2). In another experiment laboratory-prepared potato dextrose agar (LPDA) containing 0.1% glucose supported recoveries of heat-stressed cells similar to recoveries on PCA, while LPDA containing 2.0% glucose supported recoveries of heat-stressed cells similar to those obtained with commercially prepared PDA (Table 3). The pH of LPDA with 0.1% glucose was 6.9 and LPDA with 2.0% glucose had a pH of 6.6.

Recovery of heat-injured cells on MM was also

Table 2. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA), potato dextrose agar (PDA), PDA containing 0.1 M Na_2HPO_4, and PCA containing 2.0% glucose.

Heat-Stress at 56 C	PCA[a]	PDA[b]	PDA + 0.1M Na_2HPO_4 [c]	PCA + 2.0% glucose [c]
(min)			(Log CFU/ml)	
0	7.56	7.54	7.58	7.57
1.5	7.28	6.62	6.82	7.08
3.0	5.74	4.36	4.49	5.08

[a] pH 6.9

[b] pH 5.6

[c] pH 6.6

Table 3. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA), potato dextrose agar (PDA), and laboratory-prepared potato dextrose agar (LPDA) containing 0.1 or 2.0% glucose.

Storage	Heat-Stress at 56 C	PCA	PDA	LPDA (0.1% glucose)	LPDA (2.0% glucose)
(hr)	(min)			(Log CFU/ml)	
0	0	7.59	7.70	7.60	7.65
	1.5	7.08	6.85	7.20	6.94
	3.0	5.54	4.63	5.51	4.90
6	0	7.58	7.62	7.60	7.56
	1.5	7.30	7.08	7.26	7.23
	3.0	5.92	5.30	6.04	5.71

related to glucose concentration. Decreasing recoveries of heat-injured cells were obtained on MM with increasing concentrations of glucose (Table 4). However, MM prepared with steam-sterilized glucose gave reduced recovery of heat-injured cells in comparison to MM prepared with filter-sterilized glucose (Table 5). Recovery of heat-injured cells on MM containing filter-sterilized glucose was similar to recovery on PCA. The pH of MM containing 2.0% glucose when filter-sterilized was 5.5 and 5.2 when steam-sterilized.

Supplementation of PDA with yeast extract or tryptone did not increase recovery of heat-stressed cells (Table 6).

Cannibalistic Growth

Variation in plate counts of nonheated cells stored in water for 21 days was minimal while growth occurred during storage in solutions containing heat-killed cells (Figure 3). The heat-killed cells were present at a concentration equal to concentrations employed in recovery experiments (3.5×10^7 cells/ml) and growth over the 21-day period reached final numbers which were 3 to 6% of the original heat-killed cell concentration. Growth was not observed during the first 12 hours after inoculation, whereas between 12 and 24 hours the increases in viable counts averaged 69 to 75% (Table 7).

In contrast, heat-stressed cells which had not

Table 4. Plate counts of heat-stressed Saccharomyces cerevisiae on minimal media (MM) containing 0.2 to 6.0% glucose.

Storage at 22 C	Heat-Stress at 56 C	MM % glucose 0.2	2.0	4.0	6.0
(hr)	(min)		(Log CFU/ml)		
0	0	7.60	7.57	7.45	7.53
	1.5	7.04	6.74	6.45	6.53
	3.0	5.26	4.94	4.89	4.13
	4.5	3.60	3.15	2.81	2.65
12	0	7.57	7.54	7.51	7.51
	1.5	7.20	7.11	7.11	7.04
	3.0	6.15	5.84	5.83	5.80
	4.5	4.68	4.38	4.27	4.20

Table 5. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA), potato dextrose agar (PDA) and minimal media (MM) containing 2.0% filter-sterilized glucose or 2.0% steam-sterilized glucose.

Storage at 22 C	Heat-Stress at 56 C	PCA	PDA	MM $2.0^{a}S^{b}$	MM $2.0^{a}F^{c}$
(hr)	(min)		(Log CFU/ml)		
0	0	7.73	7.79	7.73	7.75
	1.5	6.83	6.52	6.51	6.88
	3.0	4.95	3.90	4.08	4.83
12	0	7.56	7.59	7.54	7.49
	1.5	7.00	6.86	7.00	7.11
	3.0	5.30	4.98	5.20	5.38

a = % glucose
b = steam-sterilized glucose
c = filter-sterilized glucose

Table 6. Plate counts of heat-stressed Saccharomyces cerevisiae on plate count agar (PCA), potato dextrose agar (PDA), and PDA supplemented with 0.50% yeast extract or 0.25% tryptone.

Storage at 22 C	Heat-Stress at 56 C	PCA	PDA	PDA + Yeast Extract	PDA + Tryptone
(hr)	(min)		(Log CFU/ml)		
0	0	7.51	-[a]	-	-
	1.5	6.75	6.08	6.08	5.90
	3.0	4.71	(2.95)[b]	(2.30)	(2.70)
24	0	7.38	-	-	-
	1.5	7.08	6.95	6.91	6.88
	3.0	6.11	5.98	5.99	5.95

[a]Data not available.

[b]Data represent estimated counts calculated from plates containing <30 colonies.

Figure 3. Growth of Saccharomyces cerevisiae in water and in suspensions of heat-killed cells at 22 C. Heat killed cells were prepared by heating 3.5×10^7 S. cerevisiae cells/ml at 56 C for 30 minutes.

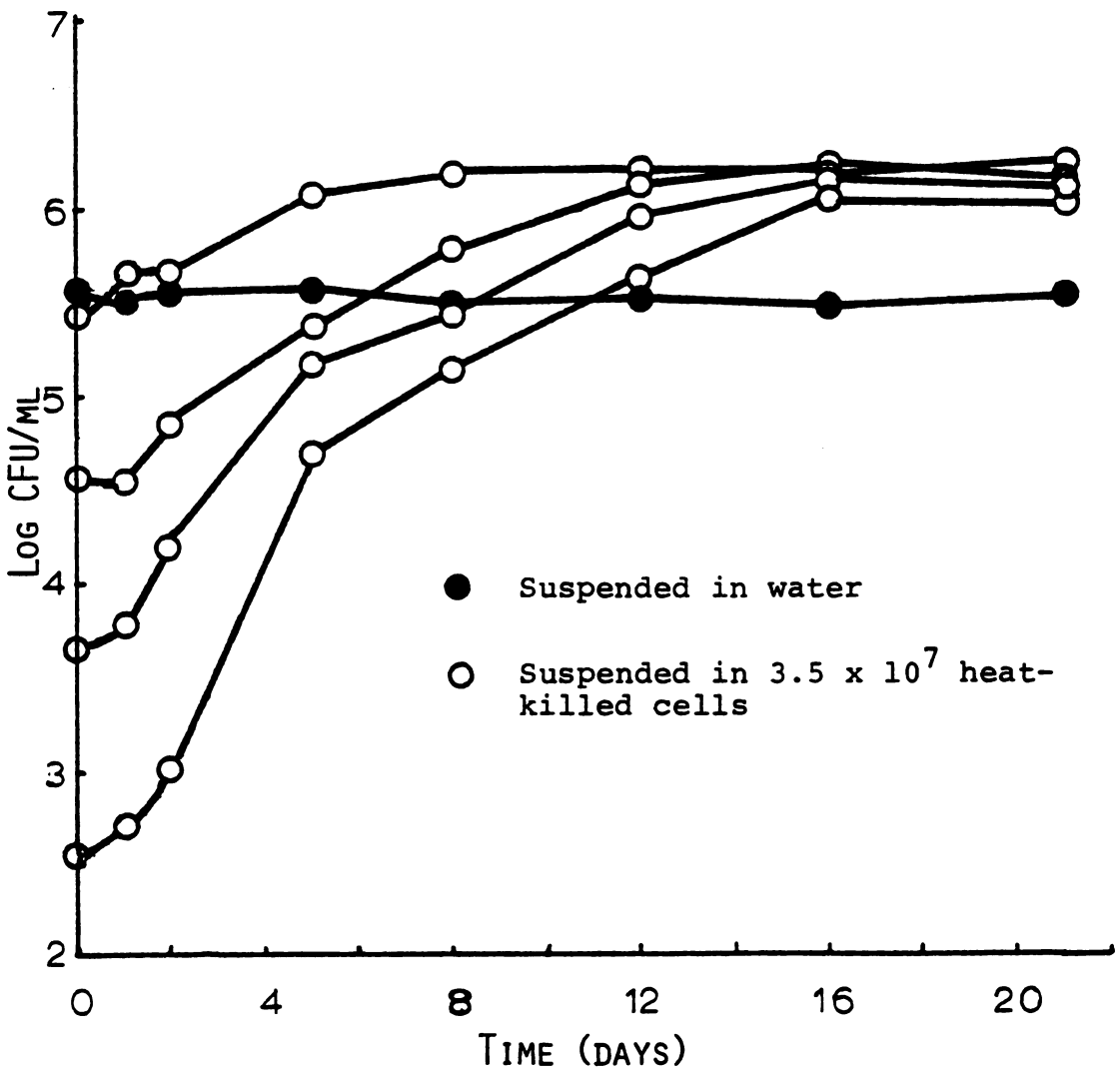


Table 7. Growth at 22 C of nonheated cells of Saccharomyces cerevisiae in suspensions of heat-killed cells. Heat-killed cells were prepared by heating 3.5×10^7 S. cerevisiae cells/ml for 30 minutes at 56 C.

Incubation Time (hr)			
0	6	12	24
(Log CFU/ml)			
4.59[a] ± .15	4.58 ± .16	4.60 ± .08	4.83 ± .26
3.50[a] ± .11	3.51 ± .09	3.55 ± .06	3.72 ± .21

[a]Inoculum levels were approximately 10^{-3} and 10^{-4} as concentrated as the heat-killed cells.

undergone complete thermal destruction showed much larger increases in plate counts during the first 24 hours of storage (Figure 4). Cells heat-stressed for 3 minutes at 56 C were held undiluted or diluted 100-fold into various concentrations of heat-killed cells. Initially the PCA count was approximately 3 $\log_{10}$- cycles lower than the original numbers of 3.5×10^7 cells/ml. Plate counts during 20 days of storage for undiluted heat-stressed cells or heat-stressed cells diluted into 100% heat-killed cells approximated numbers reached by growth of unheated cells on heat-killed cells, that is, 3 to 6% of the original concentration. However, growth of heat-stressed cells diluted into 60, 30 or 0% heat-killed cells was lower than would be anticipated if growth was in proportion to the total (live and dead) cell concentration. Nonetheless, plate counts of both undiluted or diluted cells increased approximately 10-fold during the first day after heat stress.

Recovery of Heat-Injured Cells

The effect of delayed plating on a survivor curve for *S. cerevisiae* Y25 heated at 56 C in water is shown in Figure 5. Differences between recovery of heat-stressed cells on PCA and PDA were quite apparent when samples were plated immediately after heating. After 24 hours of storage, plate counts on both PCA and PDA had increased and the differences in recoveries on the

Figure 4. Growth of Saccharomyces cerevisiae heated at 56 C for 3 minutes and stored at 22 C in water undiluted or diluted 10^{-2} with various concentrations of heat-killed cells. The heat-killed cells were prepared by heating 3.5×10^7 S. cerevisiae cells/ml at 56 C for 30 minutes and were used as storage media at 0, 30, 60 or 100%.

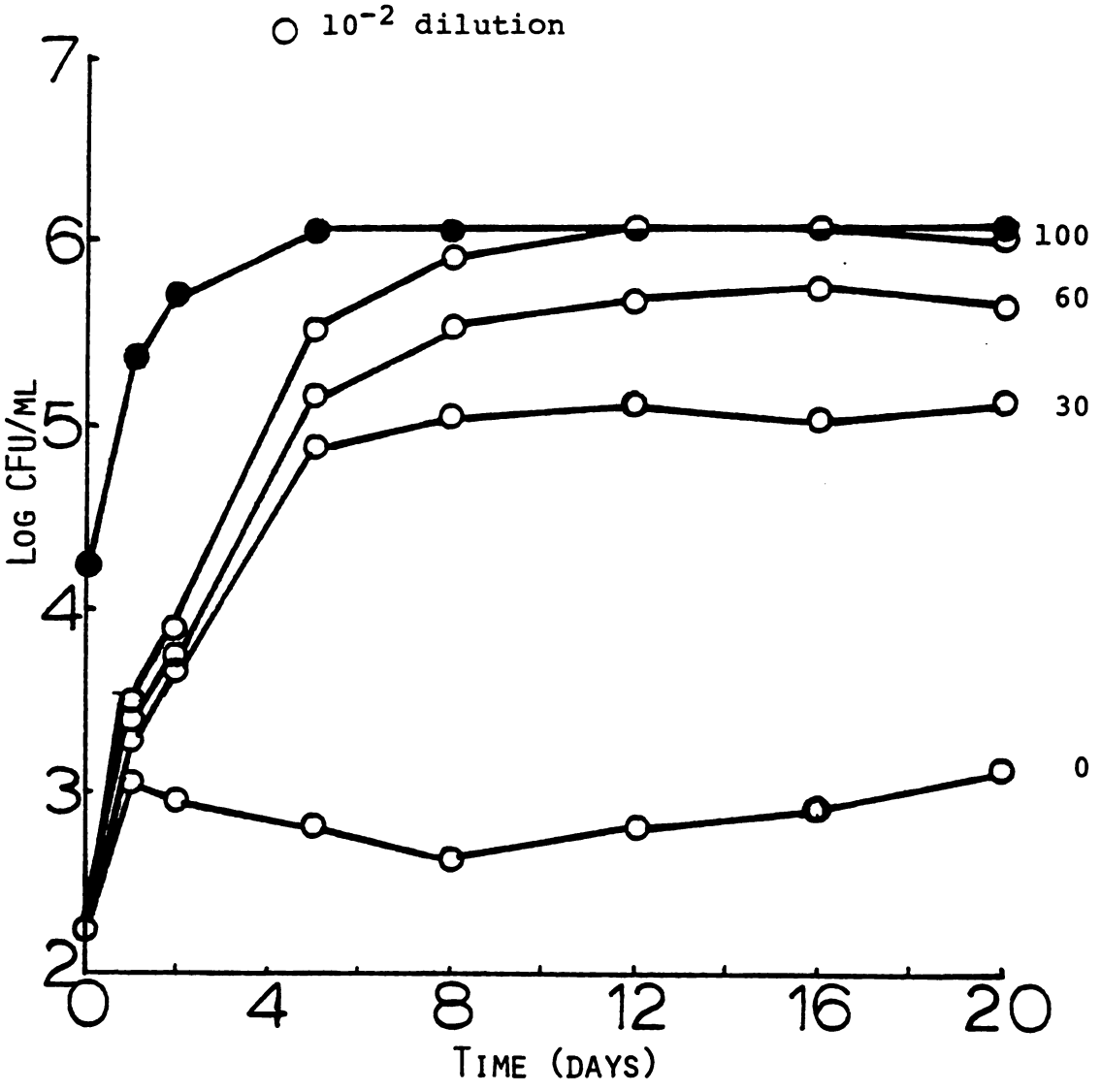
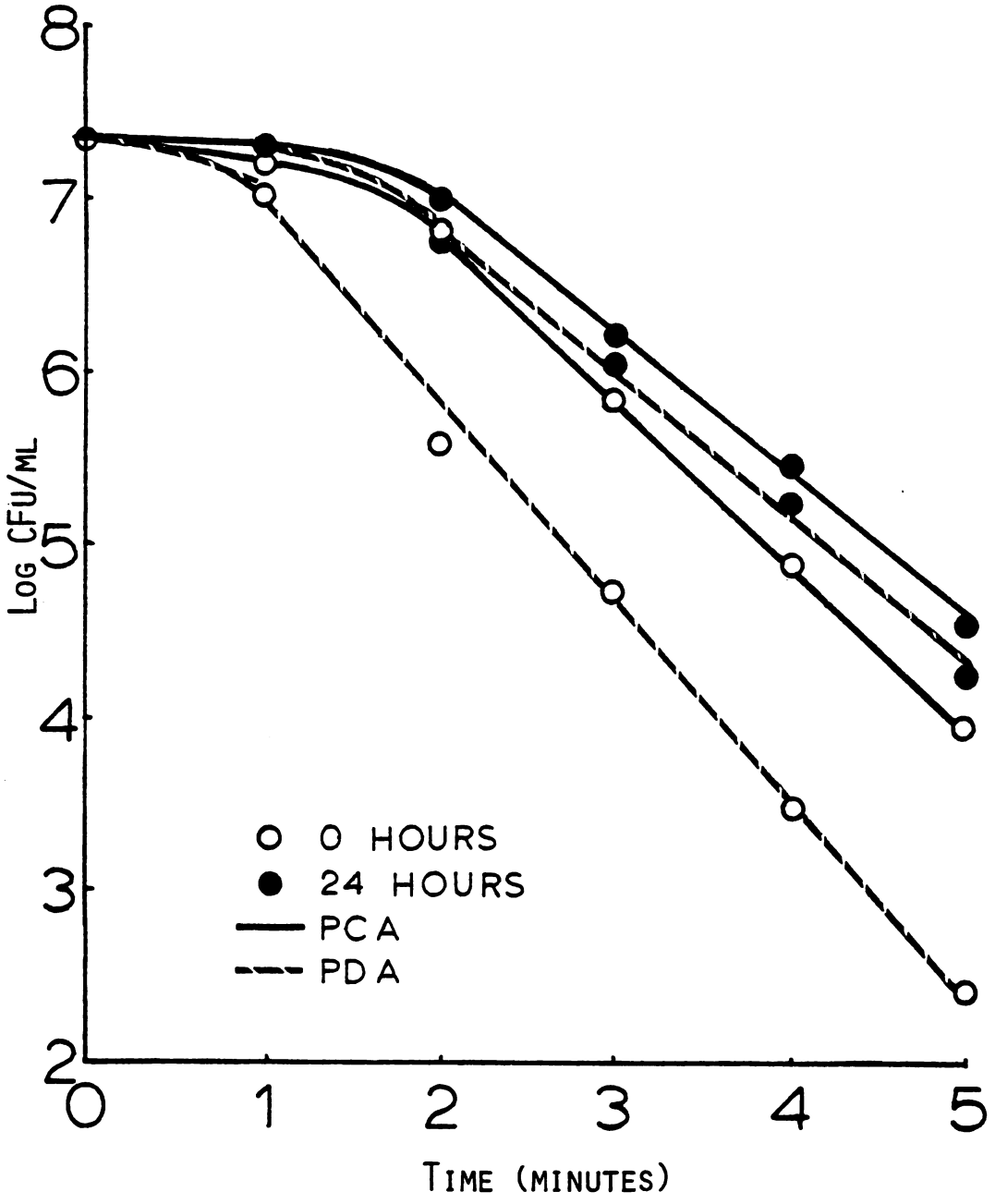


Figure 5. Survivor curves of Saccharomyces cerevisiae heated at 56 C and plated on plate count agar (PCA) and potato dextrose agar (PDA) immediately after storage in water at 22 C for 24 hours.



two media were reduced considerably.

The differences in recovery of heat-stressed cells between PCA and PDA were reduced substantially during the first six hours of storage (Figure 6). Plate counts on PCA and PDA increased considerably during the first 6 hours of storage with progressively smaller increases occurring during 6 to 12 hours and from 12 to 24 hours of storage.

Effect of Metabolic Inhibitors on Recovery of Heat-Stressed Cells

Repair of heat-injured cells was not inhibited during storage in the presence of cycloheximide (Figure 7), chloramphenicol (Table 8), chloramphenicol plus cycloheximide (Table 9), actinomycin D (Table 10), and hydroxyurea (Table 11). Apparently protein synthesis, RNA synthesis and DNA synthesis were not required for recovery of colony-forming ability of heat-injured cells. These experimental conditions also preclude increases in viable counts during storage resulting from cannibalistic growth since the concentrations of hydroxyurea, cycloheximide and actinomycin D were high enough to inhibit growth.

Storage in the presence of DNP prevented recovery of colony-forming ability of heat-injured cells (Figure 8). Viability of unheated cells was not affected by the presence of 0.1 mM DNP. Potato dextrose agar plate

Figure 6. Effect of storage in water at 22 C on plate counts of heat-stressed Saccharomyces cerevisiae. Cells were heated at 56 C and plated on plate count agar (PCA) and potato dextrose agar (PDA).

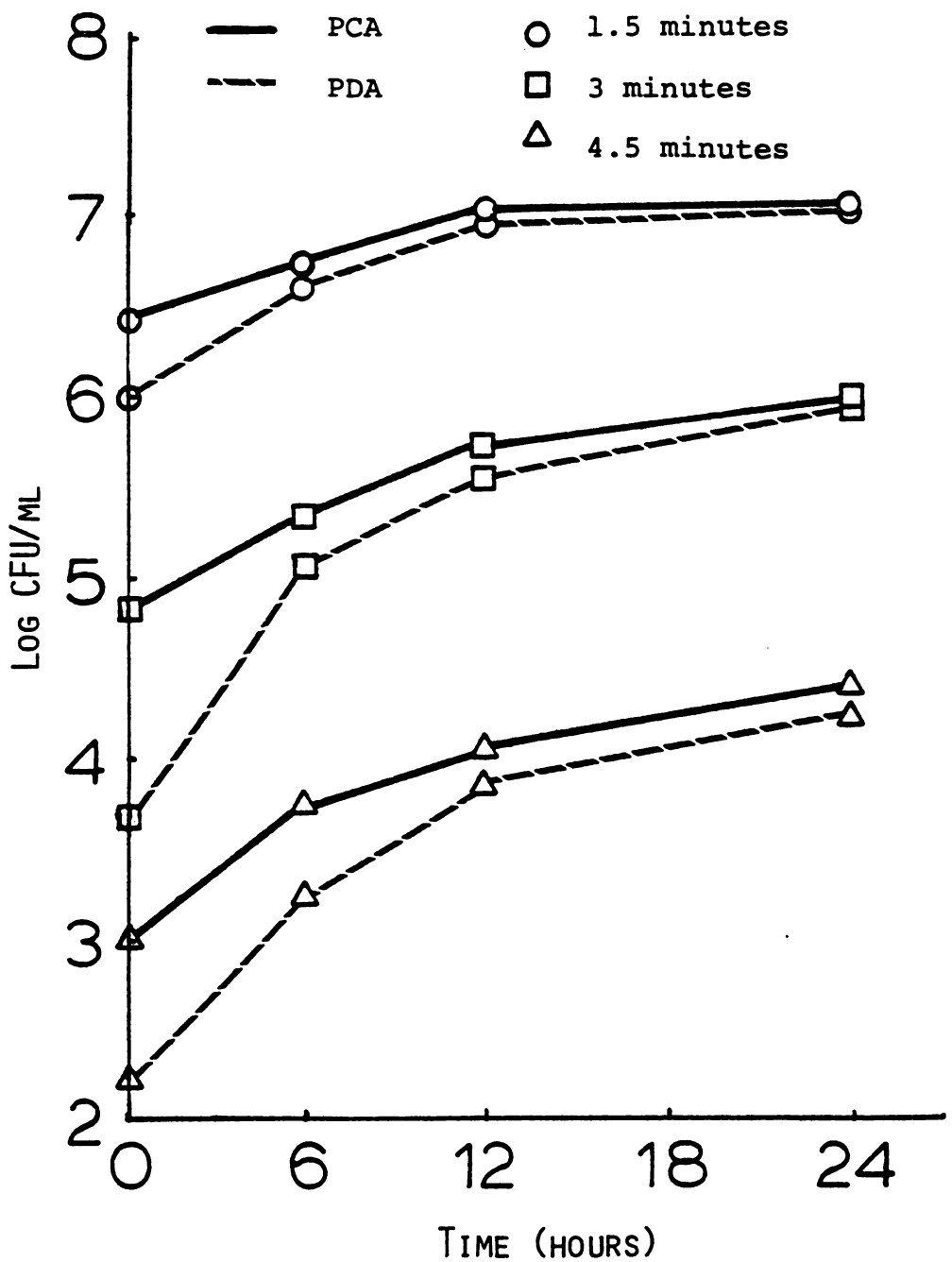


Figure 7. Effect of storage at 22 C in water with or without 1.0 µg/ml cycloheximide on plate counts of heat-stressed Saccharomyces cerevisiae. Cells were heated at 56 C and plated on plate count agar (PCA) and potato dextrose agar (PDA).

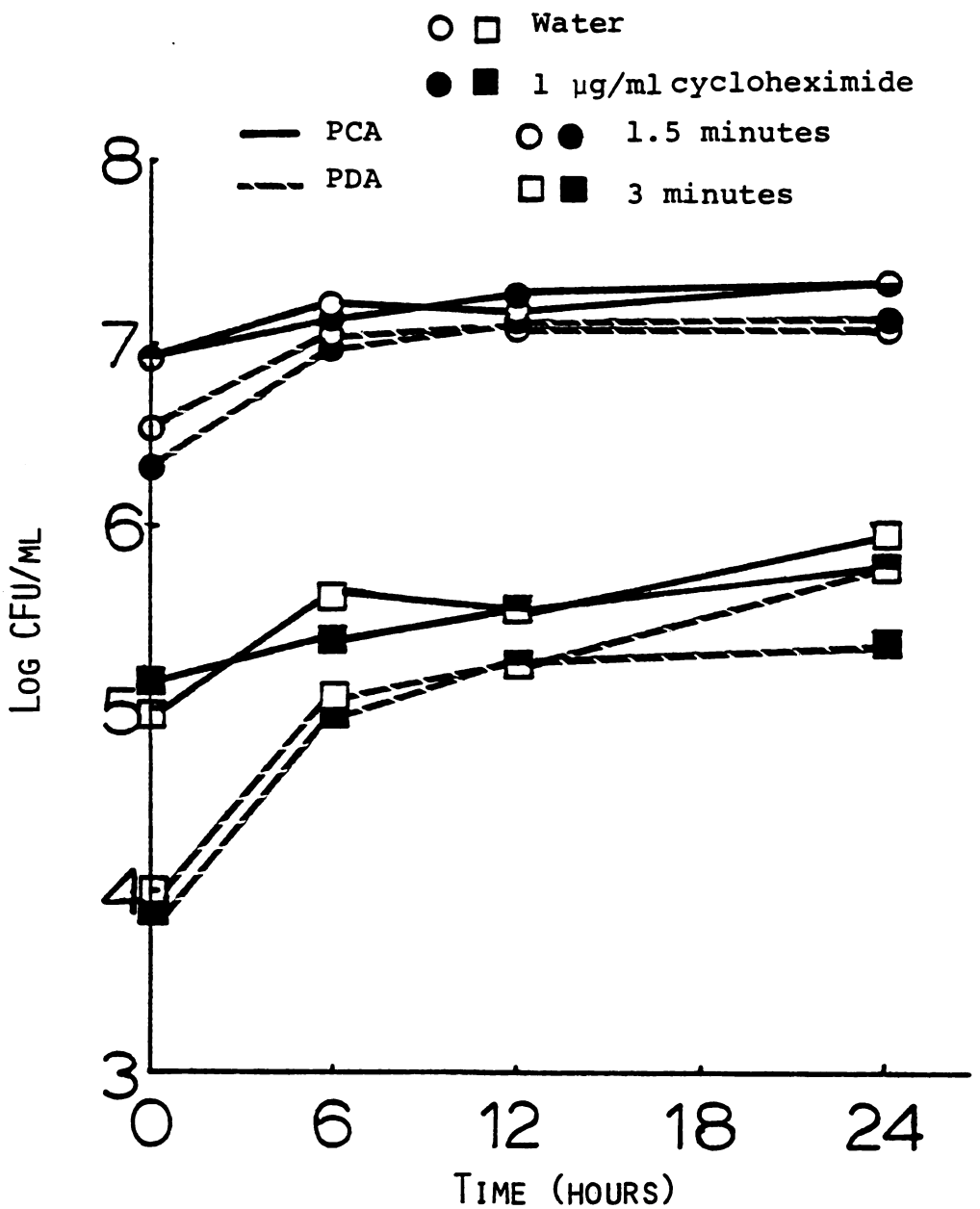


Table 8. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) of heat-stressed Saccharomyces cerevisiae stored in water with or without 5 mg/ml of chloramphenicol.

Heat-Stress at 56 C (min)	Stored with Chloramphenicol	Medium	Storage Time (hr) 0 (Log CFU/ml)	6
0	-	PCA	7.38	7.40
		PDA	7.40	7.32
	+	PCA	7.40	7.42
1.5	-	PCA	5.99	6.91
		PDA	5.15	6.86
	+	PCA	5.90	6.72
		PDA	5.15	6.52
3.0	-	PCA	3.69	5.23
		PDA	(2.60)[a]	5.04
	+	PCA	3.77	5.15
		PDA	(2.48)	4.92

[a]Data represent estimated counts calculated from plates containing <30 colonies.

Table 9. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) of heat-stressed Saccharomyces cerevisiae stored in water with or without 5 mg/ml chloramphenicol and 10 µg/ml cycloheximide.

Heat-Stress at 56 C (min)	Stored with Chloramphenicol + Cycloheximide	Medium	Storage Time (hr) 0 (Log CFU/ml)	6
0	-	PCA	7.51	7.58
		PDA	7.53	7.56
	+	PCA	7.51	7.52
1.5	-	PCA	6.57	7.04
		PDA	6.11	6.67
	+	PCA	6.45	7.04
		PDA	6.11	6.72
3.0	-	PCA	5.30	5.56
		PDA	4.31	5.26
	+	PCA	5.08	5.67
		PDA	4.26	5.18

Table 10. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) of heat-stressed Saccharomyces cerevisiae stored in water with or without 0.10 mg/ml actinomycin D.

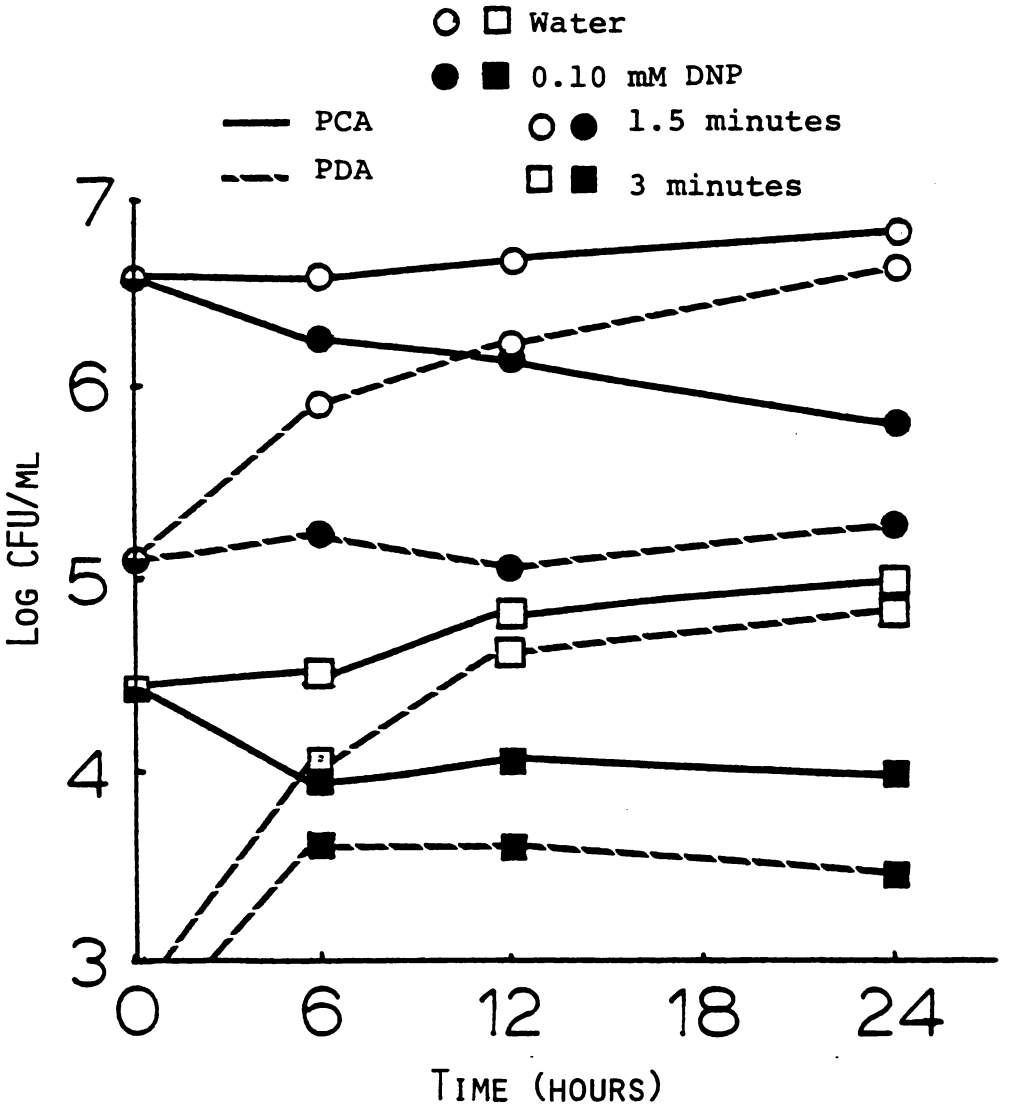
Heat-Stress at 56 C (min)	Stored with Actinomycin D	Medium	Storage Time (hr) 0 (Log CFU/ml)	6 (Log CFU/ml)
0	-	PCA	7.53	7.51
		PDA	7.49	7.57
	+	PCA	7.42	7.46
		PDA	7.42	7.51
1.5	-	PCA	7.15	7.28
		PDA	6.69	7.11
	+	PCA	7.18	7.18
		PDA	6.70	7.08
3.0	-	PCA	5.92	6.08
		PDA	4.82	5.76
	+	PCA	5.92	6.08
		PDA	4.85	5.78
4.5	-	PCA	4.23	7.78
		PDA	2.89	4.18
	+	PCA	4.30	4.72
		PDA	2.85	4.11

Table 11. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) of heat-stressed Saccharomyces cerevisiae stored in water with or without .075 M hydroxyurea.

Heat-Stress at 56 C (min)	Stored with Hydroxyurea	Medium	Storage Time (hr) 0 (Log CFU/ml)	Storage Time (hr) 6 (Log CFU/ml)
0	-	PCA	7.34	7.32
		PDA	7.36	7.32
	+	PCA	7.36	7.34
1.5	-	PCA	6.30	7.04
		PDA	5.78	6.90
	+	PCA	6.30	6.76
		PDA	5.81	6.60
3.0	-	PCA	4.04	5.28
		PDA	(2.00)[a]	5.15
	+	PCA	4.08	5.30
		PDA	(2.60)	5.15

[a]Data represent estimated counts calculated from plates containing <30 colonies.

Figure 8. Effect of storage at 22 C in water with or without 0.10 mM 2,4-dinitrophenol (DNP) on plate counts of heat-stressed Saccharomyces cerevisiae. Cells were heated at 56 C for 1.5 or 3.0 minutes and plated on plate count agar (PCA) and potato dextrose agar (PDA).



counts of heat-stressed cells which were stored in the presence of DNP did not increase or increased slightly during storage. Plate counts on PCA of heat-stressed cells stored in 0.1 mM DNP decreased during storage.

Effects of Temperature on Recovery

Storage Temperature. Storage at 4 C prevented recovery of colony-forming ability of heat-injured cells (Figure 9). Plate counts on PDA of heat-stressed cells decreased during the first 6 hours of storage, and then increased slightly through 24 hours of storage. The PCA counts of heat-stressed cells held at 4 C remained constant during storage.

Incubation Temperature. Little difference was noted in PCA or PDA plate counts of unheated cells of *S. cerevisiae* Y25 when incubated at 20, 25, 30, or 35 C. However, plate counts of heat-stressed cells were affected by incubation temperature (Figure 10). Plate counts on PCA of heat-stressed cells were not substantially affected by temperature, but plate counts on PDA decreased with increasing temperature and the effect was in proportion to the severity of the heat treatment. For example, differences between log PDA counts at 20 and 35 C were 0.44 and 1.31 after heating for 1.5 and 4.5 minutes, respectively. Storage for 24 hours at 22 C prior to plating allowed restoration of colony-forming ability at all temperatures (Table 12).

Figure 9. Effect of storage in water at 4 C or 22 C on plate counts of heat-stressed Saccharomyces cerivisiae. Cells were heated at 56 C for 1.5 or 3.0 minutes and plated on plate count agar (PCA) and potato dextrose agar (PDA).

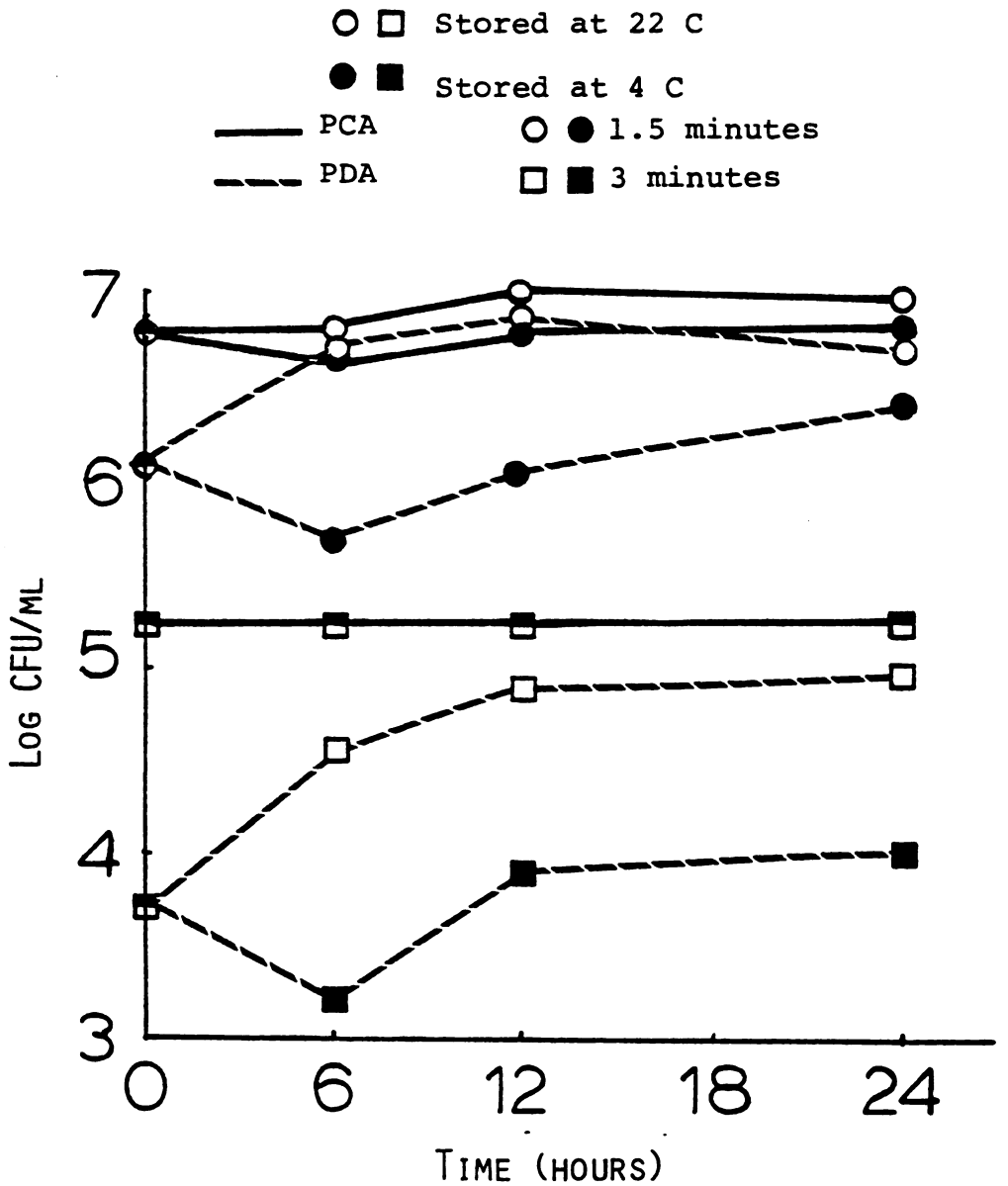


Figure 10. Effect of incubation at 20, 25, 30, or 35 C on plate counts of heat-stressed Saccharomyces cerevisiae. Cells were heated for 1.5, 3.0, or 4.5 minutes at 56 C and plated on plate count agar (PCA) and potato dextrose agar (PDA).

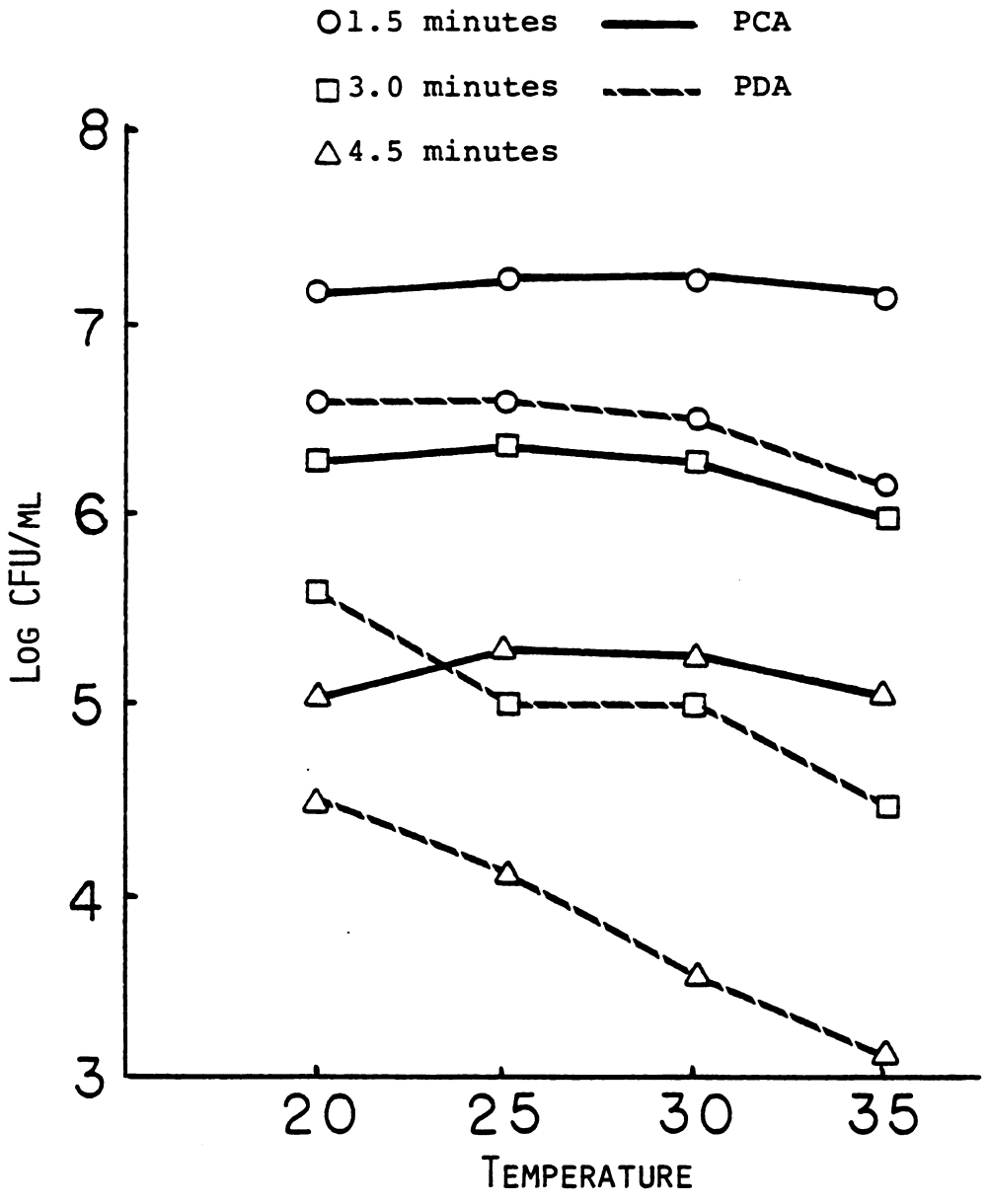


Table 12. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) incubated at 20, 25, 30, and 35 C of heat-stressed Saccharomyces cerevisiae stored for 24 hours at 22 C in water.

Heat-Stress at 56 C	Medium	Incubation Temperature (C) 20	25	30	35
(min)			(Log CFU/ml)		
1.5	PCA	7.28	7.26	7.18	7.18
	PDA	7.20	7.15	7.11	7.08
3.0	PCA	6.43	6.46	6.43	6.42
	PDA	6.23	6.18	6.15	6.15
4.5	PCA	5.51	5.56	5.49	5.57
	PDA	5.52	5.48	5.45	5.43

Respiration of Heat-stressed Cells

Differences in endogenous O_2-uptake between heat-stressed and nonheated cells of S. cerevisiae Y25 were noted. Oxygen-uptake of cells of heat-stressed at 56 C for 1 or 2 minutes was considerably higher than that of nonheated cells (Figure 11). Cells heated for 1 minute at 56 C had a high initial uptake of O_2 which declined shortly after measurements were started. Cells heat-stressed for 2 minutes at 56 C also had a high initial rate of O_2-uptake which continued through the first hour of measurement. The prolonged high rate of O_2-uptake was associated with thermal injury as indicated by reduced recovery of heat-stressed cells on PDA and PCA (Table 13). Plate counts on PCA and PDA of unheated cells and cells heated for 1 minute were similar, but plate counts of cells heated for 2 minutes were initially lower on PDA in comparison to controls. When cells were heat-stressed for 2 minutes and stored for 20 hours, plate counts on PCA and PDA were similar.

Table 14 summarizes endogenous O_2-uptake rates of unheated and heated cells for up to 4 hours after heating. Nonheated cells had an initial Q_{O_2} (µl O_2/mg dried yeast/hr) of 2.0 which declined slightly during the four hours to a Q_{O_2} of 1.8. Cells heat-stressed for 1 minute had an average initial Q_{O_2} of 40.6 which declined to 7.5 during the second hour and declined gradually to 5.4 during the fourth hour of measurement. Cells heated for 2 minutes

Figure 11. Endogenous respiration at 30 C of heat-stressed Saccharomyces cerevisiae in water. Cells were heated for 0, 1, or 2 minutes at 56 C.

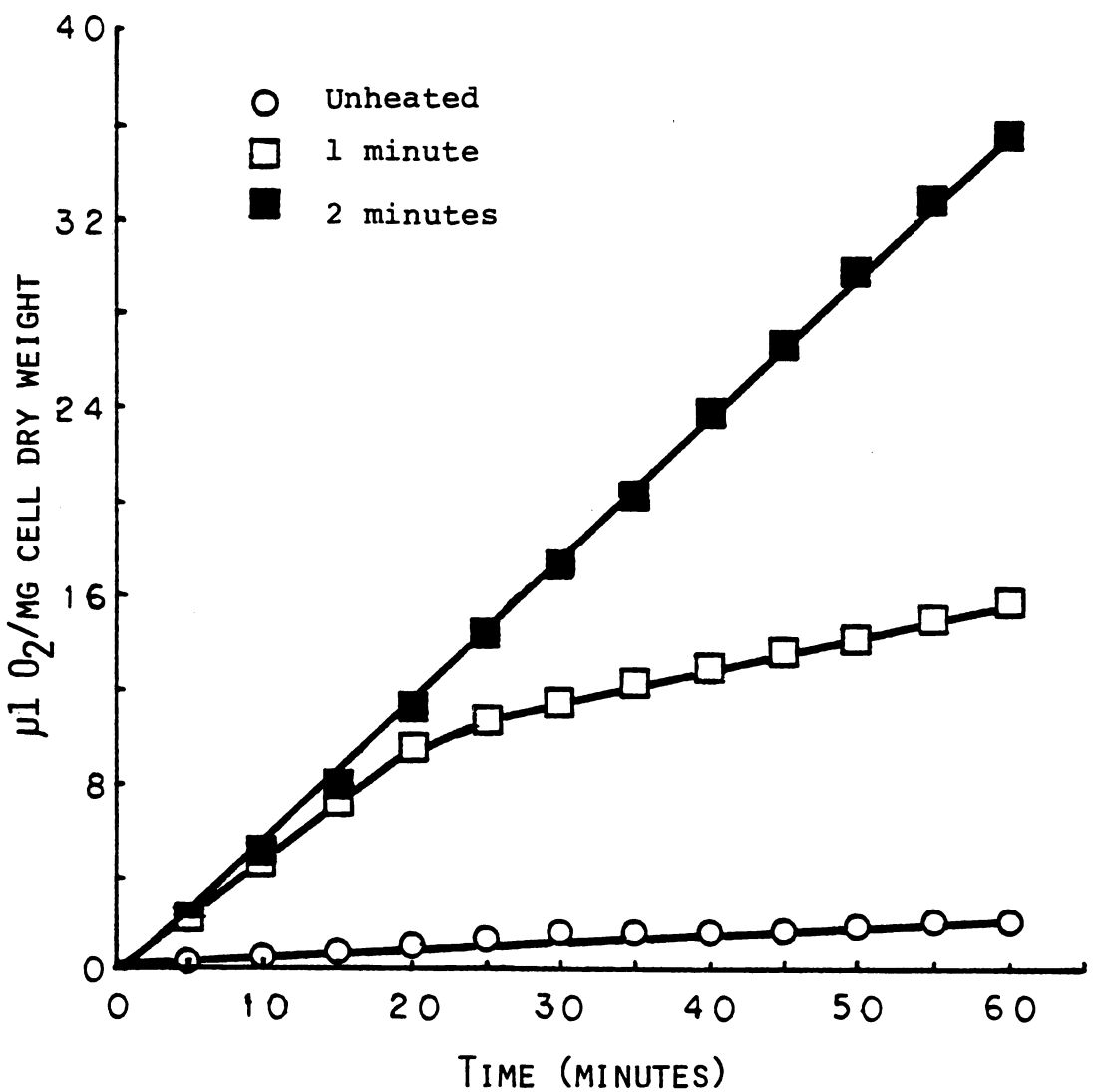


Table 13. Plate counts on plate count agar (PCA) and potato dextrose agar (PDA) of heat-stressed Saccharomyces cerevisiae immediately after heating and after 20 hours of storage in water at 22 C.

Heat-Stress at 56 C (min)	Medium	Storage Time (hr) 0 (Log CFU/ml)	Storage Time (hr) 20 (Log CFU/ml)
0	PCA	8.15	8.13
	PDA	8.16	8.15
1	PCA	8.13	8.16
	PDA	8.10	8.15
2	PCA	7.99	8.06
	PDA	7.81	8.04

Table 14. Rates of endogenous O_2-uptake in water at 30 C for nonheated and heat-stressed Saccharomyces cerevisiae.

Heat-Stress at 56 C	Time after Heating (hr)			
	0	1 - 2	2 - 3	3 - 4
(min)	Q_{O_2} (µl O_2/mg dried yeast/hr)			
0	2.0	1.9	1.8	1.8
1	40.6	7.5	6.4	5.4
2	39.7	33.5	16.7	6.0

had an initial Q_{O_2} of 39.7 which declined to 33.5 during the second hour and then gradually declined to 6.0 during the fourth hour. Although not shown in Table 14, it was interesting to note that after 20 hours of storage, endogenous O_2-uptake of heated cells was actually lower than unheated cells. The Q_{O_2} of unheated cells was 2.0 and of cells heated for 1 and 2 minutes was 0.8 and 0.9, respectively.

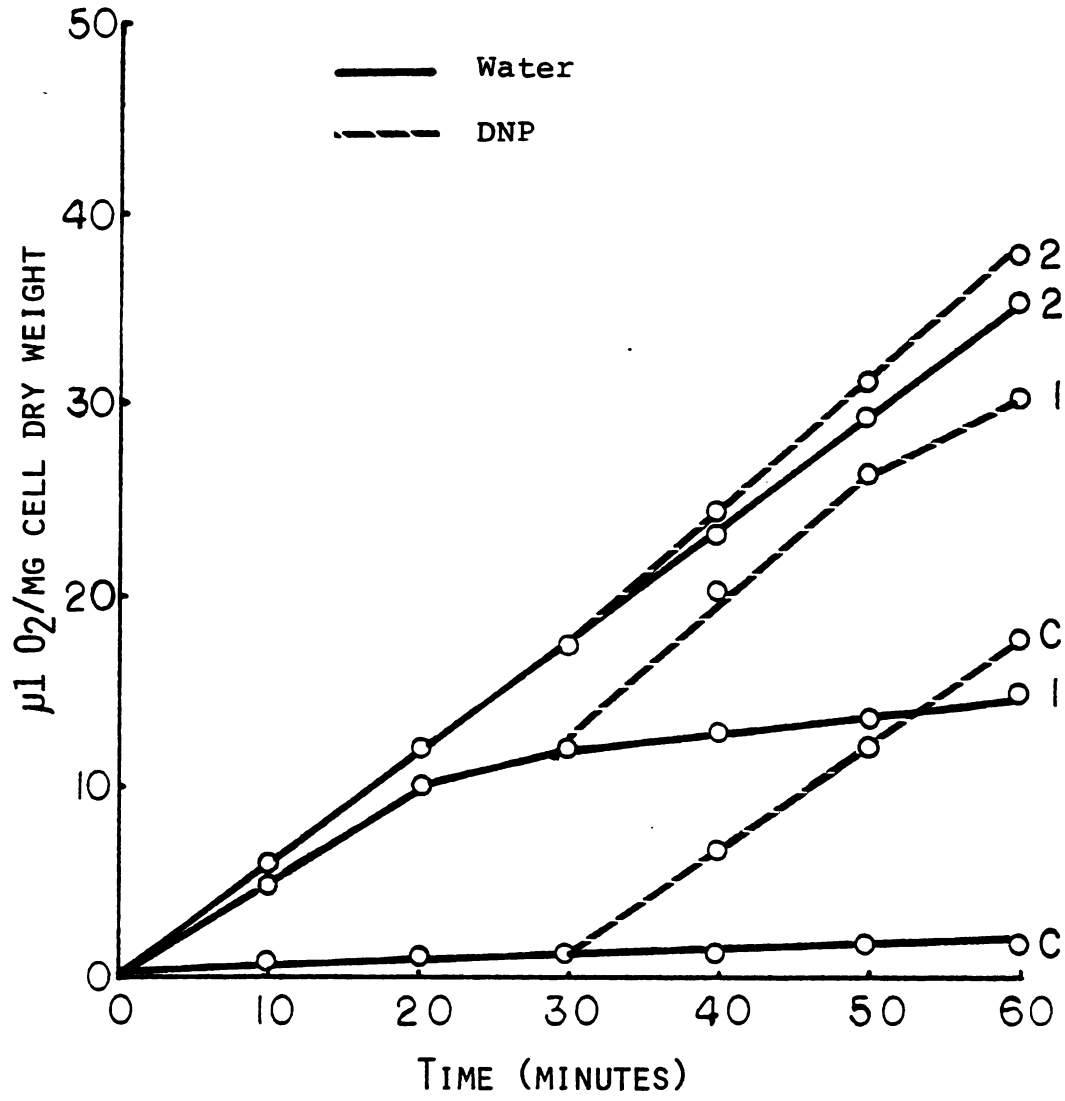
Respiratory quotients (R.Q.) of heat-stressed cells were also different from unheated cells (Table 15). The respiratory quotient is defined as the ratio of CO_2 produced/O_2 consumed. Unheated cells had R.Q. values at or near 1.00 during the first 3 hours of measurement. Cells heat-stressed for 1 minute had an initial R.Q. of 0.70 associated with high initial rates of O_2-uptake. The R.Q. of cells heat-stressed for 1 minute then increased to 0.91 by the third hour and was 0.93 during the fourth hour. In contrast, cells heat-stressed for 2 minutes had an initial R.Q. of 1.04 which dropped to 0.72 during the second hour and increased to 0.95 and 0.93 during the third and fourth hours.

Oxygen-uptake in response to the presence of DNP was also measured in unheated and heated cells (Figure 12). Rates of oxygen-uptake in unheated cells and cells heat-stressed for 1 minute increased when DNP was added. However, oxygen-uptake in cells heat-stressed for 2 minutes declined when DNP was added. The results were

Table 15. Respiratory quotients (R.Q.) in water at 30 C for endogenous respiration of non-heated and heat-stressed Saccharomyces cerevisiae.

Heat-Stress at 56 C	Time after Heating (hr)			
	0	1 - 2	2 - 3	3 - 4
(min)	R.Q. (µl CO_2/µl O_2)			
0	1.00	.98	1.00	.80
1	.70	.78	.91	.93
2	1.04	.72	.95	.93

Figure 12. Effect of addition of 0.10 mM 2,4-dinitrophenol (DNP) on endogenous respiration at 30 C of heat-stressed Saccharomyces cerevisiae in water. Cells were heated for 0, 1, or 2 minutes at 56 C and DNP was added after 30 minutes.



similar when DNP was added immediately after heating.

Addition of glucose (2.2 μmoles or 11 μmoles) stimulated oxygen-uptake in unheated cells and cells heat-stressed for 1 minute, however, little stimulation in rate of oxygen-uptake was observed in cells which were heat-stressed for 2 minutes (Figure 13). This latter result probably was due to the relatively high rate of O_2-uptake in cells heat-stressed for 2 minutes. In addition, after 4 hours the total O_2-uptake resulting from addition of glucose was more than 2 times greater for cells heat-stressed for 2 minutes than it was for nonheated cells or cells heat-stressed for 1 minute.

Leakage of Intracellular Constituents

Leakage of materials absorbing at 260 and 280 nm was minimal after heating for 5 minutes at 56 C (Table 16). The supernatant from unheated cells had an absorbance of 0.047 at 260 nm and 0.037 at 280 nm. The supernatant from a cell suspension heated for 5 minutes at 56 C had an absorbance of 0.097 at 260 nm and 0.063 at 280 nm.

Figure 13. Effect of addition of 1.0 mM glucose on respiration at 30 C of heat-stressed Saccharomyces cerevisiae in water. Cells were heated for 0, 1, or 2 minutes at 56 C and glucose was added after 30 minutes.

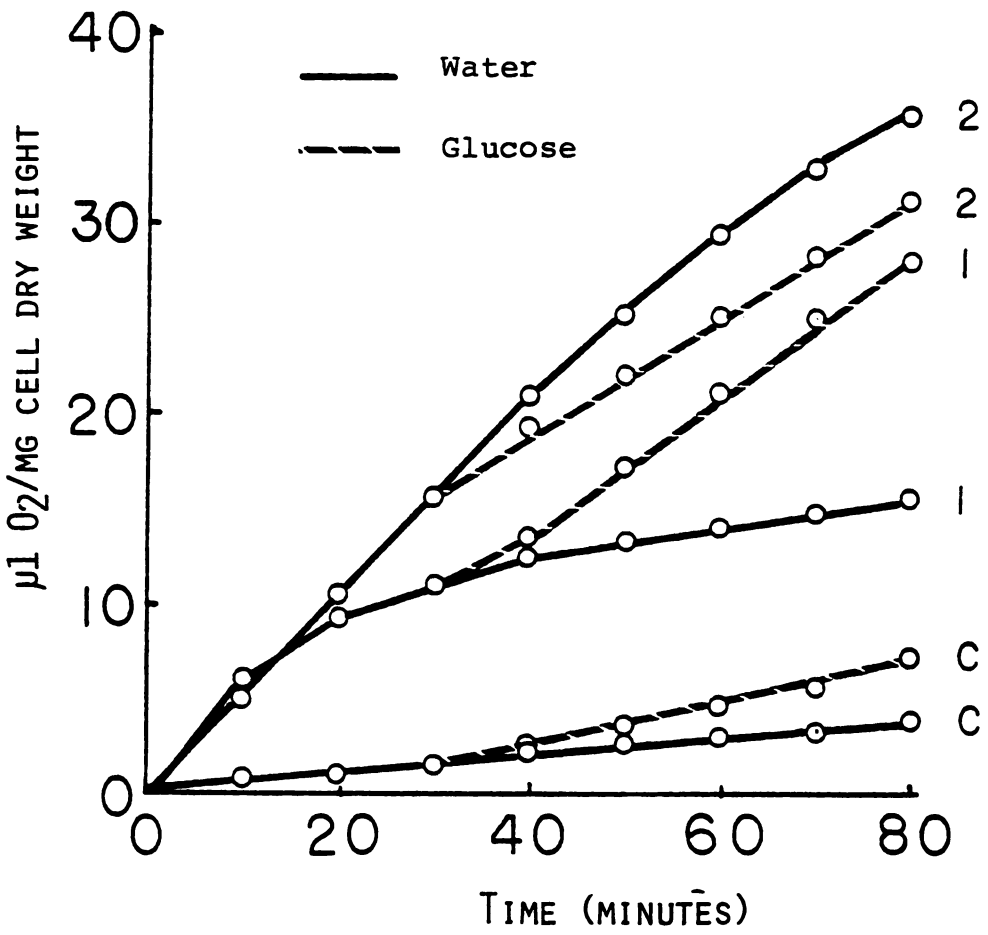


Table 16. Quantitation of materials which absorb at 260 and 280 nm in Saccharomyces cerevisiae suspensions heated at 56 C for 0-5 minutes. The suspensions contained 1.8 x 10^8 cells/ml.

Heat-Stress at 56 C (min)	Absorbance of Supernatant at 260 nm	at 280 nm
0	.047	.037
1	.070	.055
2	.070	.055
3	.087	.065
4	.090	.060
5	.097	.063

DISCUSSION

Investigations of thermal injury and recovery of microorganisms have typically utilized minimal or stress media for the demonstration of thermal injury. Although APDA is recommended for the enumeration of yeasts and molds from food products (APHA, 1976), thermal injury in Saccharomyces cerevisiae Y25 was demonstrated by reduced plate counts on PDA as compared to PCA. The injury was repairable during storage in water at 22 C and was distinguishable from cryptic or cannibalistic growth.

Recovery of Heat-Stressed Cells on PCA and PDA

Even though plate counts of nonheated cells on PCA and PDA were similar, thermal stress at 56 C resulted in reduced colony-forming ability on PDA, as compared to PCA, for a substantial portion of the heat-stressed yeast population. The proportion of survivors affected was related to the severity of heat treatment. In addition, the intercept of -0.45 of the $\log_{10}$ PCA plate counts vs. $\log_{10}$ PDA plate counts indicated an initial heat-induced sensitization to growth on PDA when cells had been heated for 1.5 minutes or more. Previous reports have attributed reduced recovery on PDA to pH sensitivity of yeasts and molds which had been subjected to environmental stress. Nelson (1972) reported maximum recovery of

heat-stressed (51 C for 20 min) S. cerevisiae near pH 8; recovery of heat-stressed cells was markedly reduced on PDA with pH values above and below this pH. A pH sensitivity was also noted in yeasts and molds isolated from food products (Skidmore and Koburger, 1966; Mace and Koburger, 1967; Koburger, 1970, 1971, 1972, 1973; Jarvis, 1973; Ladiges et al., 1974). Recently, Koburger and Farhat (1975) reported use of non-acidified PDA (pH 5.6) plus antibiotics gave recoveries of fungi from foods similar to PCA plus antibiotics.

Effect of Media Composition on Recovery

Stevenson and Richards (1976) concluded differences in recovery of heat-stressed S. cerevisiae Y25 on PCA and PDA were not based on pH since acidification of PCA to pH 5.6 did not alter differences in recovery between the two media. In addition, PDA and APDA gave similar recoveries despite differences in pH. The results of this investigation confirm and extend these observations. Recovery on PDA neutralized to pH 6.6 or on LPDA with 2.0% glucose (pH 6.6) did not appear to differ from recovery on PDA. Also, supplementation of PDA with yeast extract or tryptone did not increase recovery. In contrast, supplementation of a minimal medium with yeast extract improved recovery of thermally injured Candida nivalis (Nash and Sinclair, 1968).

One medium component found to influence recovery of

thermally injured cells in this investigation was glucose. Decreasing the glucose concentration improved recovery on LPDA. Similar effects due to glucose concentration were noted on recovery of heat-stressed cells using MM containing 0.2 to 6.0% steam-sterilized glucose. However, recovery of heat-stressed cells on MM with 2.0% filter-sterilized glucose (pH 5.5) was considerably higher than recovery on MM with 2.0% steam-sterilized glucose (pH 5.2). The small difference in pH between the two media did not appear to account for the large differences in recovery. Plate count agar containing 2.0% glucose also gave reduced recoveries. Thus, it appears that inhibitory products produced during steam sterilization by the interaction of glucose with other constituents of the medium were responsible for the reduced recoveries obtained with increasing concentrations of glucose (Tanner, 1944).

Baldy et al. (1970) reported storage in 10^{-1} to 10^{-3} M glucose prevented recovery of sublethally heat-injured conidia of Penicillium expansum. No difference in recovery of heat-stressed conidia immediately after heating was noted between PDA, ammonium acetate minimal medium, glucose-NH_4 minimal medium (1.0% glucose) and a yeast hydrolysate-neopeptone medium (2.0% glucose). However, acetate was also reported to inhibit recovery during storage. The acetate inhibition and similar glucose concentrations of the other media may account for lack of differences in recovery among the media.

Hagler and Lewis (1974) reported exposure of yeast to glucose during or immediately after thermal stress at 44 C resulted in leakage of intracellular materials. Leakage in suspensions containing 0.4 to 10.0% glucose was similar but in a suspension containing 0.2% glucose leakage was reduced. Little or no leakage was measured when yeasts were suspended in water and exposed to similar temperatures. They concluded thermal injury to the yeast cytoplasmic membrane was enhanced in the presence of glucose. In contrast, Meyer (1975) found exposure to glucose after thermal stress to have no effect on recovery of Candida P25; however, thermal injury in the presence of glucose was irrepairable.

Recovery versus Growth

Postgate (1967) suggested cryptic or cannibalistic growth may interfere with viability measurements of stressed organisms. In response to that suggestion, cannibalistic growth of S. cerevisiae Y25 was studied in this investigation. With conditions and concentrations of cells similar to those utilized in recovery experiments, growth of unheated cells on heat-killed cells was found to occur during a 21-day period. However, growth did not occur during the first 12 hours after heating and slight growth occurred from 12 to 24 hours. In contrast, heat-stressed cells had much greater increases in plate counts during the same period of time. Thus, the large increases

in plate counts of heat-stressed cells observed during the initial 24 hours of storage were the result of repair of thermal injury rather than cannibalistic growth.

Using exponential phase cells of S. cerevisiae, Schenberg-Frascino (1972) reported no cannibalistic growth or resorption of released materials was expected during storage of heat-stressed cells since the cells were washed prior to storage. Near maximal recoveries were noted within the first 48 hours of storage at 28 C in water.

Baldy et al. (1970) noted mycelium formation of heat-stressed P. expansum conidia during storage at 23 C in water if spore concentrations were greater than 10^7 spores/ml. Subsequently concentrations of 10^7 spores/ml were utilized for studies of recovery of colony-forming ability during storage. Maximum recovery was observed after storage for 3 days.

Recovery of Heat-Injured Cells

Increases in plate counts up to 10-fold or more over those obtained upon immediate plating on PCA were noted within the first 6 hours of storage in water at 22 C. In addition, much of the difference in recovery between PCA and PDA was resolved during this time. Smaller increases in plate counts on PCA and PDA occurred between 6 and 12 hours and between 12 and 24 hours.

Fries (1969, 1970, 1972) reported thermally induced

salt sensitivity of Ophiostoma multiannulatum and Rhodotorula glutinis was repairable as demonstrated by increased plate counts after storage for several hours. Tsuchido et al. (1972a) also noted recovery from thermally induced salt sensitivity of Candida utilis, and the recovery was almost complete after 6 hours of storage.

Recovery of colony-forming ability on "non-stress" media was reported to require longer periods of time. Baldy et al. (1970) reported maximal recovery of colony-forming ability on PDA of heat-stressed conidia of P. expansum required 3 days. Plate counts of conidia heat-stressed at 54 C for 1 hour were observed to increase up to 20-fold over values obtained with immediate plating. Schenberg-Frascino (1972) stored heat-stressed cells of S. cerevisiae for up to 5 days after heating; however, near maximal recoveries were reached after 2 days of storage. Increases in plate counts during the first 24 hours of storage were up to 100-fold more than plate counts immediately after heating when a yeast extract-peptone-glucose agar (2.0% glucose) medium was utilized.

Effect of Inhibitors on Repair

Storage in the presence of cycloheximide, chloramphenicol, hydroxyurea, or actinomycin D had no effect on the recovery of colony-forming ability of heat-stressed cells. Plate counts immediately after heating and during storage were not markedly affected by the presence of

some metabolic inhibitors. Apparently, protein synthesis, DNA synthesis, and RNA synthesis were not required for repair of thermal injury as demonstrated by differences in recovery on PCA and PDA or by increases of plate counts on both media during storage.

Baldy et al. (1970) reported similar results concerning repair of sublethal thermal injury of P. expansum conidia. Recovery of colony-forming ability during storage of heat-stressed conidia was not affected by the presence of cycloheximide or 5-fluorouracil, inhibitors of protein synthesis and RNA synthesis, respectively.

In contrast, Schenberg-Frascino (1972) found inhibition of protein synthesis by cycloheximide or fluorophenylalanine prevented recovery of heat-injured exponential phase cells of a haploid strain of S. cerevisiae. Similar inhibition of repair was noted if the yeast was incubated before heating with cycloheximide; however, the heat resistance of the yeast was considerably increased. The increased thermal resistance may have resulted from a shift from exponential to stationary phase due to the inhibition of growth by cycloheximide. In addition, recovery of salt tolerance of C. utilis was prevented by inhibition of protein and RNA synthesis (Tsuchido et al., 1972ab). Storage of thermally stressed C. utilis in the presence of cycloheximide or 8-azaadenine prevented recovery of salt tolerance.

Storage of heat-stressed cells in the presence of

0.1 mM DNP was found in this investigation to prevent recovery from thermal injury. Plate counts on PDA of heat-stressed cells stored in the presence of DNP increased slightly or not at all and plate counts on PCA decreased. A similar inhibition of recovery by DNP was reported in heat-stressed conidia of P. expansum (Baldy et al., 1970). Interestingly, Fries (1972) reported DNP induced salt sensitivity similar to that induced by heat-shock in O. multiannulatum. The salt sensitivity was interpreted as being a consequence of injury to cellular membranes, particularly those of the mitochondria, and to impaired oxidative phosphorylation. In a related investigation, Hagler and Lewis (1974) reported DNP increased glucose-induced leakage of intracellular constituents of heat-stressed yeasts.

Effect of Temperature on Recovery

Storage at 4 C prevented recovery of colony-forming ability of heat-injured cells in this investigation. Plate counts on PDA of heat-stressed cells stored at 4 C decreased during the first 6 hours of storage and increased only slightly after 24 hours. Plate counts on PCA did not increase when heat-stresed cells were stored at 4 C. Similar results were reported by Baldy et al. (1970) and Schenberg-Frascino (1972). Storage at 4 C is used as an indicator of the involvement of metabolic activity in repair. Since storage at 4 C or in the

presence of DNP prevented recovery, metabolic activity was apparently required for repair of injury.

Incubation temperature also influenced expression of thermal injury. Although plate counts of heat-stressed cells on PCA were not substantially affected by incubation temperature, plate counts on PDA decreased as temperatures increased from 20 to 35 C. The influence of incubation temperature on PDA plate counts was also related to the severity of the heat treatment. Since PDA plate counts of heat-stressed cells decreased with increasing temperature, this may reflect variations in degree and/or types of thermal injury. Fries (1963, 1964, 1970, 1972), Fries and Soderstrom (1963) and Gibson (1973) have also reported thermosensitivity of fungal growth resulting from thermal injury. Fries (1964) discovered a similar thermosensitivity was induced by exposure of O. multiannulatum to DNP. He interpreted these results as indicating heat stress and DNP caused damage to mitochondrial membranes with an accompanying decrease in generation of ATP.

Respiration of Heat-Stressed Cells

Rates of endogenous respiration appeared to reflect thermal injury. Prolonged high rates of endogenous respiration were related to evidence of differencial recovery of heat-stressed cells on PCA and PDA. After 20 hours of storage when plate counts on PCA and PDA of

heat-stressed cells were similar, rates of oxygen-uptake were diminished to levels approximating or lower than those of unheated cells.

Brandt (1941) reported a high endogenous oxygen-uptake in cells of S. cerevisiae heat-stressed at 50 C. Evidence of trehalose disappearance from cell reserves concomitant with high rates of oxygen-uptake was presented. While attempts were not made to measure trehalose in this investigation, the initial respiratory quotient of cells heat-stressed for 2 minutes would appear to reflect carbohydrate utilization.

Baldy et al. (1970) reported endogenous O_2-uptake of nonheated and heated spores was similar; however, viability of heated spores measured for O_2-uptake was less than 1.0% of nonheated spores. In contrast, viability of heated cells utilized in this investigation was only slightly reduced in comparison to nonheated cells even though endogenous rates of O_2-uptake were drastically different.

Meyer (1975) reported declines in endogenous O_2-uptake during exposure to supramaximal temperatures which correlated with decreases in viability of heat-stressed Candida P25. Damage to respiratory activity was repairable when cells were heat-stressed in the presence of glucose. In addition, decreases in viability were greater when cells were heat-stressed in the presence of glucose.

Changes in the respiratory quotient of heat-stressed cells may have reflected changes in endogenous substrate utilization or assimilation of substrates for repair of injury. Although the theoretical R.Q. is 1.0 for carbohydrates, 0.9 for amino acids and proteins, and between 0.7 and 0.8 for lipids (Geise, 1962), "oxidative assimilation" of substrates may result in less than theoretical oxygen-uptake (Dawes and Ribbons, 1962).

Spiegelman and Nozawa (1945) concluded endogenous respiration of S. cerevisiae utilized carbohydrate reserves. However, respiratory quotients of 0.99, 1.01, 0.92 and 0.72 were observed when respiration of S. cerevisiae was measured over a period of 7 hours. This investigator found a similar trend since the R.Q. of unheated cells declined to 0.80 after 3 hours. In contrast, heat-stressed cells were observed to undergo fluctuations of R.Q. Cells heat-stressed for 1 minute demonstrated no detectable injury with respect to difference in plate counts on PCA and PDA; however, the R.Q. was 0.70 initially and increased to 0.93 after 3 hours of storage. Furthermore, cells heat-stressed for 2 minutes, which sustained thermal injury as demonstrated by differences in plate counts on PCA and PDA, were observed to have a R.Q. of 1.04 immediately after heating. The R.Q. dropped to 0.72 during the second hour of storage, and then increased to 0.93-0.95 during the third and fourth hours of storage.

It would appear that cells heat-stressed for 2 minutes may have incurred a loss of respiratory control similar to that observed with uncoupling of oxidative-phosphorylation. Since respiratory quotients of cells heat-stressed for 1 and 2 minutes return to values near 0.95 after being at values near 0.70 during storage, this may represent utilization of a different substrate during a portion of the repair process. The initial R.Q. of 1.04 plus the high Q_{O_2} of cells heat-stressed 2 minutes then appears to be uncontrolled respiration which resulted from thermal injury similar to that proposed by Fries (1972). Ward (1968a) postulated uncoupling of respiration as an explanation for anomalous respiratory activity in the presence of DNP and reduced incorporation of substrate of Sclerotinia borealis exposed to maximal temperatures for growth. Obviously, without further evidence such as studies of substrate utilization and P/O ratios the above must remain as speculation.

Further evidence of thermal injury was provided by the rates of oxygen-uptake observed in the presence of DNP. Addition of DNP to unheated cells or cells heat-stressed for 1 minute resulted in increased O_2- uptake or typical uncoupler activity. On the other hand, oxygen-uptake in cells heat-stressed for 2 minutes was depressed by the addition of DNP. Membrane damage might also account for the decreased oxygen consumption in the presence of DNP for cells which were heat-stressed for

2 minutes. One possible explanation is that intracellular concentrations of DNP were increased due to enhanced entrance of DNP into cells with damaged cytoplasmic membranes. Alternatively, low concentrations of DNP may uncouple oxidative phosphorylation of thermally injured mitochondria in a manner similar to that observed at higher concentrations of DNP with mitochondria of non-stressed cells.

Lee (1970) determined that stimulation or inhibition of respiration of S. cerevisiae by DNP was dependent on DNP concentration, pH, and metabolic state of the cells. Inhibition of respiration was observed at high concentrations of DNP, 5×10^{-4} M or higher, and a pH of 5.0 or lower. Stimulation of respiration was observed at lower concentrations of DNP or at higher pH. The inhibition was also dependent on the metabolic state of the cells. Glucose-induced respiration of rapidly metabilizing cells was inhibited; however, glucose-induced respiration of starved cells was not affected.

Oxygen-uptake in response to addition of glucose was related to thermal stress in this investigation. Although O_2-uptake due to glucose addition was not appreciably stimulated initially in cells heat-stressed for 2 minutes, the high rate of oxygen-uptake in those cells may have precluded or masked its effect. However, glucose did increase the total O_2-uptake of cells heat-stressed for 2 minutes in comparison to unheated cells

and cells heat-stressed for 1 minute. The increased consumption of O_2 may reflect utilization of added glucose to meet increased energy demands of heat-stressed cells. Alternatively, Hagler and Lewis (1974) reported glucose addition resulted in damage to cytoplasmic membranes after thermal stress. Thus, addition of glucose may have increased oxygen consumption due to further injury. Baldy et al. (1970) also reported glucose inhibited recovery from thermal injury in P. expansum conidia.

Cell Leakage

Although leakage of cellular constituents, and in particular UV-absorbing materials, is often associated with thermal injury, leakage of materials absorbing at 260 and 280 nm from cells in this investigation was minimal after heating for 5 minutes at 56 C in water. It would appear that extensive damage to the cytoplasmic membrane was not present in thermally stressed cells; however, the amount of leakage in some suspending media may not represent losses in viability or thermal injury. Despite the decreased salt tolerance of thermally stressed C. utilis, Shibasaki and Tsuchido (1973) reported minimal leakage of materials absorbing at 260 nm after heating cells for 10 minutes at 55 C in phosphate buffer, although viability was reduced by a factor of 10^4; however, heating C. utilis at 60 C for 5 minutes resulted in considerably more leakage with a 10^5-fold reduction in

viability. Hagler and Lewis (1974) reported leakage of yeast in the presence of glucose occurred at temperatures even below the maximum temperature for growth; yet leakage in water was minimal at supramaximal temperatures despite reductions in viability. Meyer (1975) noted similar findings upon exposure of the psychrophile Candida P25 to 30 C.

CONCLUSIONS

1. Sublethal thermal injury of heat-stressed Saccharomyces cerevisiae Y25 resulted in reduced and differential recovery on plate count agar (PCA) and potato dextrose agar (PDA). The proportion of the population affected was related to the severity of heat stress.
2. Storage of heat-stressed cells at 22 C in water allowed recovery from injury. The plate counts of heat-stressed cells on both PCA and PDA increased during storage. Increases in colony-forming ability during the first 12 hours were related to recovery from injury rather than cryptic or cannibalistic growth as shown by cannibalistic growth studies and recovery in the presence of growth inhibitors.
3. Recovery from thermal injury was prevented by storage at 4 C or in the presence of DNP, but not by storage in the presence of cycloheximide, chloramphenicol, hydroxyurea, or actinomycin D. Apparently protein synthesis, RNA synthesis, and DNA synthesis were not required for recovery of colony-forming ability of thermally injured cells.
4. Reduced recovery of thermally injured cells on PDA in comparison to PCA was related to glucose concentrations in the media rather than pH. Recovery of thermally stressed cells on a minimal medium (MM, yeast

nitrogen base plus glucose) was also influenced by the glucose concentration and the method of sterilization. Recovery on MM with filter-sterilized glucose was improved in comparison to MM steam-sterilized with glucose.

5. Respiratory activity of heat-stressed cells reflected the severity of heat stress and injury. Oxygen-uptake due to utilization of endogenous and exogenous substrates was increased and respiratory quotients were altered after heat stress. In addition, endogenous respiration of thermally stressed cells in response to addition of DNP was different than that of unheated cells.

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