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Influence of Solute, pH, and Incubation Temperature on Recovery of Heat-Stressed *Wallemia sebi* Conidia

L. R. BEUCHAT†* AND J. I. PITT

Division of Food Processing, Commonwealth Scientific and Industrial Research Organisation, North Ryde, New South Wales 2113, Australia

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The influences of glucose, sorbitol, and NaCl in a basal enumeration medium at water activities (a_w) from 0.82 to 0.97 on colony formation by sublethally heat-stressed *Wallemia sebi* conidia were determined. Over this a_w range, glucose and sorbitol had similar effects on recovery, whereas at an a_w of 0.82 to 0.92, NaCl had a detrimental effect. Colony diameters were generally largest on media containing sorbitol and smallest on media containing NaCl. Maximum colony size and viable population of heat-stressed conidia were observed on media at an a_w of ca. 0.92. When the recovery incubation temperature was 20°C, the number of uninjured conidia detected at an a_w of 0.82 was reduced compared with the number detected at 25°C, while at 30°C, the number recovered at an a_w of 0.97 was reduced. The effect on heat-stressed conidia was magnified. This suggests that *W. sebi* conidia may be more tolerant of a_w values higher than the optimum 0.92 when the incubation temperature is decreased from the near optimum of 25°C and less tolerant of a_w values greater than 0.92 when the incubation temperature is higher than 25°C. The sensitivity of heat-stressed conidia increased as the pH of the recovery medium was decreased from 6.55 to 3.71. *W. sebi* conidia dispersed in wheat flour at a_w values of 0.43 and 0.71 and stored for up to 65 days at both 1 and 25°C neither lost viability nor underwent sublethal desiccation or temperature injury. These results indicate that media routinely used to enumerate molds in foods and feeds are not suitable for enumerating heat-stressed *W. sebi* conidia.

Wallemia sebi (Fries) von Arx (previously known as *Sporendonema sebi* Fries or *Sporendonema epizoum* Fries) is a xerophilic mold which sporulates rapidly and profusely. This organism commonly occurs in cereals, flour, bread (11), and soybeans (13) and has been reported to cause spoilage of these foods as well as fruit jams (14), dried fruits (6, 10), and fish (8). Like other xerophiles, *W. sebi* undoubtedly often goes undetected when foods are analyzed for fungal populations because the water activity (a_w) of media used for routine enumeration is considerably higher than the optimum for growth of *W. sebi*.

The probability of detecting xerophiles in foods is further reduced when secondary stresses such as high hydrogen ion concentration in recovery media and suboptimal incubation temperatures are imposed upon propagules which are already sublethally injured by exposure to adverse physical or chemical environments. While the injury and repair of several genera of fungi have been documented (5), information on the susceptibility of xerophiles to injury which may occur during processing or storage has not been reported.

The study reported here was designed to determine whether conidia of *W. sebi* undergo injury upon exposure to heat and reduced a_w during storage in wheat flour and to investigate the ability of stressed conidia to grow when subsequently exposed to a wide range of a_w and pH values in recovery media. The influence of incubation temperature on colony development by stressed conidia was also investigated.

* Corresponding author.

† Present address: Department of Food Science and Technology, University of Georgia, Agricultural Experiment Station, Griffin, GA 30223-1797.

MATERIALS AND METHODS

Organism. *W. sebi* (Fries) von Arx FRR 1471 from the culture collection of the Commonwealth Scientific and Industrial Research Organisation, Division of Food Processing, North Ryde, New South Wales, Australia, was studied. It was isolated from bread by J. I. Pitt in Sydney, Australia, in 1973.

Preparation of conidia used as test inocula. A medium containing 10.0 g of yeast extract, 50.0 g of glucose, 4.0 g of K_2HPO_4, 15.0 g of agar, and 1,000 ml of distilled water was used to culture *W. sebi*. The medium, designated as YGA (pH 6.2), was poured into 90-mm-diameter petri dishes and surface inoculated with 0.1 ml of conidial suspension prepared from a young YGA culture. After 4 days at 25°C, 3 ml of sterile 0.1% peptone in water (peptone water) was pipetted onto the surface of the culture and conidia were dislodged by gentle rubbing with a sterile, bent glass rod. Suspensions containing 1×10^7 to 3×10^7 conidia per ml served as inocula for all studies involving heat treatment. For viability and injury studies using wheat flour as a storage medium, conidia were transferred directly from the dry YGA culture surface to the flour without the use of peptone water.

Determination of sensitivity of conidia to heat. The ability of *W. sebi* conidia to survive heat treatment when suspended in peptone water at 48, 50, 52, and 54°C was determined. The conidial inoculum (0.5 ml) was mixed with 4.5 ml of peptone water adjusted to the desired heating temperature in a water bath. The suspension, contained in 13- by 100-mm test tubes, was positioned in the water bath such that its surface was at least 2 cm below the level of the constantly circulating water in the water bath. Samples were withdrawn after 10, 20, and 30 min of heat treatment, immediately serially diluted in peptone water, and surface plated (0.1 ml) in duplicate on YGA. Colonies were counted after 5 days of incubation at 25°C.

Determination of susceptibility of conidia to heat injury.

Conidia were heated in peptone water at 48°C for 10, 20, 30, 40, and 50 min as described above. Recovery media consisted of YGA (a_w, 0.97) and YGA supplemented with NaCl, glucose, or sorbitol to result in a_w values of 0.96, 0.92, 0.87, and 0.82. Colonies were counted after 6 days of incubation at 25°C.

The diameters of colonies formed by unheated *W. sebi* on YGA containing various concentrations of solutes were also recorded. Measurements were made after 9 days of incubation at 25°C.

Influence of incubation temperature and pH of recovery medium on resuscitation of heat-stressed conidia. Conidia heated in peptone water at 48°C for 0 and 30 min were surface plated on YGA (a_w, 0.97) and YGA supplemented with NaCl to yield a_w values of 0.96, 0.92, 0.87, and 0.82. Plates were incubated at 20, 25, and 30°C, and colonies were counted after 6 days.

Likewise, heated conidia were surface plated on YGA (a_w, 0.97) adjusted to pHs 6.55, 5.85, 4.49, and 3.71 with 5 M phosphoric acid; the pH adjustment was made after the YGA was heat sterilized. Colonies were counted after 6 days of incubation at 25°C.

Survival of conidia in flour. Commercial wheat flour was equilibrated to a_w values of 0.71 and 0.43 at 25°C by placing 100 g in sealed desiccators containing vessels of saturated strontium chloride and potassium carbonate, respectively, for 12 days. Inoculation with *W. sebi* conidia was achieved by inverting plates containing 7-day-old cultures (YGA, 25°C) directly over the flour and tapping the bottoms gently. After thorough mixing, portions of flour at each a_w were stored at 1 and 25°C. Enumeration of *W. sebi* conidia was done initially and after 9, 30, and 65 days of storage. Duplicate samples (5.0 g) were combined with 45 ml of peptone water in 200-ml pharmaceutical bottles, shaken vigorously, serially diluted, and surface plated (0.1 ml) on NaCl-supplemented YGA (a_w values of 0.97, 0.96, 0.92, 0.87, and 0.82). Colonies were counted after 9 days of incubation at 25°C.

Measurement of a_w. The a_w values of all media were determined with a Sina-scope instrument (Sina, Zurich, Switzerland) at 25°C. All data reported represent the means of values from at least two replicate experiments performed in duplicate.

RESULTS AND DISCUSSION

Demonstration of heat injury. The thermal inactivation of *W. sebi* conidia at 48, 50, and 52°C is illustrated in Fig. 1. Conidia were quite sensitive to heat, with about 96% being killed when held at 48°C for 30 min. A reduction of viable conidia exceeding 5 $\log_{10}$ was observed within 10 min when conidia were held at 52°C. An objective of the experiments to follow was to determine whether a portion of the conidia was sublethally injured upon exposure to heat. Since treatment at 48°C resulted in a substantial reduction in the viable population within a reasonable length of time, as determined by enumeration on YGA, a recovery medium assumed to be relatively free of secondary stress factors, this temperature was chosen to treat the conidia in all subsequent experiments involving an evaluation of recovery conditions on survival and colony formation.

The effects of supplementing YGA with various solutes on recovery of *W. sebi* heated at 48°C for up to 50 min are shown in Fig. 2. Glucose and sorbitol had very similar effects on recovery, although at any given a_w, the presence of sorbitol in YGA appears to have had a favorable effect on resuscitation.

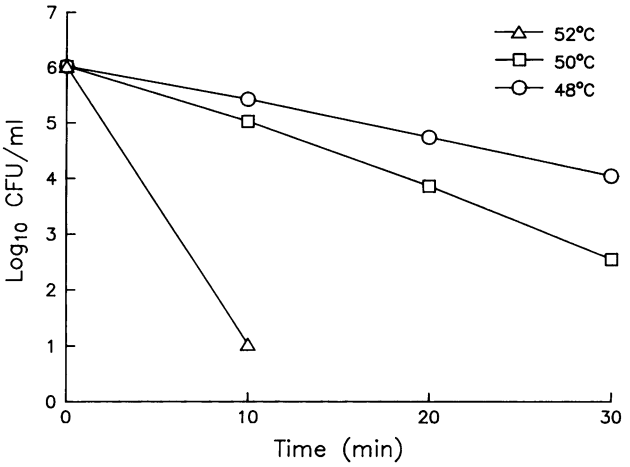

FIG. 1. Survivor curves for *W. sebi* conidia heated at 48, 50, and 52°C for up to 30 min.

Heated-stressed conidia were more sensitive to NaCl than to glucose or sorbitol. A reduction in the number of colonies formed by heat-treated conidia was observed at each analysis time and on all NaCl-supplemented YGA media when compared with the number of colonies formed on glucose- or sorbitol-supplemented YGA with the same a_w. The adverse effect of NaCl was more pronounced as the time of heating increased. Similar observations of an increased sensitivity of heat-stressed conidia of *Aspergillus flavus* and other molds to NaCl have been made (1, 4), indicating that a portion of any conidial population is susceptible to sublethal injury upon exposure to elevated temperatures.

Influence of solute on colony development. The influences of solutes on the diameters of colonies formed by unheated *W. sebi* conidia were determined (Fig. 3). Colonies were generally largest on sorbitol-supplemented YGA and smallest on YGA containing NaCl. Maximum colony size was noted on YGA at an a_w of about 0.92, regardless of the type of solute. While colonies formed on glucose- and sorbitol-supplemented YGA at this a_w were nearly equal in diameter, those formed on glucose-supplemented YGA were easier to count because of their distinct margins. Colonies formed on YGA containing sorbitol had a feathered, floccose appearance, rendering them more difficult to count, especially on plates containing more than about 100 colonies. Although the number of colonies formed on YGA supplemented with glucose, sorbitol, or NaCl at a_w values of 0.806 to 0.974 was essentially the same after 9 days at 25°C, the rate of development was much lower on NaCl-supplemented YGA. This is in agreement with observations that germination of uninjured *W. sebi* conidia, usually followed by growth, is little affected by solute type but that growth is considerably slower on NaCl-supplemented media than on sugar-supplemented media (15).

Effect of temperature on colony formation. Shown in Fig. 4 are the results from experiments designed to determine the effects of incubation temperature on colony formation by unheated and heated (30 min at 48°C) *W. sebi* conidia. It should be noted that the bars for unheated and heated conidia at a_w values of 0.82 and 0.97 (30°C recovery temperature) and heated conidia at an a_w of 0.82 (all recovery temperatures) signify populations less than 10^2 CFU/ml, the lower limit of detection. After incubation at 25°C, recovery of unheated conidia was essentially the same on all NaCl-supplemented YGA at a_w values from 0.82 to 0.97. At 20°C,

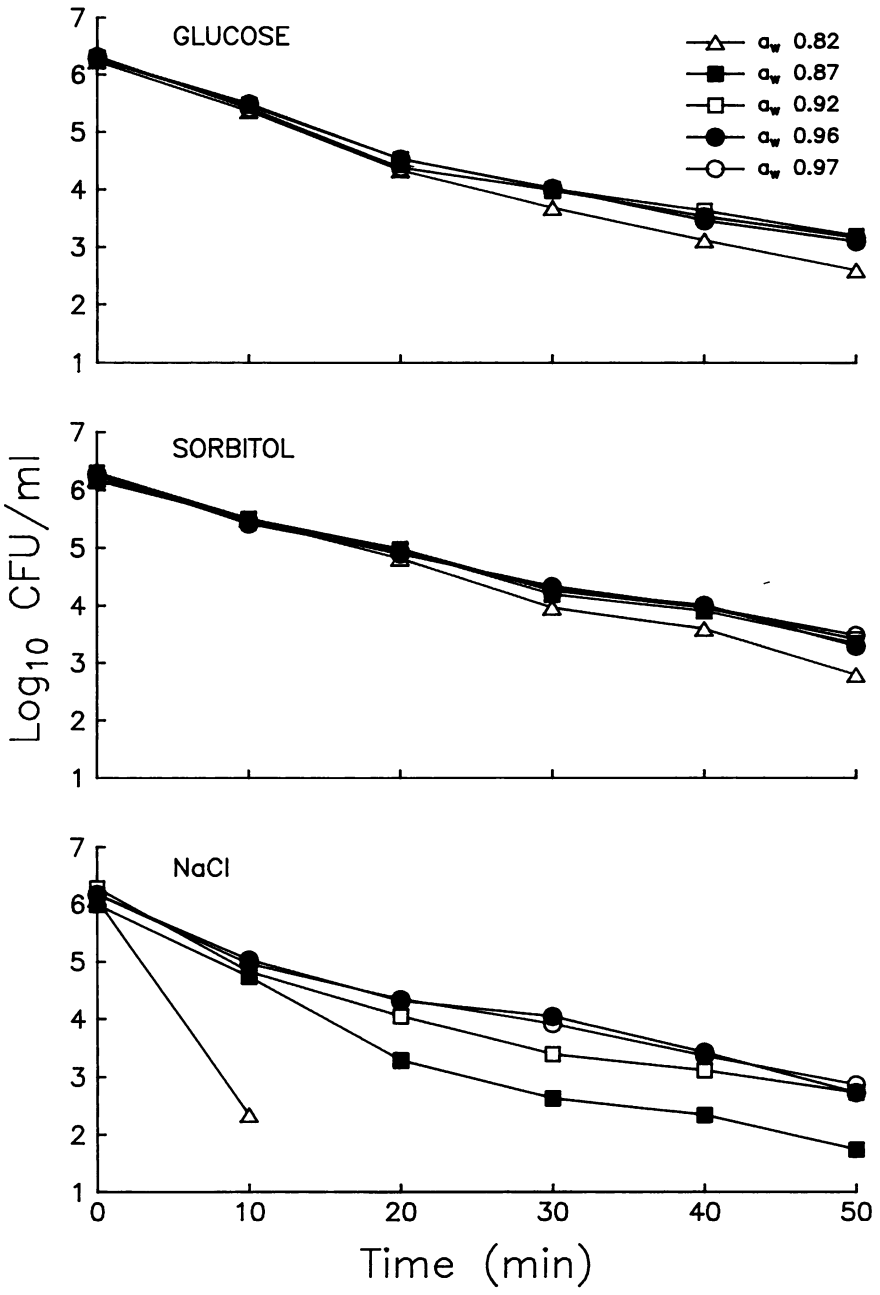

FIG. 2. Effects of supplementing YGA with glucose, sorbitol, and NaCl on recovery of *W. sebi* conidia heated at 48°C for up to 50 min.

the number of unheated conidia recovered at an a_w of 0.82 was reduced about 10-fold, while at 30°C, the number recovered at an a_w of 0.97 was reduced to less than 10^2 CFU/ml. It appears, then, that *W. sebi* conidia are more tolerant of a_w values higher than the optimum 0.92 (Fig. 2) when the incubation temperature is decreased from a near optimum of 25°C and less tolerant of a_w values greater than 0.92 when the incubation temperature is higher than 25°C. This effect was magnified for heat-stressed conidia. Incubation at 30°C was clearly unfavorable to resuscitation of these conidia, even at the lower end (a_w, 0.82) of the a_w range investigated. The suitable a_w range for recovery of heat-stressed conidia narrowed as the incubation temperature increased from 25 to 30°C.

These observations are from experiments using YGA containing NaCl, a solute causing, at a particular a_w, greater inhibition of recovery than that observed for glucose or sorbitol. Whether the same interacting effects of incubation temperature and a_w would hold true if recovery were attempted on YGA containing glucose, sorbitol, or other solutes could be determined only through additional experiments. Another isolate of *W. sebi* has been demonstrated to grow on media supplemented with equal amounts of glucose and fructose at 30 and 34°C and a_w values of 0.97 and 0.92, respectively (15). Those observations, as well as those presented here, strengthen the evidence that the range of a_w tolerated by *W. sebi* conidia narrows as the incubation temperature departs from optimum. Furthermore, the type

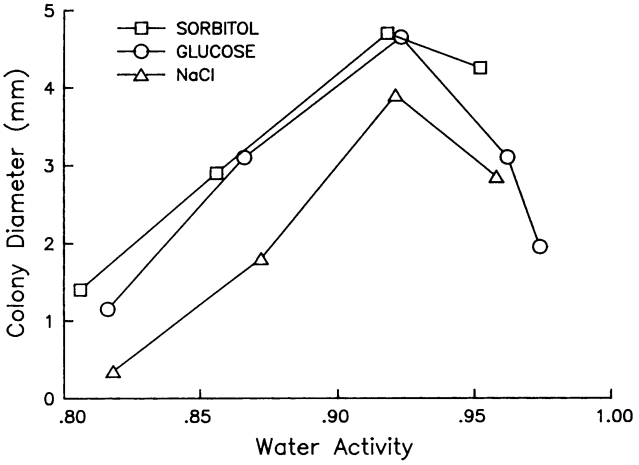

FIG. 3. Diameters of colonies formed by plating unheated *W. sebi* on YGA supplemented with glucose, sorbitol, and NaCl.

of solute used to reduce the a_w has a major effect on the ability of heated *W. sebi* conidia to germinate and grow. The effects of solutes may differ for other xerophiles. Wheeler et al. (16) reported that the halophilic molds, *Polypaecilum pisce* and *Basipetospora halophila*, grew better on low-a_w media containing NaCl than on media containing glucose and fructose at the same a_w.

Influence of pH on colony formation. The number of viable unheated *W. sebi* conidia detected on YGA (a_w, 0.97) adjusted to pH values ranging from 3.71 to 6.55 was similar when the recovery incubation temperature was 20 or 25°C (Fig. 5). An incubation temperature of 30°C resulted in populations of fewer than 10^2 CFU/ml. This confirms the data presented in Fig. 5 showing that colony formation on YGA (pH 6.2; a_w, 0.97) does not occur at 30°C. The inability of YGA at an a_w of 0.97 to support growth of *W. sebi* was not remedied by an adjustment of pH.

The sensitivity of heat-stressed conidia increased as the pH of YGA was decreased from 6.55 to 3.71. This effect was more pronounced at 20°C than at 25°C, thus strengthening observations that 25°C is nearer to the optimum temperature for recovery of heat-stressed conidia. The adverse effect of low pH on recovery and colony formation by heat-stressed conidia of other molds is well documented (2, 7). The repair process is either inhibited or prevented when sublethally injured conidia are exposed to hydrogen ion concentrations which may otherwise have no adverse effect on the germination and growth of uninjured conidia.

The influence of pH on recovery at various temperatures may have been different if YGA containing elevated levels of glucose, sorbitol, or NaCl had been used to reduce the a_w. Pitt and Hocking (12) showed that *W. sebi* is relatively intolerant to pH 4.0 compared with pH 6.0 when glycerol and NaCl are used to decrease the a_w of a medium used to study the germination of conidia. *W. sebi* grew best at pH 6.5 and grew more rapidly in media containing NaCl than in media containing glucose-fructose or glycerol. The minimum a_w for spore germination of several field fungi increased by

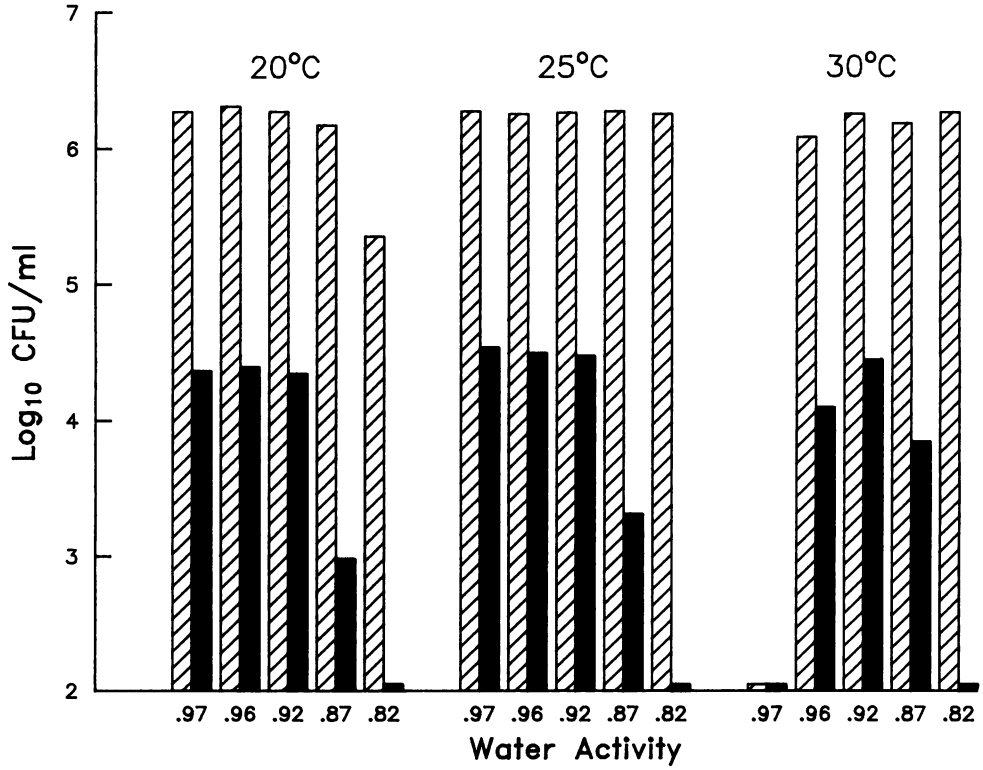

FIG. 4. Effects of incubation temperature (20, 25, and 30°C) on recovery of unheated (▨) and heated (30 min at 48°C; ■) *W. sebi* conidia on NaCl-supplemented YGA at a_w values of 0.82 to 0.97. It should be noted that bars for heated conidia at an a_w of 0.82 (all recovery temperatures) and for unheated and heated conidia at an a_w of 0.97 (30°C recovery temperature) signify populations of fewer than 10^2 CFU/ml, the lower limit of detection.

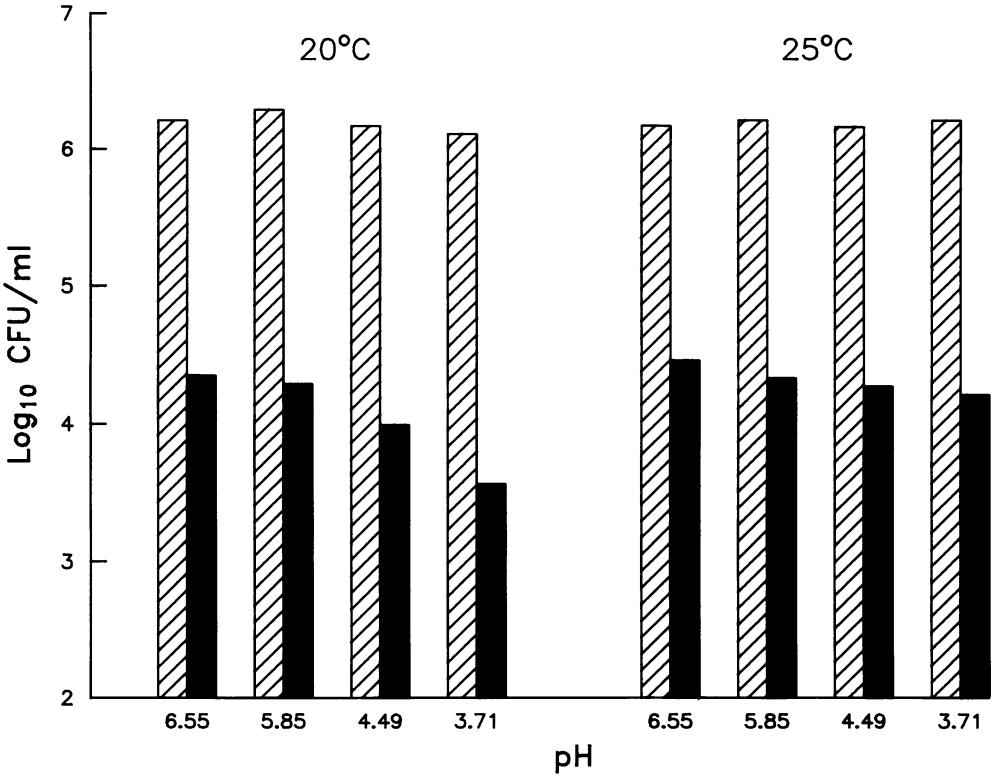

FIG. 5. Effects of pH on recovery of unheated (▨) and heated (30 min at 48°C; ■) *W. sebi* conidia on YGA (a_w, 0.97) at 20 and 25°C.

about 0.02 when the pH of the enumeration medium was decreased from 6.5 to 4.0 (9).

Viability of conidia in wheat flour. *W. sebi* conidia dispersed in wheat flour at a_w values of 0.43 and 0.71 did not lose viability when stored for up to 65 days at 1 or 25°C. Furthermore, sublethal desiccation and temperature stress were not detected in conidia subjected to any of the storage conditions used, as populations (ca. 10^5 CFU/g) remained essentially stable at a_w values of 0.82 to 0.97 throughout the experiment. These observations indicate that *W. sebi* survives well under storage conditions not unlike those practiced in the cereal and milling industries.

The composition of food in which fungal conidia are suspended during storage has been shown to influence the rate of inactivation. Viable *A. flavus* conidia, for example, are reduced by about 25% when held in peanut flour at an a_w of 0.78 for 48 weeks at 21°C compared with essentially no reduction at an a_w of 0.32 (3). Reductions greater than 10^3 CFU/g in viable conidia were observed in coconut, potato flakes, and cocoa at an a_w of 0.78 stored under the same conditions. The viability of *A. flavus* conidia in these foods at an a_w of 0.32 was relatively constant. It is difficult, therefore, to extrapolate observations on the behavior of *W. sebi* in wheat flour to other foods. Results do indicate that *W. sebi* conidia can tolerate storage a_w and temperature conditions not unlike those characterizing a wide range of food types.

In summary, results indicate that media routinely used to enumerate yeasts and molds in foods and feeds do not provide optimum conditions for enumerating heat-stressed *W. sebi* conidia. The addition of glucose or sorbitol to media to reduce the a_w to ca. 0.92 would enhance recovery and colony formation. The pH of media suitable for recovering *W. sebi* should not be less than 6.5, and the incubation temperature should be 25°C. Additional investigations are necessary to determine the suitability of media and recovery conditions for resuscitating *W. sebi* conidia injured by freezing and chemicals.

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