Sporicidal action of hydrogen peroxide

by

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DEDICATION

To my wife, Marcia Lynn, and my daughter, Rebecca Ann

INTRODUCTION

Hydrogen peroxide is used as a bactericide in foods such as milk and eggs and as a sterilant for processing equipment and packaging materials used in aseptic packaging processes. Packaging systems that utilize paper- or plastic-based packaging materials are used increasingly by the food industry because of the simplicity of operation, the lower raw-material costs, and the easier disposal of the empty containers. The nature of these packaging materials excludes the use of heat as a sterilizing agent. Hydrogen peroxide appears to be the most suitable sterilizing agent for such materials because it does not impart an off-flavor to the product and small residues on the packaging material can be tolerated without adverse effects.

Furthermore, aseptically processed foods are sterilized by high-temperature short-time sterilization. This results in products that have better taste when compared with retorted products, display superior texture and color, exhibit less damage to nutritive and vitamin properties, and show less change in heat sensitive components such as protein.

Because hydrogen peroxide is widely used as a bactericidal agent, because increased usage as such appears to be a possibility, and because bacterial spores are more resistant to hydrogen peroxide than vegetative cells, procedures used for the assessment of hydrogen peroxide as a sporicide were selected for study.

Most workers appreciate, in principle, the difficulties in recovering damaged spores but, in practice, fail to take this knowledge into

account and use conditions which are ideally suited for enumeration of undamaged spores. None of the studies conducted to determine the resistance of spores to hydrogen peroxide have addressed the problem of recovery, none have offered any rationale for the selection of media used, and none have systematically studied the effect different media have on the recovery of spores so treated. Additionally, the techniques used by others for assessment of the sporicidal action of hydrogen peroxide appear to be less than ideal for the enumeration of small numbers of spores. The ability to recover all viable spores is necessary in order to evaluate hydrogen peroxide as a bactericide. The basic purpose of this research was to improve recovery procedures used for the assessment of hydrogen peroxide as a sporicide.

The specific objectives of the study were: 1) to develop a method of maintaining spore resistance to hydrogen peroxide during storage; 2) to assess various media for recovery of hydrogen peroxide-treated spores; 3) to study the effect each constituent of the best recovery medium had on recovery; 4) to formulate an improved recovery medium; 5) to evaluate the method of hydrogen peroxide removal from treated spores; 6) to determine whether or not subculturing of spores surviving exposure to hydrogen peroxide results in an increase in the resistance of spores produced in the subculture; and 7) to determine the effects pH and metal salts have on the sporicidal action of hydrogen peroxide.

REVIEW OF LITERATURE

Credit for the discovery of hydrogen peroxide has been given to a French chemist, Baron Louis-Jacques Thenard, who in 1818 reported a method for its preparation to the Paris Academy of Sciences (Schumb et al., 1955). The individual who first recognized the bactericidal properties of hydrogen peroxide is not known. Hydrogen peroxide was introduced as an antiseptic or disinfectant in 1856; however, this occurred at a period before the necessity for asepsis was commonly recognized and based upon scientific principle (Richardson, 1891).

Preservation, Pasteurization, and Sterilization With Hydrogen Peroxide

One of the first uses of hydrogen peroxide in the food industry was suggested by Baldy in 1881 (Schumb et al., 1955). He suggested a procedure for the preservation of milk by hydrogen peroxide, and in 1883, Schrodt published on such a procedure. Today hydrogen peroxide is permitted as a bactericide in milk for the manufacture of cheddar, colby, washed curd, and Swiss cheeses, although it is not permitted as a substitute for pasteurization of milk in the United States (Food and Drug Administration, 1962). Removal of the hydrogen peroxide using catalase is required and recently Chu et al. (1975) have demonstrated the feasibility of hydrogen peroxide removal from cheese milk using immobilized catalase. The literature concerning hydrogen peroxide treatment and preservation of milk has become voluminous since Baldy's early

suggestion and a number of reviews have been written (Schumb et al., 1955; Luck, 1956; Roundy, 1958; Luck, 1962).

Preservation of numerous foods and drinks by the use of hydrogen peroxide has been attempted or proposed. These foods include fish, canned bouillon, sake, eggs, vinegar, ketchup, coffee syrup, cocoa syrup, gelatin, pickles and starch (Schumb et al., 1955) as well as soybean curd and vermicelli (Ogawa, 1970a, 1970b), cream (Collins and Dirar, 1969), and whey (Stadhouders and de Vries, 1971). Nevertheless, hydrogen peroxide is not permitted as an additive in foods in the United States (Desrosier, 1970).

Production of hydrogen peroxide by Lactobacillus species in 1 percent peptone parallels inhibition of Pseudomonas, Bacillus, and Proteus even when pH reduction is not great enough to prevent growth (Price and Lee, 1970). Dahiya and Speck (1968) have shown that neutralized culture filtrates of Lactobacillus lactis and L. bulgaricus are inhibitory to Staphylococcus aureus due to hydrogen peroxide. Gilliand and Speck (1975) have shown that an inhibitory effect of L. bulgaricus NCS1 on growth of a selected psychrotroph in a solution of nonfat milk solids is due to hydrogen peroxide. Thus hydrogen peroxide may function as a naturally occurring preservative in foods fermented by Lactobacillus species.

The effect that hydrogen peroxide has on growth, inhibition, and death of spoilage bacteria at low pH levels is not clear. Because hydrogen peroxide is most stable when acidified (Manufacturing Chemists Association, 1969), the changes in microbial flora observed in foods where

Lactobacillus species have overgrown the natural flora may be due in part to hydrogen peroxide.

Ripened honey is protected against microbial contamination by its hyperosmotic state. Dilute or unripened honey is protected against microbial spoilage by the accumulation of hydrogen peroxide (White and Subers, 1963, 1964). The hydrogen peroxide is produced by glucose oxidase which is shed from the hypopharyngeal glands of the honey bee (Gauhe, 1941).

Hydrogen peroxide utilization in the pasteurization of egg whites is allowed in two processes in the United States (USDA ARS Pub., 1969). Low concentrations (0.05 percent and 0.0875 percent) are added to the egg white and removed after pasteurization by treatment with catalase. By these procedures, salmonellae are eliminated by a milder heat treatment than when heat alone is used for pasteurization (125°F for 3.5 min vs 134°F for 3.5 min); thus, the desirable functional properties of the egg whites are preserved. A process for the pasteurization of whole egg magma has been patented (Rogers et al., 1972). In this procedure, the magma is heated in the temperature range of about 130-150°F (preferably 134-136°F) in the presence of about 0.025-0.15 percent hydrogen peroxide for the last 10 seconds of heating.

Hydrogen peroxide is effective in reducing the bacterial load in precooked frozen shrimp (Siegenthaler, 1972); and, in Japan it is used as a bleaching and sterilizing reagent in fish paste products and boiled noodles (Ishiwata et al., 1971). According to the Food Sanitation Law of Japan the maximum allowable residual concentration of hydrogen peroxide

in fish paste and noodles is 30 and 100 ppm, respectively (Ochiai, 1971).

The first use of hydrogen peroxide as a sterilant in the food industry was described by Wooldrige in 1916. He described its use for sterilizing pipes, filters and other equipment in the brewery. Recently, similar uses of hydrogen peroxide have been recommended. Kawamata et al. (1971) suggested that wooden containers used in the brewing of sake be sterilized with 1-3 percent hydrogen peroxide.

At the present time, hydrogen peroxide is employed for microbiological destruction on packaging material being used in food packaging processes such as the Pure-Pak, Tetra-Brik and Tetra-Pak packaging systems (O'Keefe, 1974). In these processes the product is sterilized via a high temperature/short-time (HTST) process, cooled, filled into a presterilized container and hermetically sealed within a bacteria-free environment. Products packaged by these processes must be distributed under refrigeration. When these products are compared with retorted products, they apparently have better taste and display superior texture and color, exhibit less damage to nutritive and vitamin properties, and show less change in such heat-sensitive components as protein (Brody, 1971).

Packaging systems that utilize paper- or plastic-based packaging materials are used increasingly by the food industry because of the simplicity of operation, the lower raw-material costs, and the easier disposal of the empty container (Toledo, et al., 1973). Most of these packaging systems are suitable for aseptic operation, but the nature of the packaging material excludes the use of heat as a sterilizing agent.

Hydrogen peroxide appears to be the most suitable sterilizing agent for such materials because it does not impart an off-flavor to the product and small residues on the packaging material can be tolerated without adverse effects (Hsu, 1970).

Bacterial Resistance to Hydrogen Peroxide

Bacterial spores have a greater resistance to hydrogen peroxide than vegetative cells (Nambudripad et al., 1949; Nambudripad and Iya, 1951; Roundy, 1958; Toledo et al., 1973). Consequently, where hydrogen peroxide is used as a bactericidal agent, the destruction of bacterial spores is of major concern. In the following section, factors affecting destruction of spores by hydrogen peroxide will be discussed.

Sporicidal Activity of Hydrogen Peroxide as Affected by Hydrogen Peroxide Concentration, Temperature, pH, and Salts

The sporicidal activity of hydrogen peroxide increases as its concentration increases (Curran et al., 1940; Roundy, 1958; Swartling and Lindgren, 1962; Swartling and Lindgren, 1968; Toledo et al., 1973; Ito et al., 1973), and as the temperature of a given concentration increases (Curran et al., 1940; Roundy, 1958; Swartling and Lindgren, 1962; Swartling and Lindgren, 1968; Kawasaki et al., 1970; Ito et al., 1973). Data of Swartling and Lindgren (1968) indicate that the temperature of hydrogen peroxide and the concentration of hydrogen peroxide do not interact to affect the sporicidal activity of hydrogen peroxide.

The effect of pH on the sporicidal activity of hydrogen peroxide

has received little attention. Cerf and Hermier (1972) studied the influence of pH of hydrogen peroxide on destruction of spores from two Bacillus species. They compared pH 2.9, 4.8, 6.7 and 7.7 in 15 percent hydrogen peroxide at 80°C and in 23 percent hydrogen peroxide at 26°C. In the former solution, there appeared to be a reduction in time required to destroy a given number of bacteria with a decrease in pH. However, the authors believed that the correlation was not sufficiently good to determine precisely the influence of pH. In 23 percent hydrogen peroxide at 26°C, they believed that pH did not influence the death rate.

Curran et al. (1940) observed the effect of pH on destruction of four species of Bacillus by hydrogen peroxide. One percent solutions of hydrogen peroxide were adjusted to pH 3, 6.9, and 9.0 using McIlvaine's buffer. Spores were treated in this system at 50°C and the time required for 50 percent and 100 percent reduction observed. Generally the rate of destruction decreased as the pH increased.

Dittmar et al. (1930) showed that very low concentrations of ferric and cupric ions enhance the germicidal effect of hydrogen peroxide on vegetative cells. Potassium dichromate when combined with manganous sulfate or cobaltous sulfate increases the toxicity of hydrogen peroxide for Esherichia coli approximately as much as ferric and cupric sulfates.

Metal ions enhance production of the hydroxyl radical from hydrogen peroxide (Barb et al., 1951). This radical is the most potent oxidant known to mankind (Neta and Dortman, 1968) and has been implicated as an agent of spore destruction (Powers and Cross, 1970; Powers et al., 1972). Metal ions also augment the decomposition of hydrogen peroxide

at a rate which is influenced both by pH and metal ion concentration (Schumb et al., 1955). Thus the rate of spore destruction may be influenced by a pH-metal ion interaction. Also relatively high levels of metal ions may reduce the sporicidal action of hydrogen peroxide by decomposing it.

Mechanisms of Hydrogen Peroxide Spore Destruction

That hydrogen peroxide is bactericidal has been known for a long time, but how hydrogen peroxide kills bacteria is not understood, although a significant body of related information exists. In the following sections an attempt has been made to synthesize this knowledge for the purpose of determining the mechanism of spore destruction by hydrogen peroxide.

DNA attack by hydroxyl radicals

The degradative effect of hydrogen peroxide on deoxyribonucleic acid (DNA) is well documented (Massie et al., 1972). Various workers have demonstrated that this degradation is greatly enhanced by iron salts (Scholes et al., 1949; Butler and Smith, 1950; Yagi, 1972). Iron salts not only enhance the degradation of DNA by hydrogen peroxide, but also increase the bactericidal effect of hydrogen peroxide. Concurrently the iron salts increase the rate at which hydrogen peroxide decomposes. This decomposition is accompanied by the formation of hydroxyl radicals, thus it is reasonable to assume that hydrogen peroxide destroys spores by means of a hydroxyl radical interaction with DNA.

Involvement of the hydroxyl radical in the bactericidal action of

hydrogen peroxide has been demonstrated in at least two instances. Miller (1969) studied the bactericidal effect of a mixture of ascorbic acid and hydrogen peroxide on Gram-negative bacteria, and noticed that sodium thiosulfate, a free-radical scavenger, completely inhibited bactericidal activity. Miller believed, however, the bactericidal effect resulted from modification of the outermost layers of the bacterial cell by free radicals rather than interaction with DNA. No supporting evidence was presented to substantiate this contention. Powers and Cross (1970) showed that spores of Bacillus megaterium are sensitized to radiation with increasing hydroxyl radical production; this sensitization was reversed when sufficiently high concentrations of ethanol, another hydroxyl radical scavenger, were present.

Evidence indicating that the hydroxyl radical may direct its lethal effect towards DNA of the cell has been presented. In 1951 Cassel observed apparent fusion of chromatinic bodies in cells of several Bacillus species treated with 9 ppm hydrogen peroxide. This observation provided morphological evidence that hydrogen peroxide may interact with DNA in vegetative cells. A similar penetration of spores by hydrogen peroxide might seem doubtful because bacterial spores have long been considered impermeable (Cohn, 1877) and resistant to staining (Hashimoto et al., 1959). However Scherrer et al. (1971) have shown that the spore is quite permeable and molecules with molecular weights of up to 8000 may enter by passive diffusion. A number of solutes relatively small in molecular size apparently permeate the surface with ease, including compounds such as glucose, alanine, and adenosine that induce germination (Gerhardt and

Black, 1961).

Pollard and Weber (1967) studied the effect of 6×10^{-3}M hydrogen peroxide on E. coli cells. Breakdown of DNA occurred, and the rate of breakdown increased with temperature. The sporicidal activity of hydrogen peroxide also increased with increases in temperature. At 37°C the rate constant was 1.2×10^{-5} which indicated that for a cell containing 5×10^{6} nucleotides, the treatment should cause 600 breaks in the DNA in 10 min. Yet the authors said that this treatment produced no enzymatic degradation and only a 65 percent decrease in cell viability.

From the investigations of Cassel (1951), and Pollard and Weber (1967), hydrogen peroxide interaction with DNA found within the vegetative cells was both inferred and demonstrated. However, cell death as a result of such action seems questionable because reaction of hydrogen peroxide with DNA was not directly related to loss of viability. Furthermore, apparent extensive DNA breakage was accompanied by only a modest loss of cell viability. However, limited loss of viability in cells may be due to DNA repair. Repair could mitigate loss of viability in cells where repair occurs about as fast as DNA damage arises, where the integrity of the repair system itself is not appreciably altered, and where other forms of lethal damage are minor.

DNA repair systems are known to be operative in radiation damaged cells. Repair of radiation damage in DNA has been observed in E. coli cells (Tait et al., 1974) where DNA polymerases I and III are believed to function in the repair (Youngs and Smith, 1973; Tait et al., 1974). Gass et al. (1973) stated that three DNA polymerases (I, II, III) have

been identified in Bacillus subtilis, a spore-forming species. These polymerases are said to be similar to the correspondingly numbered E. coli enzymes, two of which are known to function in the repair of radiation damaged DNA. Terano et al. (1974) demonstrated that vegetative cells of a mutant of B. subtilis which has reduced DNA polymerase activity is two to three times as sensitive to gamma rays as the wild type strain. Spores of a DNA polA mutant of B. subtilis are also more sensitive to radiation that those of its parent strain (Terano and Kadota, 1974). The capacity for rejoining radiation-induced single strand breaks of DNA in the B. subtilis mutant was much lower than that in the wild type strain. The wild type strain possessed all three DNA polymerases.

DNA ligase is another enzyme known to function in the repair of DNA. The DNA ligase reaction is known to be the final stage of enzymatic repair of damage induced in DNA by ultraviolet radiation, ionizing radiation and chemical agents (Howard-Flanders, 1968; Richardson, 1969) and a large part of the single strand breaks induced by ionizing radiation in DNA is supposed to be repaired by DNA ligase alone (Town et al., 1972a, 1972b).

Ligases require bihelical DNA structures containing single strand breaks displaying 3'-hydroxyl and 5'-phosphoryl end groups in juxtaposition. There appears to be little if any specificity for the nucleotides joined. The ligase cannot catalyze the joining or polymerization of deoxyribonucleoside monophosphates to DNA (Richardson, 1969).

Durban et al. (1973) has reported repair of radiation damage in

Clostridium botulinum spores; the repair seems to be similar to polynucleotide ligase activity. A similar repair system may explain the recovery of spores of several species of clostridia treated with gamma-radiation (Futter and Richardson, 1972). Durban et al. (1973) observed that repair of single strand breaks by DNA ligase in dormant C. botulinum spores can be inhibited by the addition of 0.02 M EDTA.

The hydroxyl radical, which has been associated with the bactericidal action of hydrogen peroxide, is formed by radiolysis of water. It is widely accepted that the main site of radiation damage in vegetative bacteria is the DNA (Hitchins et al., 1966); pyrimidines are the most radiosensitive constituents (Nofre and Cier, 1964). The hydroxyl radical formed in solution by UV light or as a result of high energy radiation is the principle radical involved in the formation of new or secondary radicals with pyrimidine bases, nucleotides, nucleosides, and yeast nucleic acid (Lagercrantz, 1973). Achey and Duryea (1974) also found that the hydroxyl radical is apparently the important species of water radiolysis in secondary radiation damage to DNA expressed as strand breaks. Similarly, treatment of DNA from Bacillus subtilis 60009 with hydrogen peroxide causes chain breakage as a result of base attack (Rhaese and Freese, 1968). Loss of UV absorption at 260 mu and a decrease in the melting temperature of DNA also were noted, and were attributed to breakage of double bonds in the bases.

Another similarity between the effects of radiation and hydrogen peroxide may be deduced from the experiments of Kashkin (1960) in which cultures of Bacillus faecalis alcaligenes (Alcaligenes faecalis)

acquired resistance to hydrogen peroxide under prolonged exposure. An increase in radiation resistance also resulted in increased resistance to hydrogen peroxide; the converse was not true for hydrogen peroxide.

Enzyme destruction by methionine oxidation

Another possible mode of sporicidal action by hydrogen peroxide is oxidation of methionine and consequently enzyme destruction. Koshland et al. (1962) reported that hydrogen peroxide is a specific reagent for methionine and it has been confirmed that no amino acid other than methionine is oxidized to a significant extent by low concentrations of hydrogen peroxide (Schochter and Dixon, 1964). Methionine is a normal constituent of enzymes and is apparently more labile to hydrogen peroxide than other amino acids. Therefore oxidation of methionine resulting in enzyme destruction is a possible mechanism of spore destruction by hydrogen peroxide.

The work of Kawasaki et al. (1970) and of Heinmets et al. (1954) lends support to this hypothesis. Kawasaki et al. (1970) compared the enzymatic activities of cell free extracts obtained from hydrogen peroxide treated cells of E. coli and Vibrio parahaemolyticus with those of control cells. Activities of all tested enzymes were inhibited by treatment with hydrogen peroxide and the inhibitory effect increased with increasing amounts of hydrogen peroxide/mg cellular nitrogen. Heinmets et al. (1954) observed that cells of E. coli B/r when sterilized with hydrogen peroxide, as determined by plating, could be revived by incubation for 24 h at 37°C in buffer containing metabolites from the citric acid cycle. A mixture of sodium pyruvate, oxalacetic acid, sodium acetate, sodium

citrate, cis-aconitic acid, isocitric acid, lactic acid, malic acid, succinic acid, delta-ketoglutaric acid, and sodium fumarate each at the 0.2 percent level gave the greatest recovery; about 10^6 cells versus none by standard plating procedures.

The implications of these observations is that enzymatic activity of vegetative cells decreases as the hydrogen peroxide concentration increases and that destruction of enzymes in the citric acid cycle may be crucial in bringing about loss of viability. Effect these metabolites have on spore recovery may be of practical importance in determining the kinetics of spore destruction in processes employing hydrogen peroxide as a bactericide.

Disruption of spore coat and/or cortex

The generation of free radicals in a treatment medium containing hydrogen peroxide and ascorbic acid may disturb cell membrane integrity. Miller (1969) has suggested this effect might kill vegetative bacteria interfering with selective permeability. Skarnes (1970) studied the bactericidal effect of L-amino acid oxidase towards staphylococci. The effect was attributed to production of hydrogen peroxide, because catalase effectively inhibited the bactericidal activity. Since staphylococci produce catalase and are quite susceptible to the L-amino acid oxidase system, an intracellular site of hydrogen peroxide action seems unlikely. Polyakov et al. (1973) showed by electron microscopy that hydrogen peroxide produced concentration-dependent damage in the cell wall and granule formation in the cytoplasm of E. coli cells. In this instance the bactericidal effect of hydrogen peroxide on vegetative

cells, in part, appears to be directed towards the cell wall. The morphological effect of hydrogen peroxide on the corresponding spore structures (spore coat and spore cortex) or for that matter on any spore structure, has not been examined.

Mechanisms of Resistance to Hydrogen Peroxide

Catalase

The toxicity of low concentrations of hydrogen peroxide on vegetative cells of a number of genera has been investigated and generally is least toxic to strains which produce catalase or have high catalase activity (McLeod and Gordon, 1923; Molland, 1947; Amin and Olson, 1968). However, Zobnina et al. (1974) found that the catalase activity of Mycobacterium carotenum was not significantly greater than the catalase activity of its carotene-free mutant, although the hydrogen peroxide resistance of the parent was greater than that of its mutant. Interestingly, however, the peroxidase activity of the mutant was significantly greater than that of the parent strain. Further emphasizing the lack of clarity concerning the role of catalase in cell resistance to hydrogen peroxide is that C. botulinum 169B is more resistant to 30 percent hydrogen peroxide than many species of Bacillus; species of clostridia are catalase negative (Ito et al., 1973).

Effect of other spore components on resistance to hydrogen peroxide

Calcium, dipicolinic acid (DPA), and cystine occur in much higher concentrations in spores than in vegetative cells; DPA is unique to spores (Levinson et al., 1961; Murrell, 1967; Vinter, 1959).

Development of radioresistance in sporulating cells appears to be related to synthesis of cystine (Vinter, 1961), although treatment of spores with reagents which rupture disulfide bonds does not increase spore susceptibility to hydrogen peroxide (King and Gould, 1969). Vinter (1961) showed that during germination, radiation resistance is lost, although coats still retain their sulfur content presumably as disulfide. Thus it appears that disulfides (cystine) probably afford spores no resistance to hydrogen peroxide.

Calcium (Ca^{2+}) and DPA incorporation into spores has long been associated with the acquisition of heat resistance in spores (Slepecky and Foster, 1959; Church and Halvorson, 1959; Levinson et al., 1961; Murrell, 1967). Hydrogen peroxide induces release of DPA from spores (Rode and Foster, 1960; Kawasaki et al., 1970); however, radioresistance, and therefore probably hydrogen peroxide resistance, is apparently not correlated with the content of DPA or Ca^{2+} or heat resistance (Yamazaki, 1971). Further, the radioresistance decreases as the Ca:DPA ratio increases where as heat resistance is directly related to the Ca:DPA ratio.

Treatment with acid alone increased the sensitivity of spores to neither inactivation nor lysis by hydrogen peroxide (King and Gould, 1969). Similar acid treatments are known to increase the sensitivity of spores to heat (Alderton et al., 1964), most probably by removing cations like calcium from spores by a cation exchange process, and can also modify the extent of spore dormancy (Lewis et al., 1965). The inference is that the exchangeable Ca^{2+} in spores is probably not involved in maintaining resistance of spores to chemical reagents like hydrogen

peroxide.

Zobnina et al. (1974) investigated the resistance to hydrogen peroxide of a carotenoid-containing strain of Mycobacterium carotenum and its white (carotenoid-free) mutant. The parent culture was more resistant than its white mutant. The level of various cellular components was determined in each strain and comparisons made. The difference in resistance between the two strains could be explained by the presence of carotene in the parent strain as the carotene pigment is known to exert a protective effect against UV radiation, ionizing radiation and ozone (Clark and Frady, 1959; Zobnina and Morkovina, 1971); however, differences in levels of other cell components between the two strains could also explain the difference in resistance. The level of cell lipid was lower in the resistant strain than in the mutant while levels of DNA, RNA and polysaccharides were higher in the resistant strain. The levels of protein in the two strains were identical.

Recovery of Damaged Bacillus Spores

According to Roberts (1970) recovery is regarded as the sequence of events from spore germination to vegetative growth; it is not intended to imply an ability to "repair" damage, but only to germinate and multiply in a suitable nutrient environment. From a practical point of view it would seem that even "ordinary" recovery procedures would not prevent repair to precede recovery. Recoverability or viability is commonly taken to mean one of three things: 1) capable of forming a macrocolony in or on agar; 2) capable of cell division to the extent of forming

turbidity in a most probable number (MPN) count; or 3) capable of causing detectable spoilage in a partial spoilage test.

Enumeration of Bacillus Spores Treated With Hydrogen Peroxide

Swartling and Lindgren (1968) used milk-starch agar to enumerate spores of B. subtilis treated with different concentrations of hydrogen peroxide at different temperatures. The medium was earlier described by Grinsted and Clegg (1955) and consisted of: 1 percent Evan's peptone; 1 percent yeast extract; 0.5 percent sodium chloride; 2 percent agar; 1 percent skim milk; and 0.1 percent soluble starch.

Powers and Cross (1970) used double strength methyl red-Voges Proskauer media with 1.25 percent Bacto Agar added. This medium is recommended for the performance of the methyl red and Voges-Proskauer tests in differentiation of the coli-aerogenes group. Cerf and Hermier (1972) used the medium of Wang et al. (1964) solidified with 15 g agar/liter for the recovery of spores treated with hydrogen peroxide. The medium contained 1 g vitamin-free casamino acids; 2.5 g anhydrous glucose; 5.0 g yeast extract; 0.1 g manganous sulfate; and 0.001 g ferrous sulfate/liter.

Media used by others for the enumeration of spores exposed to hydrogen peroxide include trypticase glucose extract agar and plate count agar by Toledo et al. (1973), glucose agar by Curran et al. (1940) and yeast-glucose agar by Gould and Hitchins (1963). No studies were made, however, to determine the suitability of these media for the recovery of spores damaged by hydrogen peroxide.

Inhibition of Damaged Spores in Recovery Media

Heat-damaged aerobic spores require lower concentrations of chemical inhibitors to prevent growth than do undamaged spores. The greater the damage, the more sensitive are the spores to subsequent inhibition by these chemicals. Such sensitization occurs after heat treatment (Roberts and Ingram, 1966; Cook and Gilbert, 1969). The latter workers observed that increasing the severity of heat treatment rendered spores of B. stearothermophilus increasingly sensitive to sodium chloride in the plating medium.

The addition of starch to overcome the inhibitory effect of fatty acids in media has been suggested by Murrell et al. (1950). They found that starch prevented the inhibitory effect on spore germination brought about by certain unsaturated fatty acids present in the medium. However, not all workers have been able to demonstrate such enhanced counts when starch was incorporated in the recovery medium (Amaha and Ordal, 1957).

The inhibitors thought most likely to be adsorbed by the starch are long-chain fatty acids reported to inhibit spore germination (Foster and Wynne, 1948), but later it was shown that cell division was inhibited and not germination, and then only when the fatty acids were oxidized (Roth and Halvorson, 1952; Halvorson, 1958). Thus, some media may contain inhibitors which are adsorbed by starch, while others contain no such inhibitors and consequently show no increased recovery upon addition of starch.

In addition to the use of starch for the removal of inhibitors in

recovery media, several workers have noted that charcoal and serum albumen overcome the effect of natural inhibitors in media used for spore recovery (Olsen and Scott, 1950; Murrell et al., 1950). Reviewing recovery of spores damaged by heat or agents other than hydrogen peroxide was done because factors involved in recovery may be similar and because such information does not exist for spores damaged by hydrogen peroxide.

Enumeration of Injured Spores

Media

Besides being more sensitive to inhibitors, heat-damaged spores have more exacting nutritional requirements than unheated spores. Curran and Evans (1937) observed that recovery of spores subjected to ultraviolet light was better on nutrient agar plus blood than on nutrient agar, nutrient agar plus yeast, infusion agar, or tomato juice milk powder agar. Recovery of spores subjected to heat was best on nutrient agar plus glucose. Curran and Evans (1937) concluded that organisms which survive drastic killing factors are more fastidious in their food requirements than less resistant individuals which predominate in an unexposed portion of the culture. Campbell et al. (1965) noted that as the severity of heat treatment of spores of B. stearothermophilus increased, recovery on minimal media was considerably less than on glucose-tryptone agar indicating that heat treatment caused some change or changes in the spores, which prevented their development into colonies on minimal agar. The response of aerobic spore-forming organisms after heating

is dependent on environmental conditions; tomato juice agar and yeast extract broth stimulate recovery whereas in nutrient broth, initiation of growth is much delayed (Morrison and Rettger, 1930).

Williams (1970) used nutrient broth with three different agars for recovery of heat treated spores of B. stearothermophilus. Of the three agars, namely Oxoid Agar No. 3, Difco Agar, and Ionagar No. 2, Oxoid Agar No. 3 gave the greatest recovery. Similarly Davies (1975) has shown that slight increases in the agar concentration of media used to enumerate heat-treated spores increase recovery.

Assuming that spore injury may induce dormancy, many workers have added germinating agents to recovery media. Schmidt (1957) increased colony counts of aerobic spores with the addition to the medium of L-alanine, B-alanine and L-valine. Addition of L-alanine and D-glucose to nutrient agar enhances recovery of spores of B. subtilis treated with phenol at elevated temperatures (Russell and Loosemore, 1964). In addition to the amino acids required for germination, severely heated spores of B. subtilis required glutamic acid for colony formation (Hachisuka, 1964) and contrary to the findings of others, L-alanine, used as a germinant, appeared to have an inhibitory effect on colony formation.

Rieman and Ordal (1961) observed that both spores of aerobes and anaerobes germinated in the presence of calcium chloride and sodium dipicolinate (CaDPA) without heat activation. Two strains of B. subtilis which normally required heat activation, germinated totally in a complete medium containing CaDPA (Busta and Ordal, 1964). Some spore crops were

inhibited in CaDPA medium but were all activated similarly when the CaDPA was incorporated in the final dilution prior to plating. A medium of this type used to study thermal inactivation characteristics of B. subtilis spores at ultra-high temperatures resulted in larger D-values than in a medium without CaDPA (Edwards et al., 1965a). Additionally the concave-upwards time-survival curves obtained with standard plating medium were not observed with CaDPA supplemented medium, the higher counts in the supplemented medium falling essentially on a straight line on a log percent graph.

Spores of B. stearothermophilus germinate in the presence of Na_2DPA but do not elongate after the emergence stage of outgrowth (Fields and Frank, 1973). Vegetative growth was inhibited by Na_2DPA.

Mefferd and Campbell (1951) have shown that the addition of low levels of furfural to plating media increases the counts of unheated spores of facultative thermophiles. Spores heated in a solution containing furfural did not show greater recovery than spores subjected to heat in media containing no furfural with the exception of B. stearothermophilus spores. Cook and Gilbert (1968a) found that addition of 1×10^{-4} percent (v/v) furfuraldehyde had little effect on recovery of severely heat treated B. stearothermophilus spores.

Incubation temperature

When B. subtilis spores have been damaged by ultrahigh temperatures, more survivors are evident in a standard medium at 32°C than at 45°C, whereas, the opposite is true of unheated or slightly heated spores

(Edwards et al., 1965b). Greatest counts of unheated or heat activated (10 min at 115°C) spores of B. stearothermophilus are obtained at 50-65°C, but after severe heating (60-70 min at 115°C), optimum recovery occurred in the range of 45-50°C (Cook and Gilbert, 1968a).

Cerny (1975) showed that the survival rate of B. subtilis spores to hydrogen peroxide was highly dependent on incubation temperature and incubation time. More spores developed as colonies at 30°C than at 37°C. It was also noted that many spores apparently killed by a 5 min treatment with 30 percent hydrogen peroxide at 24°C were reactivated by subsequent heat treatment for 2-10 min at 80°C. Treatment at 100°C, however, produced irreversible inactivation. No reactivation was observed at less than 50°C. Prentice and Clegg (1974) examined the effect of incubation temperature on the recovery of spores of B. subtilis 8057. Using both plate count agar and brain-heart infusion agar, they observed that unheated spores showed similar recoveries at 16-48°C whereas heated spores had an optimum recovery temperature of 30°C.

pH of recovery media

Yokoya and York (1965) used three media for the recovery of heat treated B. coagulans spores. The effect of pH was determined by adjusting the pH of each medium to 5.0 and 6.7. In each instance pH 6.7 gave higher D-values than pH 5.0.

Spores of mesophilic species of Bacillus are not especially sensitive to small changes in the pH of the recovery medium, but B. stearo thermophilus, a thermophile, is sensitive to changes of 0.5 pH units

(Cook and Brown, 1965a). Highest counts of unheated spores were obtained in a medium of low pH value (5.9). Recovery after severe heat treatment was greatest at about 7.0-7.3 (Cook and Brown, 1965a).

Eddy and Ingram (1956) determined the range of pH which would support growth equivalent to that at pH 7.0. Using an aerobic, denitrifying Bacillus, Bacillus F, the same recovery of untreated or of irradiated spores resulted between pH 6.0 and pH 8.1. Heated survivors recovered better at alkaline pH values; however, Cook and Brown (1965b) compared the recovery of heat treated spores of B. stearothermophilus at different pH levels. A pH of 7.4 gave the greatest recovery followed in order by pH 7.9, 6.8, and 6.3. Roberts and Ingram (1966) observed that the recovery of heat treated B. subtilis spores decreased in media containing sodium chloride or sodium nitrite as the pH decreased from 7.6 to 5.6.

Plating diluent

Cook and Gilbert (1968a) showed that the diluent used in the plating procedure for determination of the heat resistance of B. stearothermophilus spores had an effect on recovery. Water was the best diluent. The use of quarter-strength Ringer's solution or water containing 0.1 percent (w/v) peptone or 1×10^{-4} percent furfuraldehyde had little effect on the recovery of spores. When water containing 0.85 percent (w/v) sodium chloride was used as diluent there was a small but significant fall in the calculated D-value as compared to water.

Effect of Spore Storage on Heat Resistance

The resistance of bacterial spores to lethal agents and their extreme longevity in specific circumstances might suggest that spores in the main are insensitive to their natural surroundings and in consequence have an assured long life expectancy. In reality, the duration of life in aging spores is not so easily predicted, conditioned as it is both by internal, hereditary considerations and by various external, environmental factors.

Reports on spore viability in relation to aging have been limited almost entirely to pathogenic species inoculated into experimental animals, body fluids, or dried materials from such sources (Frankland, 1893; Tarozzi, 1906; Koser and McClelland, 1917; Morris, 1921; Stein, 1947; Minett, 1950).

Magoon (1925) has observed that the resistance of spores to heat is not a fixed property but a variable one, the degree of resistance being influenced by the age and temperature of storage. Evans and Harris-Smith (1971) stored spores of B. subtilis var. niger in spent sporulation broth at 4°C and -20°C before testing their resistance to an 85°C treatment for 15 min. While storage at 4°C or -20°C had no appreciable effect upon viability, it produced marked differences in the abilities of the spores to withstand the heating treatment. Less than 0.1 percent of freshly harvested spores survived this treatment, but, after 10 weeks at -20°C, 70 percent survived the heat treatment compared with only 4 percent of those stored at 4°C. Cook and Brown

(1965b) observed a decrease in heat resistance of B. stearothermophilus spores impregnated on various kinds of paper strips and stored 16 weeks at room temperature. Cook and Gilbert (1968b) stored spores of B. stearothermophilus at 4°C and -16°C for 52 and 65 weeks respectively. The heat resistance of these spores decreased only slightly during storage over a one-year period.

Evans and Curran (1960) examined the influence of preheating, pH and holding temperature upon viability of Bacillus spores stored for long periods in buffered substrates. They found that prestorage heat treatment increased loss of viability during storage. Also viability was maintained much longer at pH 8 than at pH levels of 4, 5, or 6. Spores stored at 0°C maintained viability considerably longer than spores stored at 30°C or 37°C.

Edwards et al. (1965b) reported that counts of B. subtilis spores surviving 113.3°-131.1°C treatments increased after storage at 3°C by up to 10-fold. Storage in skim milk produced increases in survivor populations for about the first week of storage after which counts generally decreased. Media fortified with calcium dipicolinate gave initial counts which were higher than in unfortified media but counts decreased as the spores aged.

MATERIALS AND METHODS

Organism

Culture and maintenance

Bacillus subtilis var. *niger* American Type Culture Collection (ATCC) 9372 obtained from Mr. Ron Swank of the Cherry-Burrell Corp., Cedar Rapids, Iowa, was used in this study. The organism was selected because other researchers have shown it to be very resistant to hydrogen peroxide (Ito et al., 1973; Wardle and Renninger, 1975). Stock cultures of this organism were maintained on Nutrient Agar (Difco) slants incubated and stored at room temperature.

Production of spores

Spores of *B. subtilis* var. *niger* ATCC 9372 were produced on Trypticase Soy Agar (BBL) in 8" x 14" porcelain-coated pans containing 400 ml of medium. The inoculum used for spore production was prepared by first inoculating a Nutrient Agar (Difco) slant from the stock culture. After 24 h incubation at 30°C, the surface growth was washed from the slant using sterile, distilled water. This growth was diluted to 100 ml and agitated to homogenize the cell suspension. Twenty ml of this cell suspension was used to inoculate the agar surface of one pan. To prevent contamination, aluminum foil was used to cover the pans. After 4 days incubation at 30°C examination of the growth by dark phase microscopy indicated nearly 100 percent sporulation.

Harvesting and cleaning of spores

Spores were dislodged from the agar surface with a sterile, bent glass rod and harvested by washing the surface with sterile, distilled water. The spore suspension was pipetted into a sterile 500 ml centrifuge bottle containing glass beads and centrifuged in a refrigerated (5°C) RC2-B Sorvall centrifuge at 9000 rpm (RCF = 14,712) for 10 min in the GS-3 rotor. Glass beads allowed for the dispersion of pellets between washings. Harvested spores were washed 3 times using cold (5°C), sterile, distilled water. Spores used for storage studies were washed 9 times; the first 6 washings were made using cold, sterile, distilled water while the last 3 washings were made using cold, sterile solutions of either 0.85 percent sodium chloride, Butterfield's buffer (phosphate-buffered distilled water) or distilled water. For all other experiments the spores were washed by the latter procedure using a cold, sterile solution of 0.85 percent sodium chloride for the last 3 washings. Exposure of spores to 70°C in M/15 phosphate buffered at pH 7 for 30 min showed no decrease in cell numbers indicating the spore crops were essentially all spores.

Storage of spores

Cleaned spores were stored in distilled water in 200 ml prescription bottles at 5°C for the initial media study. For storage studies, spores were stored in either 0.85 percent sodium chloride, Butterfield's buffer, or distilled water; they were stored in each solution at -29°C or 4°C. Spores for all other experiments were stored in 0.85 percent sodium chloride at 4°C. To prevent evaporation during storage,

Parafilm (American Can Co., Neenah, Wis.) was used to seal storage containers.

Hydrogen Peroxide

Thirty percent hydrogen peroxide obtained from the Fisher Scientific Co. (Certified ACS) was used as a stock solution for preparing spore treatment solutions. The concentration of the stock solution was verified by the potassium permanganate method of DuPont (1972). The potassium permanganate solution was prepared and standardized according to the Official Methods of Analysis of the Association of Official Agricultural Chemists (Horwitz, 1965), sections 42.023 and 42.024, except that the solution of potassium permanganate was filtered with a sintered glass filter.

Preparation of hydrogen peroxide treatment solutions

Hydrogen peroxide solutions used for spore treatment were prepared aseptically. To prepare a solution containing 5 percent hydrogen peroxide, 15 ml of stock hydrogen peroxide was poured into a 400 ml beaker and made up to 99 ml using distilled water. The 5 percent solution of hydrogen peroxide was then heated to 50°C on a Pyromagnestir (Labline Inc., Chicago, Ill.) and the pH adjusted to pH 4 by the addition of hydrochloric acid and sodium hydroxide.

The electrodes used for adjusting pH of the hydrogen peroxide solutions were sterilized by soaking them for 45 min in a 0.15 percent solution of sodium hypochlorite adjusted to pH 5 using 5 N sulfuric acid. Prior to contact with the solutions of hydrogen peroxide the electrodes

were rinsed 5 times with distilled water (Pearson and Walker, 1976).

After pH adjustment, the beaker of hydrogen peroxide was clamped into a water bath maintained at 50°C and stirred continuously by a motor-driven glass rod (260 rev/min). After the temperature of the solution had equilibrated, one ml of spore suspension was added to yield approximately 3.7×10^7 spores/ml. Spores were exposed to the hydrogen peroxide for up to 15 min. The pH, volume and hydrogen peroxide concentration of the solution did not vary during spore treatment. That the hydrogen peroxide concentration did not vary was confirmed by the sodium thiosulfate method of DuPont (1972).

Enumeration of Spores Exposed to Hydrogen Peroxide

Catalase solution

After exposure of spores to solutions of hydrogen peroxide for a predetermined time, a 1 ml aliquot of the treated spores was pipetted into 9 ml of 0.05 M phosphate buffered at pH 7 (5.81 g dipotassium hydrogen phosphate and 4.54 g potassium dihydrogen phosphate/liter) or Butterfield's buffer containing 1 to 20 mg catalase (Sigma Chemical Co., St. Louis, Mo.) and held at room temperature. In certain experiments, conditions used for removal of hydrogen peroxide were altered. These alterations are described separately in subsequent sections of Materials and Methods. The catalase had an activity of 3600 Sigma units/mg (one Sigma unit decomposes 1 μ mole of hydrogen peroxide/min at pH 7.0 at 25°C). Solutions of catalase were sterilized by filtering through a 0.22 μ Millipore filter. Efficiency of the removal of hydrogen

peroxide was tested by the method of Toledo et al. (1973).

Media

The media selected for recovery of spores exposed to hydrogen peroxide were the medium of Halvorson (1957), the medium of Wang et al. (1964), Plate Count Agar (Difco), Trypticase Soy Agar (BBL), Nutrient Agar (Difco), and Trypticase Glucose Extract Agar (BBL). Contents of each medium are described in Table 1.

Standard plate count procedures were used for enumeration of surviving spores. Butterfield's buffer was used for dilution blanks. The temperature of incubation was 28°C and the plates were counted until maximum counts were attained. In an initial survey the abilities of the media to recover spores exposed to hydrogen peroxide were compared. Freshly harvested spores were used for the first replicate; spores from the same batch after 2 weeks of storage were used for a second replicate.

Examination of the Medium of Wang et al. (1964)

The formula for the medium of Wang et al. (1964) is given in Table 1. The initial survey of media indicated this medium gave the highest level of recovery, therefore the effect each constituent of the medium had on recovery was examined. Table 2 outlines the experiments.

The influence of the pH of the medium also was examined. The medium was adjusted after autoclaving to pH values of 6.4, 6.8, 7.0, 7.3, and 7.6 using sodium hydroxide and hydrochloric acid. Two aliquots of

Table 1. Composition of media used for recovery of spores exposed to hydrogen peroxide (g/l)

Constituent	Medium of Wang et al.[a] (1964)	Plate count agar (Difco)	Medium of Halvorson (1957)	Trypticase glucose extract agar (BBL)	Nutrient agar (Difco)	Trypticase soy agar (BBL)
Agar	20	15	20	15	15	15
Glucose	2.5	1	--	1	--	--
Yeast extract (Difco)	5	2.5	5	--	--	--
Vitamin-free casamino acids (Difco)	1	--	--	--	--	--
Trypticase soy broth (BBL)	--	--	5	--	--	--
Tryptone	--	5	--	5	--	--
Bacto peptone (Difco)	--	--	--	--	5	--
Phytone peptone (BBL)	--	--	--	--	--	5
Trypticase peptone (BBL)	--	--	--	--	--	15
Beef extract	--	--	--	3	3	--
Sodium chloride	--	--	5	--	--	5
Manganous sulfate	0.1	--	--	--	--	--
Ferrous sulfate	0.001	--	--	--	--	--

[a]Adjust pH to 6.8 before autoclaving.

Table 2. Variations in levels of ingredients in the medium of Wang et al. (1964) used for recovery of spores treated with hydrogen peroxide for different lengths of time

Experiment	Experiment time (min)	Medium constituent	Fixed level (g/l)[a]	Levels examined (g/l)	Number of replicates
A1	8 min	Agar	20	15, 20, 25, 30	4
A2	8 min	Glucose	2.5	0, 2.5, 5, 7.5	4
A3	8 min	Vitamin-free casamino acids	1	0, 1, 2, 3	4
A4	8 min	Yeast extract	5	0, 2.5, 5, 7.5, 10	4
A5	8 min	Ferrous sulfate	0.001	0, 0.001, 0.01, 0.1	4
A6	8 min	Manganous sulfate	0.1	0, 0.1	4
B1[b]	10 min	Agar	25	25, 30, 35	3
B2	10 min	Vitamin-free casamino acids	3	0, 3, 6, 9	3
B3	10 min	Ferrous sulfate	0.1	0, 0.1, 1	3
B4	10 min	Manganous sulfate	0.1	0, 0.1, 1	3
C1[c]	10 min	Vitamin-free casamino acids	5	4, 5, 6, 7	4
C2	10 min	Ferrous sulfate	0.1	0.1, 0.3, 0.5, 0.7	6
C3	10 min	Manganous sulfate	0.1	0.1, 0.3, 0.5, 0.7	6

[a]Fixed level indicates the concentration at which the ingredient was held when levels of the other ingredients were varied (experiment A).

[b]The levels of glucose and yeast extract were held constant at 5 g/l and 7.5 g/l, respectively in experiment B.

[c]The levels of glucose, yeast extract and agar were held constant at 5 g/l, 7.5 g/l, and 30 g/l, respectively in experiment C.

spores were exposed to hydrogen peroxide and recovered using media adjusted to each pH. This provided for two replicates of the experiment. Electrodes used for adjusting the pH of the medium were sterilized by the method of Pearson and Walker (1976).

After observations on the constituents of the medium of Wang et al. (1964) independently and after determination of levels of each ingredient that provided maximum recovery, three factorial experiments were conducted to determine a combination of alterations in the medium that might provide for an improved recovery medium. An outline of these experiments is given in Table 3.

Additional efforts to improve the medium of Wang et al. (1964) included addition of the following: L-alanine (0.1 g/l), DL-methionine (0.1 g/l), zinc sulfate (0.01 g/l), magnesium sulfate (0.01 g/l), sodium chloride (2.5 g/l), spore exudate (10 ml/l), and starch (1 g/l). The spore exudate was prepared by exposing a 1:10 dilution of spores (3.7×10^8 spores/ml) to 5 percent hydrogen perioxide for 15 min. Excess hydrogen peroxide was removed by adding catalase to the solution until the evolution of oxygen ceased. After removal of the hydrogen peroxide the mixture was autoclaved.

Effect of Storage on Spore Resistance

Cleaned spores were brought to a volume of about 75 ml with distilled water and divided into 3 equal portions. Each portion was placed in a 500 ml centrifuge bottle and 200 ml of each of the 3 storage solutions (distilled water, Butterfield's buffer, and 0.85 percent sodium chloride) was added. The spores were washed 3 more times in the

Table 3. Factorial experiments with different levels of constituents of the medium of Wang et al. (1964)

Experiment	Experimental design	Exposure time (min)	Medium constituent[a, b, c,]	Levels compared (g/l)
A	2^4 factorial	10 and 12	Glucose	2.5 vs 5
			Yeast extract	5 vs 7.5
			Agar	15 vs 30
			Vitamin-free casamino acids	0 vs 6
B	2^2 factorial	12	Ferrous sulfate	0 vs 0.1
			Manganous sulfate	0 vs 0.1
C	2^2 factorial	12	Ferrous sulfate	0 vs 0.1
			Vitamin-free casamino acids	0 vs 6

[a]The levels of ferrous sulfate and manganous sulfate were held constant at 0.1 g/l each for experiment A.

[b]The levels of glucose, yeast extract, vitamin-free casamino acids and agar were held constant at 2.5, 5, 6, and 15 g/l respectively for experiment B.

[c]The levels of glucose, yeast extract, agar and manganous sulfate were held constant at 2.5, 5, 15, and 0.1 g/l respectively for experiment C.

respective solutions and finally resuspended in 50 ml of storage solution. From each of the 3 solutions, ten 2 ml-aliquots were placed in 20 ml test tubes for storage at -29°C while the remainder of the spores were kept in prescription bottles at 5°C. Prior to storage, resistance of the spores to hydrogen peroxide was determined. The spores were exposed to hydrogen peroxide for 0, 1, 5, 8, and 10 min. Surviving spores were enumerated on the medium of Wang et al. (1964), after hydrogen peroxide removal using 20 mg catalase/9 ml Butterfield's buffer. The incubation temperature was 28°C and the plates were counted daily until maximum counts were attained.

Resistance of the spores was redetermined after 15, 35, and 56 days of storage. Thus, the experiment was a 3 x 2 x 3 factorial (storage solution x storage temperature x storage time) and was replicated 3 times. Storage treatments were applied to 3 separately produced batches of spores providing 3 replications.

Factors Influencing Enumeration of Damaged Spores

Spores were exposed to 5 percent hydrogen peroxide for 8 min; after the exposure, 1 ml aliquots of treated spores were added to a 9 ml catalase preparation. The experiment was a 2 x 2 x 2 x 2 x 5 x 2 factorial replicated 3 times. The factors were as follows:

1) temperature of catalase solution (1°C vs 22°C),
2) concentration of catalase in solution (10 mg/9 ml vs 20 mg/9 ml),
3) catalase contact time (1 hr vs 5 hr),
4) catalase solution plus 1 percent glucose vs no glucose,

5) recovery medium [medium of Wang et al. (1964) vs medium of Halvorson (1957) vs Nutrient Agar vs Trypticase Soy Agar vs Plate Count Agar] , and

6) recovery media containing no calcium dipicolinate vs media containing approximately 20 mM calcium dipicolinate.

Temperatures of catalase solutions were maintained by using an ice bath to maintain 1°C and a water bath for 22°C. A final concentration of approximately 20 mM calcium dipicolinate in the plating medium was realized by adding 0.5 ml of 0.8 M sodium dipicolinate and 0.5 ml of calcium chloride to each Petri dish before the addition of the plating medium. Sodium dipicolinate was obtained by neutralizing dipicolinic acid (Sigma Chemical Co., St. Louis, Mo.) with 2.5 N sodium hydroxide.

Additional observations were made on the following factors and their influence on the number of spores recovered after a 12-min exposure to 5 percent hydrogen peroxide.

1) . . . concentrations of catalase of 10 mg, 1 mg, and 0.1 mg/9 ml of Butterfield's buffer held at room temperature. The recovery medium was the medium of Wang et al. (1964) modified by the addition of 6 g of vitamin-free casamino acids and of 0.1 g ferrous sulfate. Three aliquots of treated spores were recovered using each concentration of catalase.

2) . . . solutions used for the suspension of catalase (1 mg/9 ml) held at room temperature: distilled water, Butterfield's buffer, 0.05 M phosphate buffered at pH 7, Butterfield's buffer with 0.1 percent DL-methionine added, Butterfield's buffer with 0.5 percent yeast extract added, Butterfield's buffer with 0.125 percent glucose and 0.25 percent yeast extract added, and Butterfield's buffer with 0.125 percent glucose and 0.05 methionine. Platings of surviving spores were made immediately and after 1 week storage at 5°C. The medium of Wang et al. (1964) was used for recovery. Three aliquots of treated spores were recovered using each solution of catalase.

Factors Influencing the Sporicidal Activity of Hydrogen Peroxide

Metal salts

Spores were exposed to solutions of 5 percent hydrogen peroxide heated to 50°C for 10 min. Various metal salts were added to the solutions including ferric chloride, ferric citrate, ferrous sulfate, cupric sulfate, zinc sulfate and chromium chloride as well as a combination of ferric chloride and cupric sulfate. The concentration of each salt was 0.1 mM. When ferric chloride and cupric sulfate were combined the concentration of each salt was 0.05 mM. The effect these salts had on the sporicidal action of hydrogen peroxide was compared to a control solution that contained only hydrogen peroxide. The sporicidal influence of ferric chloride, cupric sulfate, and a combination of both in the absence of hydrogen peroxide also were examined.

pH

Spores were treated in 5 percent hydrogen peroxide at 50°C for 10 or 12 min. McIlvaine's buffer was used for pH adjustment for one experiment (10 ml/50 ml of treatment solution) while hydrochloric acid and sodium hydroxide were used to adjust pH in another study. Buffer solutions were prepared by combining 0.1 M citric acid and 0.2 M disodium phosphate. The pH levels selected were 2.2, 3.2, 4.2, 5.2, 6.2, and 7.2. The treatment solutions were placed in 250 ml beakers and heated to 50°C. After temperature equilibration, 0.5 ml of the spore suspension was added. Three aliquots of spores were exposed to provide for 3 replications of the experiment when McIlvaine's buffer was used for pH adjustment. The experiment was replicated twice when hydrochloric acid and sodium

hydroxide were used for pH adjustment.

Selection of Spores Resistant to Hydrogen Peroxide

A suspension of spores was prepared that contained 3.7×10^9 spores/ml. The optical density of such a spore suspension was 3.605 at 540 nm on a Spectronic 20 spectrophotometer. To determine the resistance of the spores a 1:100 dilution was made in 5 percent hydrogen peroxide heated to 50°C, and a survival curve established.

The catalase activity of generation 1 spores was determined by diluting the spores 1:10 in a 0.1 N solution of hydrogen peroxide at 22°C. The concentration of hydrogen peroxide in the solution was measured every 15 min for 105 min by the method of DuPont (1972). The rate of hydrogen peroxide disappearance was taken as a function of catalase activity.

Spores were recovered after exposure to hydrogen peroxide until only a very limited number of survivors could be recovered. A colony of a "last survivor" was picked to a Trypticase Soy Agar slant and incubated at 30°C until sporulation was complete (4-7 days). The spores were harvested by washing with distilled water, placed in a 100 ml beaker and heated to 50°C. At this time hydrogen peroxide was added to yield a final concentration of 5 percent. Spores surviving the longest exposure were picked to a slant and a new generation of spores produced. This process was repeated 10 times. A large batch of spores was produced from the "last survivor" of generation 10. The spore concentration of generation 10 was standardized to that of generation 1 after which resistance to hydrogen peroxide and catalase activity were determined.

RESULTS AND DISCUSSION

Maintenance of Resistance of Spores to Hydrogen Peroxide

Before factors affecting the sporicidal action of hydrogen peroxide could be studied, a technique for the preservation of spore resistance to hydrogen peroxide had to be developed. In addition, a medium for the recovery of spores exposed to hydrogen peroxide had to be selected. The initial task was to select a medium that would yield maximal recovery of spores of B. subtilis var. niger that had been exposed to hydrogen peroxide. Six media, 4 of which had been used by others for the recovery of spores exposed to hydrogen peroxide were examined, and the number of hydrogen peroxide-treated spores recovered in each compared. Although spore resistance declined drastically during storage, a medium that tentatively appeared to be the best recovery medium was selected, namely, the medium of Wang et al. (1964) (medium W). This medium was then used as the recovery medium in subsequent experiments designed to compare the effects of various storage treatments on spore resistance to hydrogen peroxide. Storage of spores in 0.85 percent sodium chloride at 4°C was found to be the best method of storage. A detailed discussion of the work follows.

Selection of Medium for Storage Study

The numbers of hydrogen peroxide-treated spores recovered on each of 6 media were compared (Table 4). These media included the medium of Halvorson (1957) (medium H), Plate Count Agar (PCA), Trypticase Soy Agar

Table 4. Effect of recovery medium on the log of the number of spores recovered after exposure to hydrogen peroxide before and after 2 weeks of storage at 4°C in distilled water

	Time of storage	Time of exposure to hydrogen peroxide			
		1m	5m	8m	10m
TGEA	0 weeks	7.869	7.415	6.398	5.146
	2 weeks	7.146	no growth	no growth	no growth
Medium of H (Halvorson, 1957)	0 weeks	7.954	7.892	7.380	6.255
	2 weeks	7.845	7.415	no growth	no growth
PCA	0 weeks	7.869	7.531	6.869	5.623
	2 weeks	7.892	7.255	4.146	no growth
Medium of W (Wang et al., 1964)	0 weeks	7.934	7.813	7.623	too numerous to count
	2 weeks	7.898	7.204	4.771	no growth
NA	0 weeks	7.929	7.041	4.505	3.176
	2 weeks	7.792	no growth	no growth	no growth
TSA	0 weeks	7.724	6.623	4.114	3.301
	2 weeks	7.820	no growth	no growth	no growth

(TSA), Nutrient Agar (NA), Trypticase Glucose Extract Agar (TGEA), and medium W (Wang et al., 1964). PCA, TGEA, TSA, and medium W have been used by others for the recovery of hydrogen peroxide-treated spores (Cerf and Hermier, 1972; Toledo et al., 1973; and Wardle and Renninger, 1975). Medium H was selected because of its ability to support recovery of heat-damaged spores. NA was used because of its ability to support growth of Bacillus sp. Maximum recovery was attained with medium W, although spore resistance had decreased after 2 weeks of storage. Because medium W recovered the most spores, it was used in subsequent storage studies.

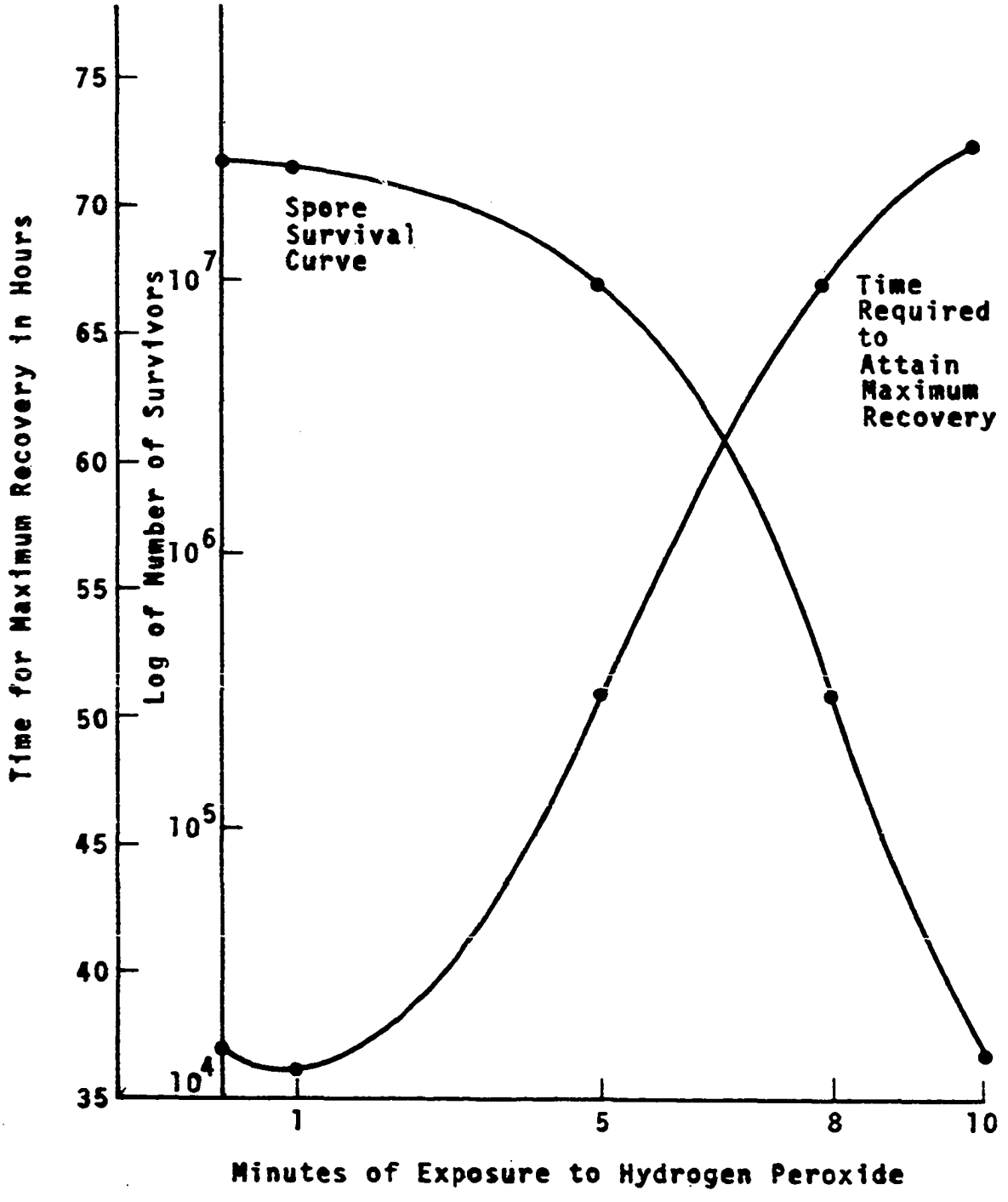
Effect of Spore Storage Conditions on Spore Resistance

Scrutiny of the data in Table 4 indicates that the resistance of the spores to hydrogen peroxide changed over the storage period of 2 weeks. Therefore a means of preserving spore resistance was needed before factors affecting resistance to this chemical sterilant could be determined. The effect that storage factors have on resistance to heat has been examined by others (Evans and Harris-Smith, 1971; Cook and Gilbert, 1968b; Cook and Brown, 1965b; Edwards et al., 1965b) but the effect that storage conditions have on the resistance of bacterial spores to hydrogen peroxide is not known.

The overall effect that length of time of exposure to hydrogen peroxide had on spore survival and rate of colony formation is presented in Figure 1. As the time of exposure to hydrogen peroxide was increased, the length of time for colonies to appear increased and the numbers of colonies that developed decreased. The time required to attain maximum counts as the time of exposure increased was determined by counting the plates periodically during incubation. Diminutive colonies appeared on the plates as numbers of survivors decreased. When extended incubation times were used the development of spreading colonies interfered with the counting of these colonies.

To further complicate recovery, illogical plate counts were frequently observed; that is, platings of serial dilutions of spores did not always result in serial reductions in the number of colonies growing in the respective plates. Instead, plate counts of low dilutions often

Figure 1. The effect of length of exposure time to 5 percent hydrogen peroxide at 50°C on spore survival and rate of colony formation


Time for Maximum Recovery in Hours
75
70
65
60
55
50
45
40
35
Log of Number of Survivors
10^7
10^6
10^5
10^4
Spore Survival Curve
Time Required to Attain Maximum Recovery
1
5
8
10
Minutes of Exposure to Hydrogen Peroxide

were inordinately higher than plate counts of high dilutions. These illogical counts had no apparent relationship to the storage treatments. This problem was resolved by adhering to the rules of counting plates as nearly as possible as spelled out in Standard Methods for the Examination of Dairy Products (Hausler, 1972).

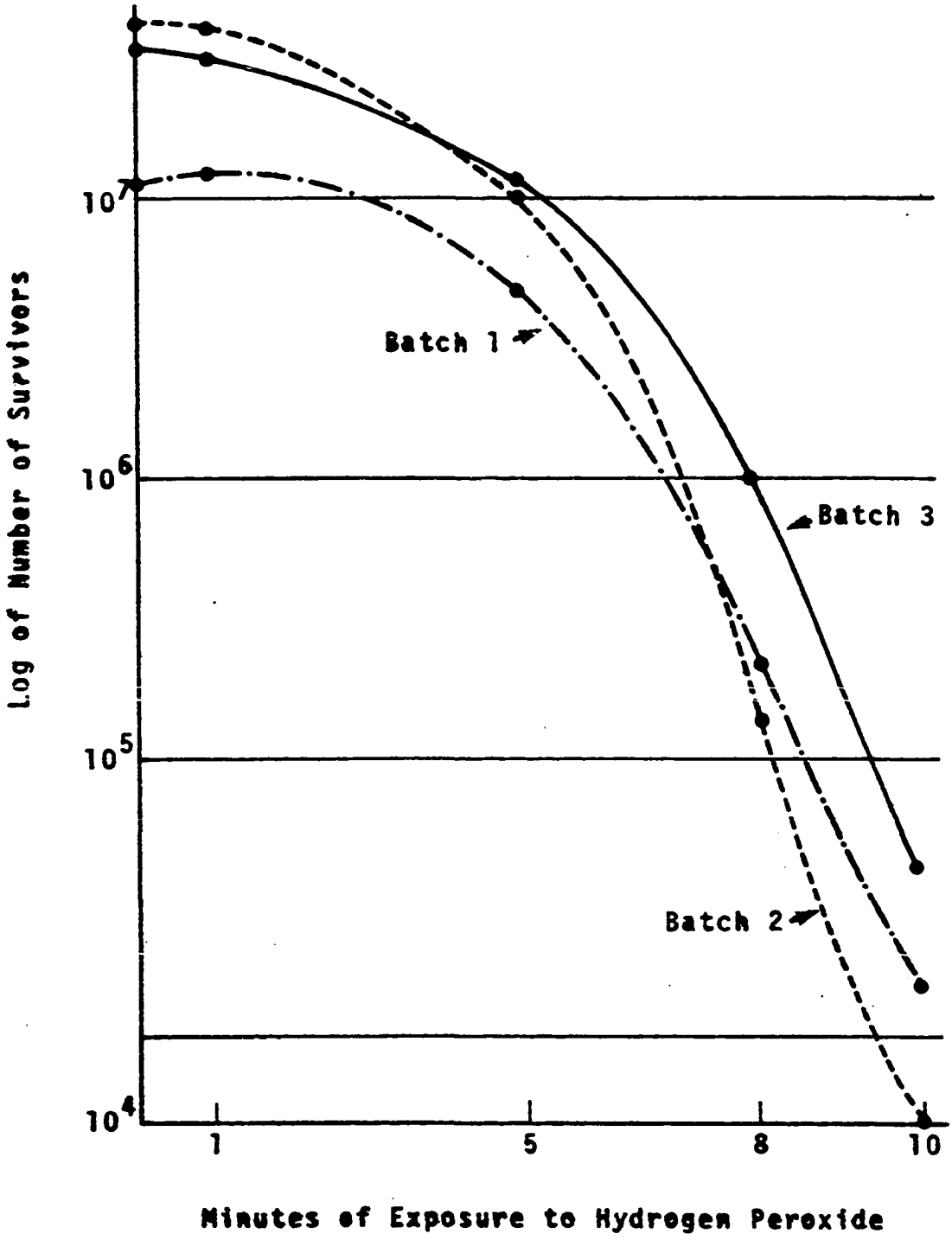
Multiple regression procedures were used to analyze the variance of the log conversions of the spore counts before and after 1 min, 5 min, 8 min, and 10 min exposures to hydrogen peroxide (Tables 35, 36, 37, 38, and 39, Appendix). Tables 40 through 44 of the Appendix show single degree of freedom comparisons made within storage solution and storage time treatments. In general, single factors such as length of time of storage, temperature of storage, and storage solution became more important as the duration of exposure increased. The influence of these factors became particularly evident in spores that had been exposed to hydrogen peroxide for 8 min or more.

Effect of Single Factors on Spore Resistance

Batch or replication

Differences in spore counts between batches, both before and after hydrogen peroxide treatment, were highly significant (Tables 35, 36, 37, 38, and 39, Appendix). Differences between batches were significant mainly because many treatments were applied to each batch. Figure 2 shows that differences between batches 1 and 3 relate primarily to the initial count; batch 2 was slightly less resistant than the other batches. The use of analysis of variance, however, enabled the separation of

Figure 2. Effect of spore batch on resistance to hydrogen peroxide


Log of Number of Survivors
10^7
10^6
10^5
10^4
Batch 1
Batch 3
Batch 2
1
5
8
10
Minutes of Exposure to Hydrogen Peroxide

variation due to batch from other factors allowing for comparisons to be made between treatments. Although spore batches were produced by equivalent procedures, variability in resistance is not unexpected due to many factors including variation between batches of media as well as variation in incubator temperatures. This type of variation from one batch of spores to another is commonly encountered by individuals who have investigated heat resistance of spores.

Length of storage time

Length of time of storage did not have a significant effect on viability of spores (Table 35, Appendix); neither did it have a significant effect on the number of spores surviving an exposure of 5 min or less Tables 36 and 37, Appendix). However, after exposure to hydrogen peroxide for 8 min and 10 min a significant quadratic effect of time of storage on spore resistance became evident (Tables 43 and 44, Appendix). Figure 3C shows that spore resistance decreased for 35 days and then regained prestorage resistance after 56 days of storage.

No satisfactory explanation is available for this phenomenon, but it occurred consistently. Others have shown that storage of and aging of spores affects resistance to other adverse environmental factors such as heat and chemicals (Magoon, 1925; Evans and Harris-Smith, 1971; Cook and Gilbert, 1968b; Evans and Curran, 1960; and Edwards et al., 1965b).

Storage temperature

Spores stored at -29°C were significantly more resistant than spores stored at 4°C (Tables 38 and 39, Appendix). Significant differences

were not observed before exposure or after 1 min and 5 min treatments (Tables 35, 36, and 37, Appendix); after both 8 min and 10 min exposures, however, the resistance of spores stored at -29°C was greater than the resistance of spores stored at 4°C (Figure 3A). Magoon (1925) and Evans and Harris-Smith (1971) also have shown that the temperature of storage affects spore resistance to heat, and in fact Evans and Harris-Smith (1971) observed an increase in spore resistance to heat after 10 weeks of storage at 4°C.

The storage temperatures of 4°C and -29°C were chosen because storage at room temperature may result in loss of resistance because of the favorable temperature for germination; whereas, when spores are refrigerated, germination is not usually a problem (Evans and Curran, 1960). Also the effect that two wide ranging storage temperatures had on spore resistance could be compared.

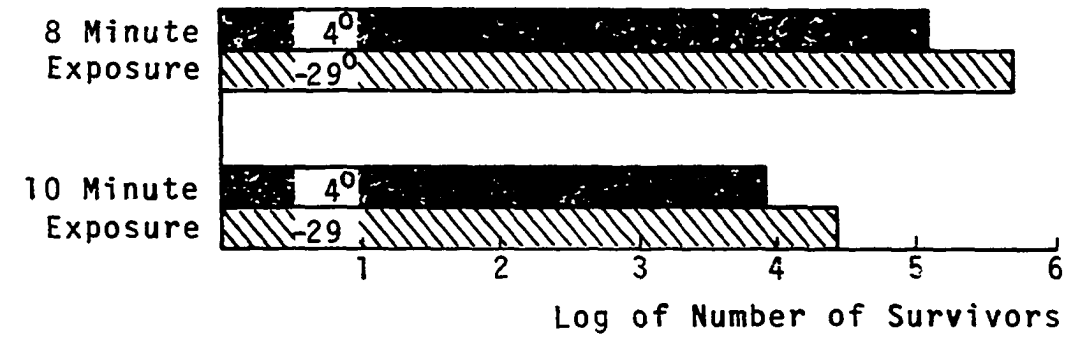
Storage solution

Three different solutions were selected for storage and for comparison of survival of spores and for maintenance of resistance to hydrogen peroxide. Physiological saline (0.85 percent sodium chloride) was used by Toledo et al. (1973) to maintain spores of a Bacillus sp. before exposure to hydrogen peroxide. Cerf and Hermier (1972) stored spores in distilled water for their studies on the sporicidal activity of hydrogen peroxide. Butterfield's buffer (0.31 mM phosphate, pH 7) is frequently used in biological work and was chosen as the third solution.

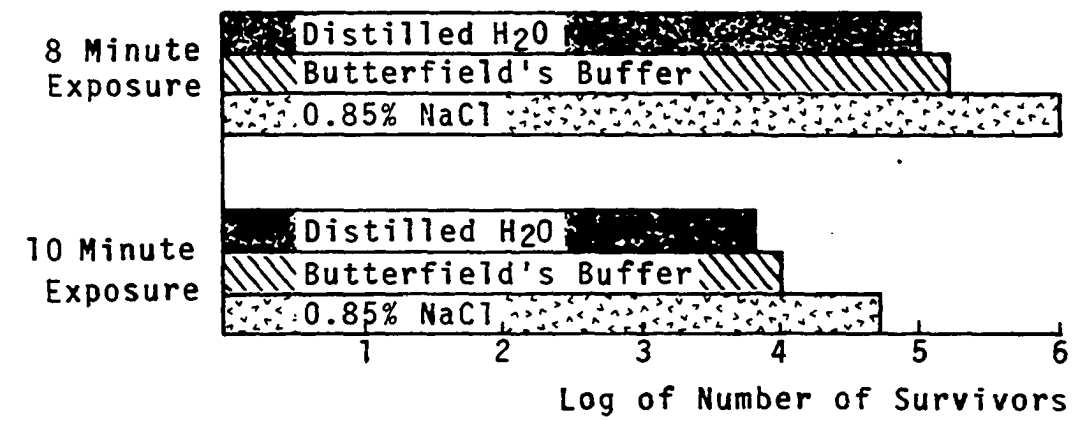
Spores stored in 0.85 percent sodium chloride were more resistant than spores stored in either of the other solutions (Figure 3B).

Figure 3. The effect of storage temperature, storage solution, and storage time on the resistance of spores B. subtilis var. niger exposed to hydrogen peroxide for 8 min and 10 min

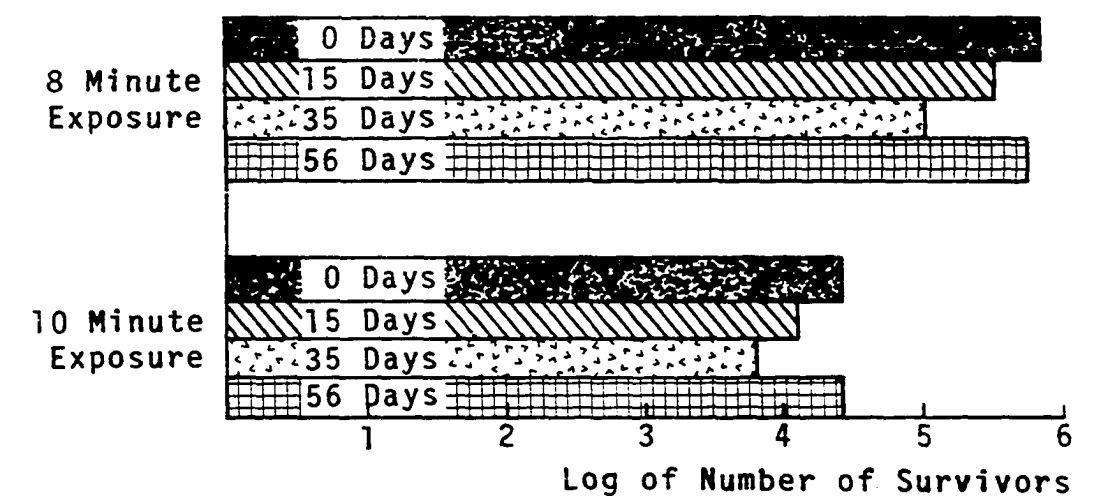
A. Storage Temperature



B. Storage Solution



C. Storage Time



Storage solutions did not have a significant effect on resistance of spores exposed to hydrogen peroxide for 5 min or less (Tables 35, 36, and 37, Appendix). After both 8 min and 10 min exposures, spores stored in 0.85 percent sodium chloride were significantly more resistant than spores stored in the other solutions (Tables 43 and 44, Appendix). The resistance of spores that had been stored in distilled water was not significantly different from the resistance of spores that had been stored in Butterfield's buffer (Tables 40, 41, 42, 43, and 44, Appendix). Storage solutions had little effect on resistance immediately after harvest (Figure 4) therefore inhibition or enhancement of the sporicidal action of hydrogen peroxide due to the storage solutions per se was negated. Apparently time was required for the different solutions to affect spore resistance.

In establishing ionic equilibrium between the spore and the storage solution, the expectation is that spores stored in 0.85 percent sodium chloride would lose fewer native ions than spores stored in the other solutions. Resistance to hydrogen peroxide may be preserved by retaining native ions within the spore.

Effect of Main Factors on Rate of Recovery

Because plates were counted periodically, rate of recovery or rate of appearance of colonies as influenced by storage treatments could be examined. The rate of appearance of colonies is little affected by storage temperature as may be noted in Figure 5. Similar results were noted for storage solution, storage time, and spore batch. Thus, only

Figure 4. Effect of storage solution on spore resistance to hydrogen peroxide before storage

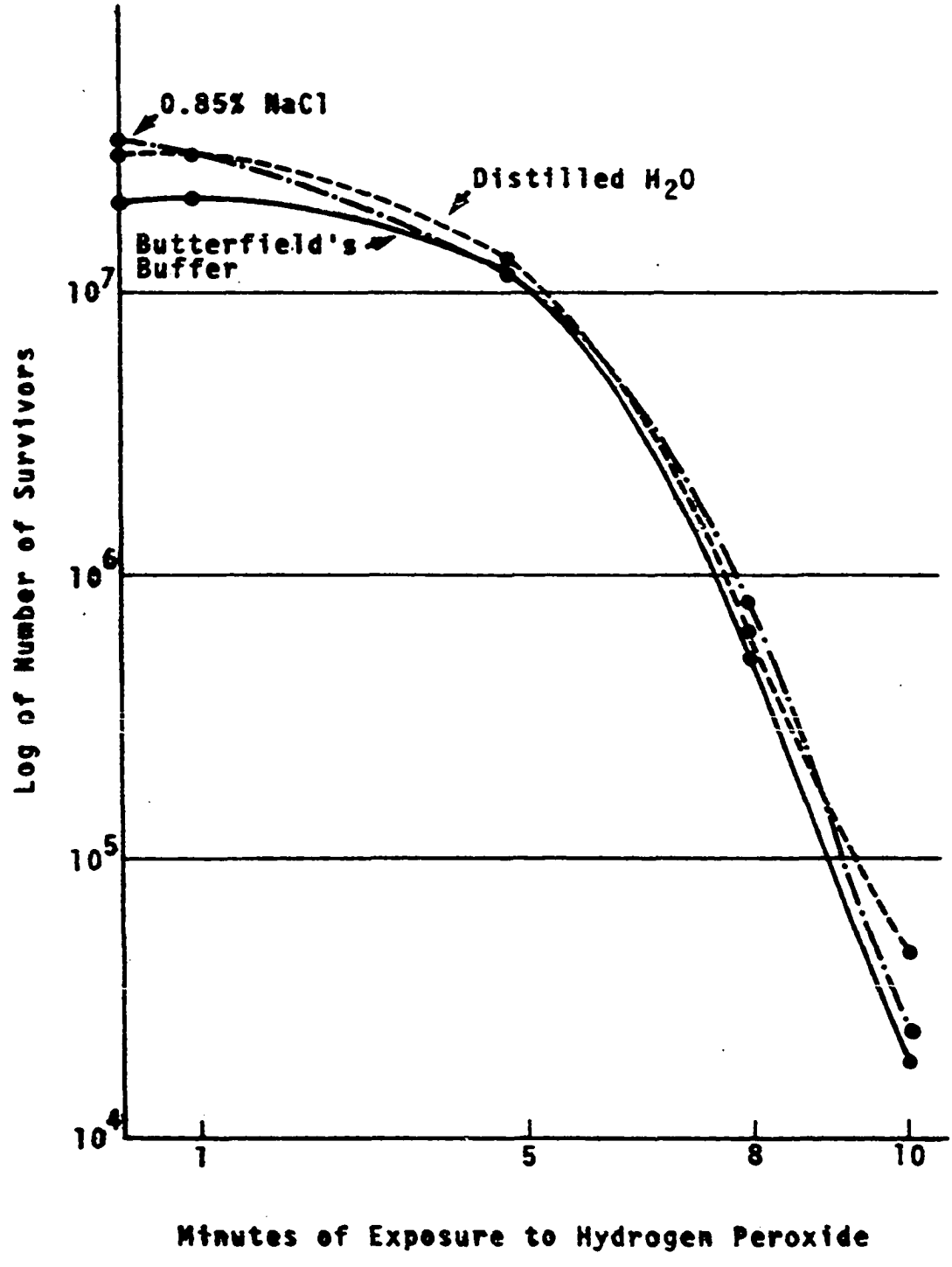
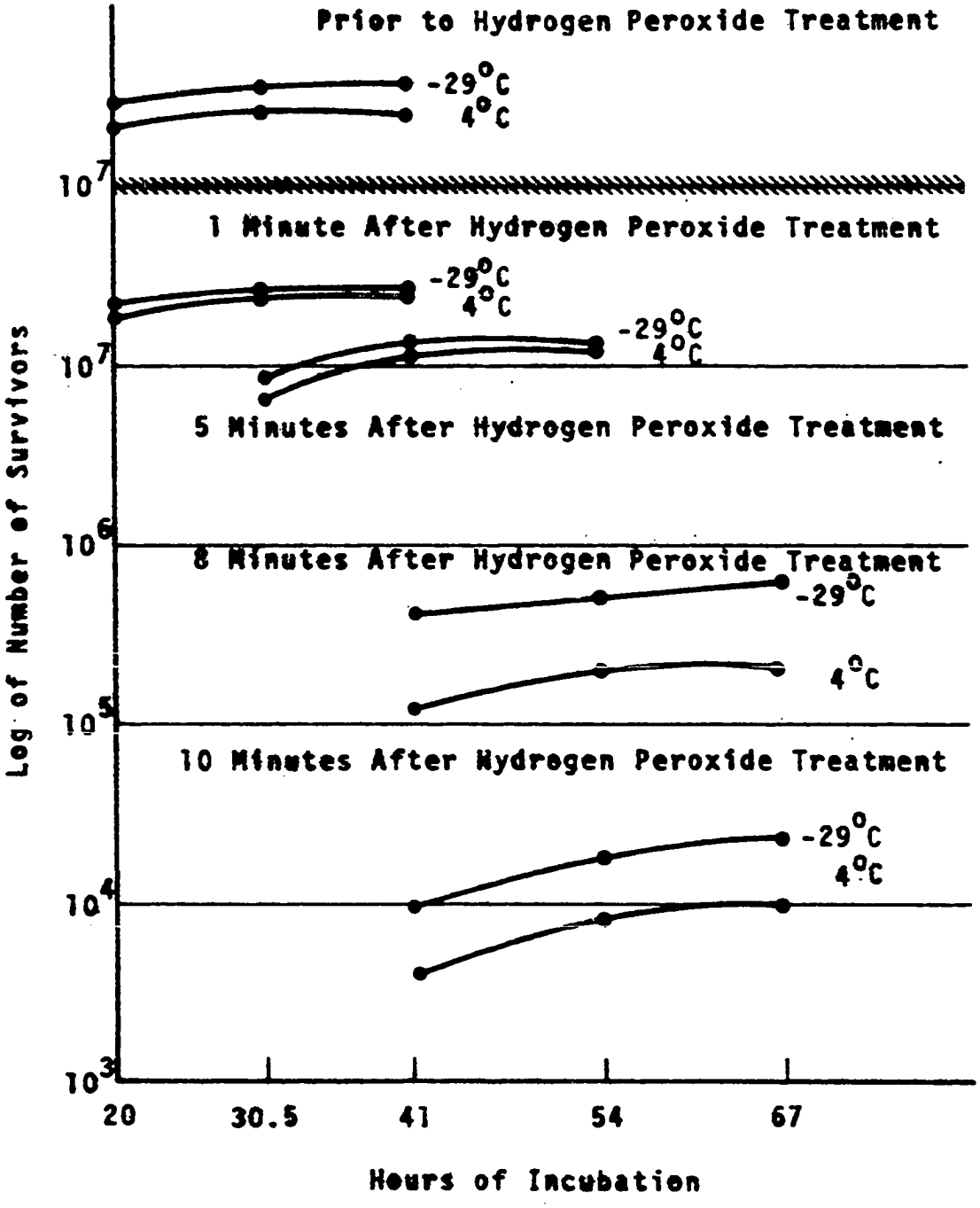

0.85% NaCl
Distilled H_2O
Butterfield's Buffer
10^7
10^6
10^5
10^4
Log of Number of Survivors
1
5
8
10
Minutes of Exposure to Hydrogen Peroxide

Figure 5. Effect of exposure to hydrogen peroxide on the rate of recovery of spores stored at 4°C and -29°C


Prior to Hydrogen Peroxide Treatment
-29°C
4°C
1 Minute After Hydrogen Peroxide Treatment
-29°C
4°C
-29°C
4°C
5 Minutes After Hydrogen Peroxide Treatment
8 Minutes After Hydrogen Peroxide Treatment
-29°C
4°C
10 Minutes After Hydrogen Peroxide Treatment
-29°C
4°C
Log of Number of Survivors
10^7
10^7
10^6
10^5
10^4
10^3
20
30.5
41
54
67
Hours of Incubation

the number of spores susceptible to hydrogen peroxide is influenced by storage treatment; whereas, the ability of the survivors to multiply and give rise to colonies is unaffected.

The effect of the interaction of two or more storage factors on spore resistance

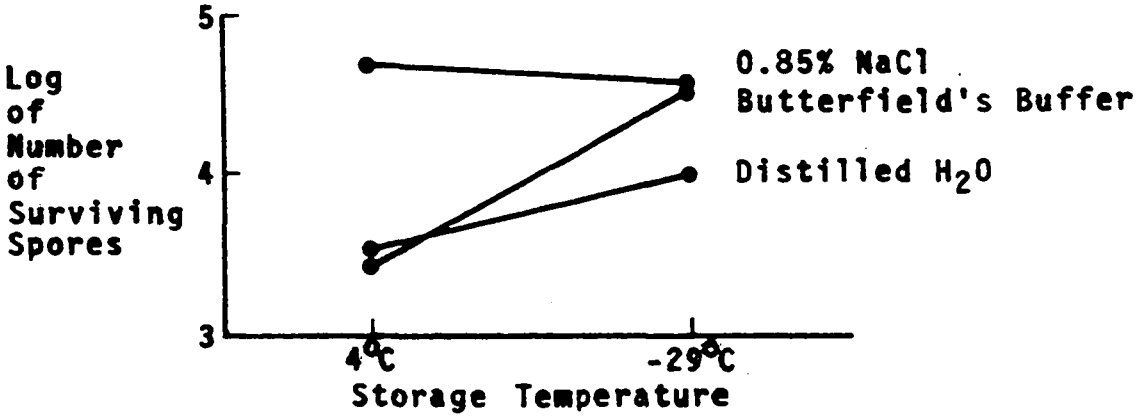
A significant storage temperature by storage solution interaction was observed after a 10 min exposure to hydrogen peroxide (Table 39, Appendix). Decreasing the storage temperature from 4°C to -29°C had a positive effect on the resistance of spores stored in distilled water and Butterfield's buffer; decreasing the storage temperature had a negative effect on the resistance of spores stored in 0.85 percent sodium chloride (Figure 6A).

The overall storage time by storage solution interaction was not significant although during the final 20 days of storage the resistance of spores stored in 0.85 percent sodium chloride or Butterfield's buffer increased while the resistance of spores stored in distilled water decreased (Figure 6B). Throughout the storage period the resistance of spores stored in 0.85 percent sodium chloride was greater than and parallel to the resistance of spores stored in Butterfield's buffer. The storage temperature by storage time (Figure 6C) and the storage temperature by storage time by storage solution interactions were not significant.

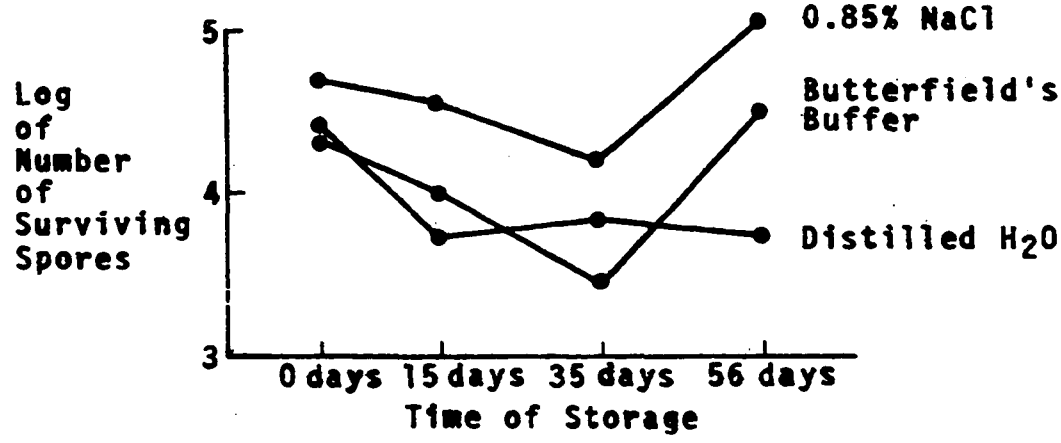
Storage of spores in 0.85 percent sodium chloride maintained greater spore resistance than did storage in other solutions regardless of storage time. Variability in spore resistance during the storage in 0.85

Figure 6. The effect of two factor interactions (A. Storage temperature by storage solution; B. Storage time by storage solution; C. Storage time by storage temperature) on the resistance of spores exposed to hydrogen peroxide for 10 min

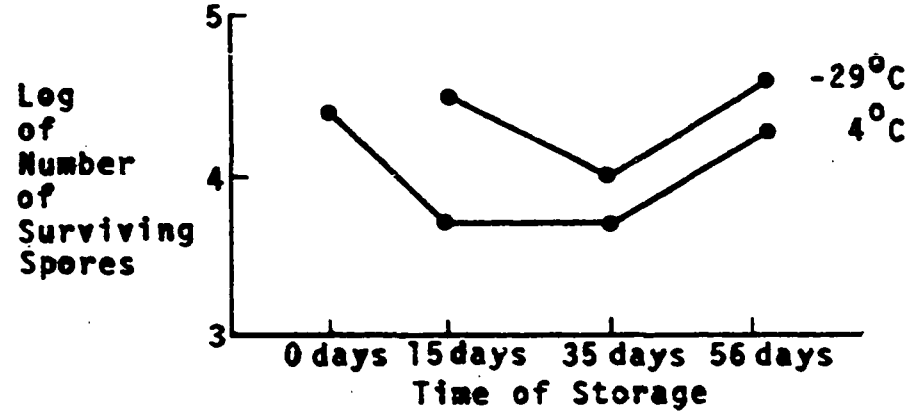
A. Storage Temperature by Storage Solution Interaction



B. Storage Time by Storage Solution Interaction



C. Storage Time by Storage Temperature Interaction



percent sodium chloride was less for spores stored at 4°C than for spores stored at -29°C. Thus in subsequent studies spores were stored in 0.85 percent sodium chloride at 4°C.

Factors Affecting Recovery of Spores Exposed to Hydrogen Peroxide

Upon the development of a satisfactory procedure for storage of spore preparations, factors affecting spore recovery were examined. Beef liver catalase was used to remove hydrogen peroxide from treated spores before plating. The effects that catalase concentration, temperature of the catalase solution, solution used for the suspension of the catalase solution, and time of catalase contact had on recovery in each of 5 media were examined. Medium W was confirmed as the best recovery medium. For this medium, increasing the catalase concentration from 10 mg/9 ml to 20 mg/9 ml, lowering the temperature of the catalase solution from 22°C to 1°C, and increasing the time of spore contact with the catalase from 1 hr to 5 hr all had positive effects on recovery. A detailed scrutiny of the above work follows.

Media

The principal source of variation in recovery of spores exposed to hydrogen peroxide was media (Table 45, Appendix). Duncan's multiple range test (Steel and Torrie, 1960) was used to test for differences between media (Table 5). Medium W was the best recovery medium. Medium H and PCA were almost as good as medium W. Recovery in medium W was 100-fold greater than recovery in TSA and 300-fold greater than recovery in NA.

Table 5. Effect of media on the time required to attain maximum recovery and on the number of spores recovered after an 8 min exposure to hydrogen peroxide

Media	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)	Time needed to attain maximum recovery
Medium W (Wang et al., 1964)	7.171	+	3.5 days
Plate count agar	6.954	┬	5.0 days
Medium H (Halvorson, 1957)	6.854	┴	5.0 days
Trypticase soy agar	5.176	+	10.2 days
Nutrient agar	4.646	+	14.2 days

[a]See Table 45 of the Appendix for error degrees of freedom and error mean square.

Examination of the formulae of the media shows that the 3 media (medium W, PCA, and Medium H) supporting greatest recovery of spores contained yeast extract; whereas, the 2 media (TSA and NA) supporting least recovery contained no yeast extract. Morrison and Rettger (1930) found that yeast extract broth stimulated the recovery of heat-damaged spores, and that, in nutrient broth, initiation of growth of heat-injured spores was much delayed. The similarity that the effect yeast extract has on outgrowth of both heat-damaged and hydrogen peroxide-damaged spores suggests a common site of injury. Also the two best media, medium W and PCA, contained added glucose, 2.5 g/l and 1 g/l respectively; the other media contained no added glucose. This observation suggests that glucose also is important in the recovery of spores damaged by hydrogen

peroxide. Other ingredients unique to medium W such as vitamin-free casamino acids (VFCA), manganous sulfate and ferrous sulfate may also account for the superior recovery observed in this medium.

Temperature of catalase solution

Table 6 shows the mean levels of spore recovery at each catalase temperature for each medium as well as the overall averages. Probabilities of the mean levels of recovery being equal also are given.

Table 6. Effect of temperature of catalase solution on the log of the number of spores recovered in 5 bacteriological media after an 8 min exposure to hydrogen peroxide

Media	Catalase solution temperature 1°C	22°C	Probability of equal means[a]
Medium W (Wang et al., 1964)	7.238	7.104	<.01
Plate count agar	6.961	6.947	.59
Medium H (Halvorson, 1957)	6.716	6.994	.03
Trypticase soy agar	5.357	4.994	<.01
Nutrient agar	5.011	4.281	<.01
Overall average	6.257	6.064	<.01

[a]Tables 45, 46, 47, 48, 49, and 50 of the Appendix.

The overall effect that lowering the temperature of the catalase solution had was positive. Analyses of variance for each medium showed that at the low temperature significantly more spores were recovered with each medium except for PCA and medium H. Significantly more spores were recovered at the high temperature with medium H. Temperature of the catalase solution had no significant effect on the number of spores recovered in PCA.

The sporicidal action of hydrogen peroxide decreases as temperature decreases (Swartling and Lindgren, 1968). Therefore when the catalase solution is held at 1°C, the sporicidal activity that occurs after addition of the sample to the catalase solution but before all of the hydrogen peroxide is removed should be somewhat less than what it would be if the temperature of the catalase solution were 22°C. Also, the breakdown of hydrogen peroxide by catalase is exothermic, therefore maintenance of the catalase solution as a low temperature may be of added importance. Although catalase activity at 1°C is approximately equal to catalase activity at 22°C (Beers and Sizer, 1952), activity at 22°C may be lower due to an increase in temperature caused by the exothermic reaction. For this reason, better recovery is attained by holding the catalase solution at 1°C because hydrogen peroxide is less lethal at this temperature and because the hydrogen peroxide may actually decompose faster at 1°C than at 22°C.

Catalase concentration

The effect that 2 different concentrations of catalase had on recovery of spores in each of 5 media was examined. A concentration of

10 mg/9 ml of Butterfield's buffer was selected because it removed the hydrogen peroxide from .1 ml of 5 percent hydrogen peroxide in 1 min; Toledo et al. (1973) successfully recovered hydrogen peroxide-treated spores using this criterion (1 min for removal of hydrogen peroxide). Recovery with a higher concentration of catalase (20 mg/9 ml) was selected to see whether or not this criterion was adequate.

The overall effect of increasing the catalase concentration was positive. Increasing the catalase concentration had a positive effect for each medium except nutrient agar where no significant effect was observed (Table 7). The high concentration of catalase probably removes hydrogen peroxide quicker and therefore stops the lethal action of hydrogen peroxide more quickly than does the low concentration of catalase.

Table 7. Effect of catalase concentration on the log of the number of spores recovered in 5 media after an 8 min exposure to hydrogen peroxide

Media	Catalase concentration 10 mg	20 mg	Probability of equal means[a]
Medium W (Wang et al., 1964)	7.123	7.218	.03
Plate count agar	6.916	6.993	<.01
Medium H (Halvorson, 1957)	6.785	6.924	.25
Trypticase soy agar	5.060	5.291	.03
Nutrient agar	4.647	4.646	.99
Overall average	6.106	6.214	.05

[a]See Tables 45, 46, 47, 48, 49, and 50 of the Appendix.

In a subsequent experiment, the numbers of spores recovered using 0.1, 1.0, and 10.0 mg of catalase/9 ml of solution were compared. After a 12 min exposure no spores were recovered using 0.1 mg catalase/9 ml of solution. Student's t-test (Snedecor and Cochran, 1967) indicated that there was no significant difference between the levels of recovery attained with 1.0 mg and 10 mg catalase/9 ml of solution.

Just why increasing the catalase concentration from 10 mg/9 ml to 20 mg/9 ml had a positive effect on recovery when increasing the catalase concentration from 1 mg/9 ml to 10 mg/9 ml had zero effect on recovery is not clear. Because the time required to filter sterilized solutions of catalase increases drastically as the concentration of catalase increases from 1 mg/9 ml to 20 mg/9 ml, use of a low concentration of catalase (1 mg/9 ml) appears to be a more efficient and workable method. Thus in subsequent experiments the concentration of catalase used was 1 mg/9 ml.

Contact time for catalase

Since it was known that injured spores required more time to give rise to colonies than uninjured spores (Figure 1), it was hypothesized that spore repair occurred during this delay in colony formation and that delays in colony formation had a negative effect on recovery. To test this hypothesis the number of spores recovered immediately after removal of hydrogen peroxide (1 hr maximum contact time for catalase) was compared with the number of spores recovered after 5 hrs of contact time for catalase before plating in each of 5 media (Table 8).

The overall effect of contact time for catalase on recovery was not

Table 8. Effect of contact time for catalase on the log of the numbers of spores recovered in 5 media after an 8 min exposure to hydrogen peroxide

Media	Contact time for catalase 1 hr	5 hr	Probability[a] of equal means
Medium W (Wang et al., 1964)	7.125	7.216	.04
Plate count agar	6.969	6.939	.24
Medium H (Halvorson, 1957)	6.981	6.728	.04
Trypticase soy agar	5.081	5.270	.08
Nutrient agar	4.681	4.611	.54
Overall average	6.168	6.153	.79

[a]See Tables 45, 46, 47, 48, 49, and 50 of the Appendix.

significant. However a significant effect was noted for two media; medium H and medium W. Increasing the contact time for catalase had a negative effect on recovery with medium H and a positive effect on recovery with medium W.

It could be argued that the positive effect noted for medium W was due to spore outgrowth and multiplication. However, this did not occur; at 22°C, a temperature at which multiplication might be expected, the number of spores recovered decreased after 5 hrs whereas at 1°C, a temperature at which multiplication does not occur, there was an increase in the number of spores recovered.

Because increasing the contact time for catalase from 1 hr to 5 hr had a positive effect on recovery in medium W, the effect of increasing the contact time for catalase further (1 week) was examined. Also the

effect of the addition of nutrients was examined. Nutrients such as yeast extract and glucose were added because they seem to be important for the recovery of hydrogen peroxide-treated spores. Methionine is very susceptible to hydrogen peroxide (Koshland et al., 1962) therefore DL-methionine was added to replenish spore methionine oxidized by hydrogen-peroxide.

One week of storage at 4°C had a negative effect on recovery (Table 51, Appendix). No significant differences between levels of recovery were observed when the solutions used for storage were compared (Table 9).

Table 9. The effect of each of 7 solutions containing 1 mg of catalase/9 ml on the number of spores recovered after a 12 min exposure to hydrogen peroxide

Solution used for catalase suspension	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
Butterfield's buffer containing 0.1 percent DL-methionine	4.640	+
Butterfield's buffer containing 0.125 percent glucose and 0.25 percent yeast extract	4.563	|
Butterfield's buffer containing 0.5 percent yeast extract	4.540	|
0.05 M phosphate buffered at pH 7	4.512	|
Butterfield's buffer containing 0.05 percent methionine and 0.125 percent glucose	4.462	|
Distilled water	4.438	|
Butterfield's buffer	4.372	+

[a]See Table 51 of the Appendix for error degrees of freedom and error mean square.

Apparently storage of spores before plating in solutions containing nutrients does not improve recovery and is not recommended.

Although increasing the contact time for catalase from 1 hr to 5 hrs had a positive effect on the recovery of spores exposed to hydrogen peroxide for 8 min, extending the contact time for catalase to 1 week had a negative effect on the recovery of spores exposed to hydrogen peroxide for 12 min. This may indicate that the ability to repair damage caused by hydrogen peroxide is retained after 8 min of exposure but that it is lost after 12 min of exposure. It is also possible that for spores exposed 12 min, repair occurred soon after hydrogen peroxide removal but that viability was lost during the week of storage.

Glucose concentration in the catalase solution

Glucose was implicated as a nutrient important for spore recovery because recovery was better in media that contained glucose. Thus loading the spore with glucose before plating via addition of glucose to the catalase solution seemed like a reasonable way to improve recovery by allowing for repair to occur before plating.

The overall effect of adding glucose to the catalase solutions was positive. Mean levels of spore recovery attained in each of the 5 media with and without glucose added to the catalase solution are given in Table 10. For media which already contained glucose, such as medium W (2.5 g/l) and PCA (1 g/l), the effects of adding glucose to the catalase solutions were respectively zero and positive. In the other media which contained no added glucose, addition of glucose to the catalase solution had a positive effect on recovery. Recalling that after a 1 min

Table 10. Effect of the addition of 1 percent glucose to the catalase solution on the log of the number of spores recovered in each of 5 media after an 8 min exposure to hydrogen peroxide

Media	No added glucose	1 percent added glucose	Probability[a] of equal means
Medium W (Wang et al., 1964)	7.176	7.166	.79
Plate count agar	6.928	6.981	.05
Medium H (Halvorson, 1957)	6.762	6.947	.13
Trypticase soy agar	5.059	5.292	.03
Nutrient agar	4.487	4.806	.01
Overall average	6.082	6.238	.01

[a]See Tables 45, 46, 47, 48, 49, and 50 of the Appendix.

exposure to hydrogen-peroxide there was very little difference between the mean levels of recovery for each of the media examined (Table 4) suggests that the energy requirements of injured spores are much greater than those of uninjured spores. In a subsequent section the effect of glucose in medium W is shown to be very important.

Effect of Interactions on Recovery

Catalase concentration by time of catalase contact

In the overall analysis of variance there was not a significant catalase concentration by catalase contact time interaction (Table 45, Appendix). Analyses of variance of each medium indicated a significant interaction for only one medium; medium W which was the best recovery

medium (Table 46, Appendix). With a low concentration of catalase, increasing the time of catalase contact had a negative effect on recovery; whereas, with a high concentration of catalase increasing the time of catalase contact had positive effect on recovery (Table 11). The overall averages indicated the same trends. This evidence suggests that extending the time of catalase contact with catalse concentrations of 20 mg/9 ml or higher may further increase the number of spores recovered. With the high concentration of catalase the hydrogen peroxide concentration may be reduced to a level low enough to allow spore repair to occur whereas with the low concentration of catalase the hydrogen peroxide concentration may not be lowered to a level which permits repair to occur.

Table 11. Effect of the catalase concentration by catalase contact time interaction on the log of the number of spores recovered in medium W and the overall effect of the interaction after an 8 min exposure to hydrogen peroxide

	Catalase concentration/catalase contact time			Significant interaction[a] (5 percent level)
		1 hr	5 hrs	
Medium W	10 mg/9 ml	7.128	7.122	Yes
	20 mg/9 ml	7.118	7.314	
Overall	10 mg/9 ml	6.137	6.076	No
	20 mg/9 ml	6.198	6.230	

[a]See Tables 45 and 46 of the Appendix for analyses of variance.

Catalase concentration by glucose concentration

A significant overall catalase concentration by glucose concentration interaction was observed (Table 45, Appendix). Without glucose added to the catalase solution the effect of increasing the catalase concentration was negative but after adding 1 percent glucose to the catalase solution, increasing the catalase concentration had a positive effect (Table 12). Examination of the interaction for each medium indicated that with added glucose the effect of increasing the catalase concentration was positive for all media except nutrient agar. For the best recovery medium, medium W, increasing the catalase concentration had a more positive effect on recovery with glucose added to the catalase solution than when it was not added, however the interaction was not significant.

Table 12. Effect of the catalase concentration by glucose concentration interaction in medium W and the overall effect of the interaction on the log of the number of spores after 8 min exposure to hydrogen peroxide

	Glucose concentration	Catalase concentration		Significant interaction[a] (5 percent level)
		10 mg/9 ml	20 mg/9 ml	
Medium W	0 g/l	7.165	7.187	Yes
	1 g/l	7.082	7.249	
Overall	0 g/l	6.083	6.082	No
	1 g/l	6.129	6.347	

[a]See Tables 45 and 46 of the Appendix for analyses of variance.

The effect that each preplating treatment had on recovery of spores in medium W after exposure to hydrogen peroxide for 8 min is presented in Table 13. The best recovery was attained when the temperature of the catalase solution was 1°C, the concentration of the catalase solution was 20 mg/9 ml, the time of contact for catalase was 5 hr, and the concentration of glucose in the catalase solution was 1 percent.

To facilitate sterilization of the catalase solutions, a low concentration of catalase (1 mg/9 ml) is recommended. Addition of glucose to the catalase solution is not recommended because it did not significantly improve recovery in medium W. Increasing the time of catalase contact from 1 hr to 5 hr had a positive effect on recovery when the catalase concentration was 20 mg/9 ml and a negative effect on recovery when the catalase concentration was 10 mg/9 ml, indicating that with an even lower concentration of catalase an increase in the time of contact for catalase would have a negative effect on recovery. Thus a minimal contact time for catalase is recommended. Holding the catalase solution at 1°C is recommended not only because it had a positive effect on recovery but also because control of temperature is easier at 1°C than at room temperature. With high concentrations of hydrogen peroxide temperature control is particularly important because the breakdown of the hydrogen peroxide is exothermic.

Calcium dipicolinate

Because dipicolinic acid is leached from spores both by heat treatment and by hydrogen peroxide-treatment (Kawasaki et al., 1970) and because recovery of heat treated spores is increased by addition of calcium

Table 13. Effect of preplating treatment on the recovery of spores in medium W after exposure to hydrogen peroxide for 8 min

Temperature of catalase solution	Concentration of catalase (mg/9 ml)	Percent concentration of glucose	Time of contact for catalase (hr)	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
1°C	20	1	5	7.396	
1°C	20	0	5	7.318	
22°C	20	1	5	7.276	
1°C	20	1	1	7.270	
22°C	20	0	5	7.268	
1°C	10	0	1	7.239	
1°C	10	1	5	7.228	
1°C	20	0	1	7.211	
22°C	10	0	1	7.196	
1°C	10	1	1	7.157	
22°C	10	0	5	7.139	
1°C	10	0	5	7.084	
22°C	20	1	1	7.055	
22°C	10	1	5	7.023	
22°C	20	0	1	6.952	
22°C	10	1	1	6.918	

[a]See Table 45 of the Appendix for error degrees of freedom and error mean square.

dipicolinate (CaDPA) (Edwards et al., 1965a), the effect that addition of CaDPA to 5 different media had on recovery of hydrogen peroxide-treated spores was studied. The concentration of CaDPA used in the media was 20 mM; a concentration at which maximum germination of Bacillus megaterium is known to occur (Alderton et al., 1964).

Addition of the CaDPA caused crystal formation in each of the media and thereby made it impossible to accurately count plates. There appeared to be less growth in media that contained CaDPA than in the plain media.

Effect of Ingredients of Medium W (Wang et al., 1964) on Spore Recovery

The effect that each constituent of medium W has on spore recovery was examined as described in Tables 2 and 3. The pH of the media used in these studies was adjusted to 6.8 after autoclaving unless stated otherwise. From these studies the main effects of each medium constituent were learned. In the following sections the results of these experiments are discussed.

Yeast extract

Yest extract is a very important constituent of the medium for recovery of spores. As the concentration of yeast extract in the medium was increased from 0 g/l to 10 g/l the number of spores recovered increased exponentially. Spore recovery fit an exponential pattern because the data fit a fourth degree polynominal in which all terms were significant, and because the linear and cubic effects were positive and the quadratic and quartic effects were negative (Table 52, Appendix).

Recovery without yeast extract was very poor; maximum spore recovery was attained at a concentration of 7.5 g/l. Significantly more spores were recovered with a concentration of 7.5 g/l than with a concentration of 5 g/l (Table 14); this effect was later confirmed in other experiments. (See Tables 66 and 67 in the Appendix.)

Table 14. Effect of varying the concentrations of yeast extract in medium W on the recovery of spores exposed to hydrogen peroxide for 8 min

Concentration of yeast extract (g/l)	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
7.5	7.550	†
10.0	7.474	† †
5.0	7.398	†
2.5	6.810	†
0.0	4.065	†

[a]See Table 52 of the Appendix for error degrees of freedom and error mean square.

Media not containing yeast extract yielded plate counts of uninjured spores as high or higher than media which contain yeast extract; however, for recovery of spores injured by hydrogen peroxide, media which contain yeast extract are superior to media void of yeast extract (Tables 1 and 4). Damage of spores caused by hydrogen peroxide may increase the demand for one or several of the nutrients supplied by yeast extract. Yeast extract contains a variety of compounds (Baltimore Biological

Laboratory, Inc., 1968) essential for cell growth and perhaps most importantly, compounds necessary for repair.

Glucose

Glucose like yeast extract was an important constituent of the medium for assuming maximum spore recovery. Without glucose in the medium numbers of spores recovered were low; addition of 2.5 g of glucose/l increased the level of recovery 1000-fold for spores exposed 8 min (Table 15). As was noted earlier the requirements for an energy source by hydrogen peroxide-injured spores are considerably greater than for uninjured spores; addition of glucose to medium W greatly improves recovery. Russell and Loosemore (1964) observed higher counts for B. subtilis spores exposed to heat and phenol in nutrient agar containing glucose than in plain nutrient agar.

Table 15. Effect of varying the concentration of glucose in medium W on the recovery of spores exposed to hydrogen peroxide for 8 min

Concentration of glucose	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
5.0	7.434	+
7.5	7.426	+
2.5	7.398	+
0.0	4.479	+

[a]See Table 53 of the Appendix for error degrees of freedom and error mean square.

There was not a significant increase in recovery when the concentration of glucose was increased to 5 g/l but the number of spores recovered after an 8 min exposure was usually somewhat higher at the 5 g/l level than at the 2.5 g/l level. Increasing the glucose concentration from 2.5 g/l to 5 g/l significantly decreased the number of spores recovered after a 10 min exposure (Table 66, Appendix) but after a 12 min exposure the effect was not significant (Table 67, Appendix). Glucose requirements of spores changed as the time of exposure increased; apparently nutrient requirements of the spores changed as the degree of spore injury increased.

Vitamin-free casamino acids

The effect of varying the concentration of vitamin-free casamino acids (VFCA) from 0 g/l to 9 g/l in medium W had a positive and significant linear effect on the number of spores recovered (Tables 54 and 55, Appendix). Maximum recovery was attained with the addition of 6.0 g of VFCA/l (Table 16). The positive effect that increasing the VFCA concentration from 0 g/l to 6.0 g/l had was confirmed later in other experiments (Tables 65, 66, and 67, Appendix).

VFCA provides amino acids not found in yeast extract, namely, aspartic acid, glycine, glutamic acid, and proline (Baltimore Biological Laboratory, Inc., 1968). The balance of amino acids attained by adding both VFCA and yeast extract may benefit recovery of spores exposed to hydrogen peroxide. Also 6.0 g of VFCA/l supplies 2.2 g of sodium chloride/l which may help establish an osmotic pressure in the medium that is optimal for spore recovery.

Table 16. Effect of varying the concentration of vitamin-free casamino acids in medium W on recovery of spores exposed to hydrogen peroxide for 10 min

Experiment[a]	Concentration of vitamin-free casamino acids (g/l)	Log of number of spores recovered	Duncan's multiple range test[b] (5 percent level)
B2	6	7.334	†
	9	7.326	|
	3	7.290	† †
	0	7.235	†
C1	6	7.313	†
	4	7.283	† †
	7	7.245	† †
	5	7.240	†

[a]See Table 2 of the Materials and Methods.

[b]See Tables 55 and 56 of the Appendix for error degrees of freedom and error mean squares.

Agar

Within the concentration range examined (15 g/l to 35 g/l), agar did not have much of an effect on recovery. Increasing the concentration from 15 g/l to 30 g/l had a positive and significant linear effect on recovery of spores exposed for 8 min (Table 57, Appendix). But for spores exposed 10 min increasing the concentration from 25 g/l to 35 g/l had a negative and significant linear effect on recovery (Table 58, Appendix). The medium containing 35 g of agar/l was so viscous that good mixing of the spore samples and media was prevented. This is probably the reason lower counts were attained with this concentration of agar. In another experiment the negative effect that increasing the

the agar concentration had on the recovery of spores exposed 10 min was confirmed (Table 66, Appendix); increasing the agar concentration also had a negative effect on the recovery of spores exposed for 12 min (Table 67, Appendix).

In experiments A and B1 (Tables 2 and 3) when the concentration of agar was varied, the concentration of ferrous sulfate was held constant at 0.1 g/l. Under these conditions, the influence of increasing the agar concentration was negative; however, when the concentration of iron was reduced to 0.001 g/l (Table 2, Experiment A1), increasing the agar concentration enhanced recovery.

Spores injured by hydrogen peroxide have heightened requirements for glucose and yeast extract suggesting that injured spores require more energy and nutrients for outgrowth than do uninjured spores. An increase in glucose demand would require an increase in the oxygen demand. Under these circumstances a high rate of oxygen diffusion into the medium and maintenance of a high oxidation-reduction potential within the medium would be important for the recovery of spores of an aerobe such as B. subtilis var. niger. Increasing the agar concentration within the medium and thereby decreasing the rate of oxygen diffusion into the medium could have a negative effect on spore recovery particularly if the oxidation-reduction potential were already poised at a relatively low level due to a high concentration of ferrous sulfate (Hewitt, 1950). The contrasting effects that increasing the agar concentration had when the concentration of ferrous sulfate was increased from 0.001 g/l to 0.1 g/l may have been due to a lowering of the oxidation-reduction

potential by the ferrous sulfate.

Manganous sulfate

The effect that manganous sulfate had on spore recovery in medium W was examined. Addition of 0.1 g of manganous sulfate/l of medium resulted in the recovery of significantly more spores than when it was left out of the medium (Tables 17 and 18). The number of spores recovered when the manganous sulfate concentration was 1 g/l was not significantly different from the number of spores recovered when the manganous sulfate concentration was 0.1 g/l (Table 18). However, increasing the concentration of manganous sulfate above 0.1 g/l significantly reduced the number of spores recovered when the pH of the medium was adjusted to 6.8 before autoclaving (Table 19); before adjustment, pH of the medium was 6.2. The negative effect that increasing the manganous sulfate concentration above 0.1 g/l had when pH adjustment was done before autoclaving may be due to the formation of toxic compounds, or to the destruction of essential nutrients or both.

Table 17. Analysis of variance of the effect manganous sulfate (0 g/l vs 0.1 g/l) in medium W had on the recovery of spores exposed to hydrogen peroxide for 8 min

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.198	0.066	.01
Manganous sulfate[a]	1	0.028	0.028	.02
Error	3	0.004	0.001	
Corrected total	7	0.230		

[a]Log number of spores recovered without manganous sulfate = 7.279; with = 7.398.

Table 18. Effect of varying the concentration of manganous sulfate in medium W on the recovery of spores exposed to hydrogen peroxide for 10 min

Concentration of manganous sulfate (g/l)	Log of the number of spores recovered	Duncan's multiple range test[a] (5 percent level)
0.1	7.328	+
1.0	7.279	+
0.0	6.927	+

[a]See Table 59 of the Appendix for error degrees of freedom and mean square.

Table 19. Effect of varying the concentrations of manganous sulfate in medium W on the recovery of spores exposed to hydrogen peroxide for 10 min. (pH adjusted to 6.8 before autoclaving)

Concentration of manganous sulfate (g/l)	Log of the number of spores recovered	Duncan's multiple range test[a] (5 percent level)
0.1	7.314	+
0.3	7.190	+
0.5	6.523	+
0.7	5.501	+

[a]See Table 60 of the Appendix for error degrees of freedom and error mean square.

The results indicate that addition of 0.1 g of manganous sulfate/l of medium W benefits the recovery of spores exposed to hydrogen peroxide. This beneficial effect may be due to replenishment of manganous ions lost during hydrogen peroxide treatment; manganous ions are required by certain enzymes (Davis et al., 1973). Concentrations of manganous sulfate greater than 0.1 g/l did not improve recovery and actually had a

negative effect on recovery if the pH of the medium was adjusted before autoclaving. Autoclaving of the manganous sulfate apart from the other ingredients of medium W is recommended; adjustment of pH could then be made before autoclaving which would eliminate the problem of adjusting pH aseptically after autoclaving.

Ferrous sulfate

Increasing the concentration of ferrous sulfate from 0 g/l to 0.1 g/l had a positive and significant linear effect on recovery (Table 61, Appendix). The positive effect that ferrous sulfate has in this concentration range is probably due to replenishment of iron lost during hydrogen peroxide treatment. An aerobe such as B. subtilis var. niger requires both ferrous and ferric ions for electron transport (Davis et al., 1973).

Recovery attained with a ferrous sulfate concentration of 1 g/l was not significantly different from the recovery attained with a concentration of 0.1 g/l (Table 20). However, as was noted with manganous sulfate, increasing the ferrous sulfate concentration above 0.1 g/l had a negative effect when the pH of the media was adjusted before autoclaving (Table 21). Because increasing the ferrous sulfate concentration above 0.1 g/l did not have a negative effect if the pH was adjusted after autoclaving, but did have a negative effect if the pH was adjusted before autoclaving, formation of toxic products or destruction of nutrients essential for recovery or both due to chemical reaction(s) with iron during autoclaving of the media is implied.

Addition of ferrous sulfate to the media after autoclaving would

Table 20. Effect of varying the concentration of ferrous sulfate in medium W on the recovery of spores exposed to hydrogen peroxide for 10 min

Concentration of ferrous sulfate (g/l)	Log of the number of spores recovered	Duncan's multiple range test[a] (5 percent level)
0.1	7.282	†
1.0	7.244	†
0.0	7.112	+

[a]See Table 62 of the Appendix for error degrees of freedom and error mean square.

Table 21. Effect of varying the concentration of ferrous sulfate in medium W on the recovery of spores exposed to hydrogen peroxide for 10 min. (pH adjusted to 6.8 before autoclaving)

Concentration of ferrous sulfate (g/l)	Log of the number of spores recovered	Duncan's multiple range test[a] (5 percent level)
0.1	7.231	†
0.3	7.213	†
0.7	6.558	+

[a]See Table 63 of the Appendix for error degrees of freedom and error mean square

forestall any deleterious reactions between ferrous sulfate and other ingredients of the medium. It would also allow for adjustment of the pH of the medium before autoclaving. Thus addition of ferrous sulfate after autoclaving is recommended.

Interactions

Ferrous sulfate by manganous sulfate

No significant ferrous sulfate by manganous sulfate interaction on spore recovery in medium W was observed (Table 74, Appendix). However, more replications of the experiment may have revealed an interaction because without ferrous sulfate in the medium addition of 0.1 g of manganous sulfate/l had a negative effect on recovery but after addition of 0.1 g of ferrous sulfate/l, addition of 0.1 g manganous sulfate/l had a positive effect on recovery (Table 22). Such an interaction is in want of a biological explanation.

Table 22. Effect of the ferrous sulfate by manganous sulfate interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 12 min

		Concentration of ferrous sulfate (g/l)	
		0.0	0.1
Concentration of manganous sulfate (g/l)	0.0	5.600	5.262
	0.1	4.629	5.970

Ferrous sulfate by vitamin free casamino acids

The effect of the ferrous sulfate by VFCA interaction on spore recovery in medium W was nearly significant ($p = .07$; Table 65, Appendix), thus, mean levels of recovery may be of interest. Adding ferrous sulfate in the absence of VFCA had a negative effect on recovery;

whereas, adding ferrous sulfate when VFCA were present had a positive effect on recovery (Table 23). Ferrous sulfate may be required for the maximum utilization of nutrients found in VFCA.

Table 23. Effect of a ferrous sulfate by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 12 min

		Concentration of ferrous sulfate (g/l)	
		0.0	0.1
Concentration of vitamin-free casamino acids (g/l)	0.0	6.238	6.170
	6.0	6.617	6.754

Glucose by vitamin-free casamino acids

No significant glucose by VFCA interaction was noted for recovery of spores after a 10 min exposure to hydrogen peroxide; increasing the glucose concentration from 2.5 g/l to 5.0 g/l had a negative effect on recovery with or without VFCA in the medium (Table 24). But after a 12 min exposure a significant interaction was observed; increasing the glucose concentration in media not containing VFCA had a positive effect on recovery; whereas, in media containing VFCA the effect was negative (Table 24).

An increase in energy demand in the form of glucose as the degree of spore injury increases could explain the contrasting effects of this interaction noted between 10 min and 12 min exposures. Another explanation is that the initial spore population was not homogeneous. If

this were true, nutrient requirements of the spores surviving hydrogen peroxide treatment could change as the number of survivors decreased.

Table 24. Effect of the glucose by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide 10 min and 12 min

Length of time of exposure to hydrogen peroxide	Concentration of vitamin-free casamino acids (g/l)	Concentration of glucose (g/l)		Significant[a] interaction (5 percent level)
		2.5	5.0	
10 min	0.0	7.140	7.050	No
	6.0	7.280	7.194	
12 min	0.0	5.932	6.005	Yes
	6.0	6.358	6.327	

[a]See Tables 66 and 67 of the Appendix for analyses of variance.

Yeast extract by vitamin-free casamino acids

A significant yeast extract by VFCA interaction was noted for spores treated both 10 min and 12 min (Table 25). Regardless of the length of time of exposure, the effect of increasing the yeast extract concentration in the absence of VFCA was negative and the effect of increasing the yeast extract concentration when VFCA was in the medium was positive (Table 25).

The cause of the negative effect that increasing the concentration of yeast extract had in the absence of VFCA may be due to an ingredient or combination of ingredients reaching an inhibitory concentration.

Table 25. Effect of yeast extract by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 10 min and 12 min

Length of time of exposure to hydrogen peroxide	Concentration of vitamin-free casamino acids (g/l)	Concentration of yeast extract		Significant interaction[a] (5 percent level)
		5.0	7.5	
10 min	0.0	7.104	7.086	Yes
	6.0	7.204	7.271	
12 min	0.0	5.974	5.963	Yes
	6.0	6.301	6.384	

[a]See Tables 66 and 67 of the Appendix for analyses of variance.

The positive effect that increasing the concentration of yeast extract had when VFCA was added may be due to an improvement in the balance of amino acids that was noted earlier. The positive effect of this balance may then overcome the negative effect that increasing the concentration of yeast extract had in the absence of VFCA.

Agar by vitamin-free casamino acids

No significant agar by VFCA interaction was noted for spores exposed 10 min; increasing the concentration of agar had a negative effect both with and without VFCA. For spores exposed 12 min a significant interaction was detected; the negative effect that increasing the agar concentration had was more pronounced without than with VFCA (Table 26).

In the medium void of VFCA, injured spores may have been more

Table 26. Effect of the agar by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 10 min and 12 min

Length of time of exposure to hydrogen peroxide	Concentration of vitamin-free casamino acids (g/l)	Concentration of agar		Significant[a] interaction (5 percent level)
		15	30	
10 min	0.0	7.133	7.056	No
	6.0	7.267	7.208	
12 min	0.0	6.022	5.915	Yes
	6.0	6.348	6.338	

[a]See Tables 66 and 67 of the Appendix for analyses of variance.

exacting in their oxygen requirements due to a lack of or imbalance of nutrients. Hence, increasing the agar concentration, which would reduce the rate of oxygen diffusion into the medium, would have a more negative effect on recovery in a medium void of VFCA than it would have if VFCA was added to the medium.

Yeast extract by agar by vitamin-free casamino acids

No significant yeast extract by agar by VFCA interaction was noted for spores exposed 10 min; with or without VFCA the effect of increasing the concentration of agar was negative at both the high and low levels of yeast extract. A significant interaction was noted for spores exposed 12 min; without VFCA the effect of increasing the concentration of agar was negative at both the high and low levels of yeast extract; with VFCA the effect of increasing the concentration of agar was negative

at the low level of yeast extract and positive at the high level of yeast extract (Table 27). Increasing the agar concentration generally had a negative effect on recovery; what accounts for the positive effect that increasing the agar concentration has when both the yeast extract and VFCA levels are high is not clear.

Table 27. Effect of the agar by yeast extract by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 10 min and 12 min

Length of time of exposure to hydrogen peroxide	Concentration of vitamin-free casamino acids (g/l)	Concentration of yeast extract	Concentration of agar (g/l)		Significant interaction[a] (5 percent level)
			15	30	
10 min	0.0	5.0	7.129	7.080	No
		7.5	7.138	7.033	
	6.0	5.0	7.248	7.160	
		7.5	7.286	7.256	
12 min	0.0	5.0	6.010	5.938	Yes
		7.5	6.034	5.893	
	6.0	5.0	6.332	6.270	
		7.5	6.363	6.406	

[a]See Tables 66 and 67 of the Appendix for analyses of variance.

Glucose by agar by vitamin-free casamino acids

There was no significant glucose by agar by VFCA interaction for spores exposed to hydrogen peroxide for 10 min. Increasing the glucose concentration had a negative effect on the recovery of spores exposed 10 min with or without VFCA at both the high and low levels of agar. For spores exposed 12 min there was a significant interaction; increasing the concentration of glucose had a positive effect on recovery without VFCA at both agar levels, and with VFCA, increasing the glucose concentration had a positive effect on recovery at the low concentration of agar but a negative effect on recovery at the high concentration of agar (Table 28).

Table 28. Effect of agar by glucose by vitamin-free casamino acids interaction in medium W on the log of the number of spores recovered after exposure to hydrogen peroxide for 10 min and 12 min

Length of time of exposure to hydrogen peroxide	Concentration of vitamin-free casamino acids (g/l)	Concentration of agar (g/l)	Concentration of glucose (g/l)		Significant interaction (5 percent level
			2.5	5.0	
10 min	0.0	15	7.197	7.070	No
		30	7.082	7.030	
	6.0	15	7.296	7.238	
		30	7.265	7.150	
12 min	0.0	15	6.008	6.036	Yes
		30	5.856	5.974	
	6.0	15	6.343	6.353	
		30	6.374	6.302	

[a]See Tables 66 and 67 of the Appendix for analyses of variance.

The positive influence that increasing the glucose concentration had after the 12 min exposure was probably due to the heightened energy demand of injured spores. The negative effect that increasing the glucose concentration had when levels of both VFCA and agar were high may have been due to an increase in the osmotic pressure brought about by combining high concentrations of agar, glucose, and VFCA.

The effect that varying the concentrations of yeast extract, glucose, agar, and VFCA in medium W had on the recovery of spores exposed to hydrogen peroxide is presented in Tables 29 and 30. The recovery medium that gave the highest level of recovery after 10 min and 12 min of exposure contained 2.5 g of glucose/1, 7.5 g of yeast extract/1, 30 g of agar/1, and 6.0 g of VFCA/1 as well as 0.1 g of ferrous sulfate/1, and 0.1 g of manganous sulfate/1. The composition of the original medium was 2.5 g of glucose/1, 5.0 g of yeast extract/1, 20 g of agar/1, 1.0 g of VFCA/1, 0.001 g of ferrous sulfate/1 and 0.1 g of manganous sulfate/1. Increasing the concentrations of yeast extract (from 5.0 g/1 to 7.5 g/1), VFCA (from 1.0 g/1 to 6.0 g/1), ferrous sulfate (from 0.001 g/1 to 0.1 g/1) is recommended. Increasing the concentration of agar did not significantly increase recovery and therefore is not recommended.

pH of the medium

Recovery of injured spores in media adjusted to different pH levels varied widely. Significantly more spores were recovered at pH levels of 6.8 and 7.0 than at lower or higher pH levels (Table 31). Furthermore only 2 to 3 days were required for maximum recovery at pH levels

Table 29. Effect of varying the concentration of yeast extract, glucose, agar, and vitamin-free casamino acids in medium W on the recovery of spores exposed to hydrogen peroxide for 10 min

Glucose level (g/l)	Yeast extract level (g/l)	Agar level (g/l)	Vitamin-free casamino acids level (g/l)	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
2.5	7.5	30	6	7.320	
2.5	7.5	15	6	7.316	
2.5	5.0	15	6	7.274	
5.0	7.5	15	6	7.256	
2.5	7.5	15	0	7.247	
5.0	5.0	15	6	7.220	
2.5	5.0	30	6	7.210	
5.0	7.5	30	6	7.192	
2.5	5.0	15	0	7.147	
5.0	5.0	15	0	7.110	
5.0	5.0	30	6	7.109	
2.5	5.0	30	0	7.095	
2.5	7.5	30	0	7.070	
5.0	5.0	30	0	7.064	
5.0	7.5	15	0	7.029	
5.0	7.5	30	0	6.996	

[a]See Table 66 of the Appendix for error degrees of freedom and error mean square.

Table 30. Effect varying the concentration of yeast extract, glucose, agar, and vitamin-free casamino acids in medium W on the recovery of spores exposed to hydrogen peroxide for 12 min

Glucose level (g/l)	Yeast extract level (g/l)	Agar level (g/l)	Vitamin-free casamino acids level (g/l)	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
2.5	7.5	30	6	6.458	+
5.0	7.5	15	6	6.382	+ +
5.0	7.5	30	6	6.352	+
2.5	7.5	15	6	6.343	
2.5	5.0	15	6	6.342	
5.0	5.0	15	6	6.322	
2.5	7.5	30	6	6.288	+
5.0	5.0	30	6	6.250	+
5.0	7.5	15	0	6.050	+
5.0	5.0	15	0	6.021	+
2.5	7.5	15	0	6.016	
5.0	5.0	30	0	6.012	
2.5	5.0	15	0	5.998	+
5.0	7.5	30	0	5.936	+ +
2.5	5.0	30	0	5.864	
2.5	7.5	30	0	5.850	+

[a]See Table 67 for error degrees of freedom and error mean square.

Table 31. Effect of varying the pH level in medium W on recovery of spores exposed to hydrogen peroxide for 10 min

pH level	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
6.8	7.229	+
7.0	7.198	+
7.3	7.042	+
6.4	7.022	+
7.6	6.589	+
7.9	6.267	+

[a]See Table 64 of the Appendix for error degrees of freedom and error mean square.

of 6.8 and 7.0 whereas at other pH levels 3 to 7 days were required before maximum recovery was attained. Adjustment of the pH to 7.0 is recommended.

Others have noticed that recovery of injured spores is better at pH levels approaching neutrality than at other pH levels. Yokoya and York (1965) observed better recovery of heat-treated spores of B. coagulans at pH 6.7 than at pH 5.0. Cook and Brown (1965a) attained maximum recovery of uninjured B. stearothermophilus spores at pH 5.9 but after severe heat treatment recovery was greatest at pH 7.0 to 7.3.

Influence of Additives in Recovery Medium W

The influence of several additives in recovery medium W was examined. The effect that addition of 2.5 g of sodium chloride/1 medium W had on recovery was examined for two reasons: one, increasing the concentration of VFCA in the medium improved recovery; VFCA is 37 percent sodium chloride; two, medium H which was the third best recovery medium contained 5 g of sodium chloride/1. L-alanine was added to the medium because Russell and Loosemore (1964) had shown that recovery of spores exposed to heat or phenol is improved by the addition of L-alanine to the medium. A sterile solution of spores previously exposed to hydrogen peroxide (spore exudate) was added because it was thought that replenishment of cell constituents leached from the spores by hydrogen peroxide might improve recovery.

Addition of sodium chloride, L-alanine, or spore exudate had no significant effect on recovery (Table 32). By looking at the effect of a wide range of concentrations of each of the additives instead of a single concentration, a more precise relationship between the additives and recovery may have been ascertained.

The effect that the addition of magnesium sulfate, DL-methionine, starch, or zinc sulfate to medium W had on recovery were also examined. Because methionine is particularly susceptible to hydrogen peroxide (Koshland et al., 1962), DL-methionine was added to the medium to determine whether or not replenishment of spore methionine oxidized by hydrogen perioxide would improve recovery. The effect of starch in the medium on recovery was tried because Olsen and Scott (1946) increased

Table 32. Effect of the addition of L-alinine, spore exudate, or sodium chloride to medium W on recovery of spores exposed to hydrogen peroxide for 12 min

Additive	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
Spore exudate (10 ml/l)	6.522	+
Sodium chloride (2.5 g/l)	6.389	|
L-alanine (0.1 g/l)	6.352	|
Control (no additive)	6.126	+

[a]See Table 68 of the Appendix for error degrees of freedom and error mean square.

the recovery of heated Bacillus spores by adding starch to the recovery medium. Magnesium sulfate and zinc sulfate were added to the medium to replenish any losses brought about by the hydrogen peroxide treatment. These salts are required by DNA polymerases for DNA synthesis (Springgate et al., 1973; Low et al., 1974).

After addition of zinc sulfate to medium W no spores were recovered. Addition of starch, DL-methionine, and magnesium sulfate all significantly reduced the level of recovery attained (Table 33). Other workers have shown that addition of starch to media used for the recovery of heat-treated spores did not improve recovery (Amaha and Ordal, 1957; Briggs, 1966). The negative effect that starch had on recovery in this study may have been due to a lowering of the rate at which oxygen diffuses into the medium. By examining a wider range of concentrations

Table 33. Effect of addition of magnesium sulfate, DL-methionine, and starch to medium W on recovery of spores exposed to hydrogen peroxide for 12 min

Additive	Log of number of spores recovered	Duncan's multiple range test[a] (5 percent level)
Control (no additive)	6.366	+
Magnesium sulfate (0.01 g/l)	5.989	+
DL-methionine (0.1 g/l)	5.878	+
Starch (1 g/l)	5.331	+

[a]See Table 69 of the Appendix for error degrees of freedom and error mean square.

of each of these additives a more accurate relationship between the additives and recovery may have been ascertained.

Effect of Metal Salts on the Sporicidal Activity of Hydrogen Peroxide

Because Dittmar et al. (1930) have shown that ferric and cupric ions enhance the lethal effect of hydrogen peroxide on vegetative cells, the effects that metal salts have on the sporicidal action of hydrogen peroxide were examined. Salts such as ferric citrate, zinc sulfate, and chromium chloride were selected because their effect on the sporicidal action of hydrogen peroxide was unknown. Ferric chloride and cupric sulfate were selected because of their demonstrated ability to enhance the lethal action of hydrogen peroxide against vegetative cells; the ability of ferric chloride and cupric sulfate to enhance the sporicidal effect of hydrogen peroxide had not been studied.

The sporicidal action of a 12 min exposure to hydrogen peroxide was not significantly affected by the addition of ferric citrate, zinc sulfate, or chromium chloride to the hydrogen peroxide sultion (Table 70, Appendix). No spores were recovered after a 12 min exposure to hydrogen peroxide that contained 0.1 mM ferric chloride, or 0.1 mM cupric sulfate, or both (0.05 mM concentration of each), indicating that ferric chloride and cupric sulfate enhanced sporicidal action.

After a 10 min exposure to hydrogen peroxide that contained ferric chloride, cupric sulfate, or both a countable number of spores was recovered. There was not a significant difference among the sporicidal effects due to the addition of these salts (Table 71, Appendix). However the numbers of spores recovered were considerably smaller than the number recovered when only hydrogen peroxide was used or when ferric chloride, cupric sulfate, or both were used in the absence of hydrogen peroxide (Table 34).

Table 34. Effect of a 10 min exposure to solutions of metal salts, 5 percent hydrogen peroxide, and 5 percent hydrogen peroxide plus metal salts held at 50°C on spore survival

	Solution	Log of number of surviving spores
A)	0.05 mM ferric chloride and 0.05 mM cupric sulfate	8.041
B)	0.1 mM cupric sulfate	7.863
C)	0.1 mM ferric chloride	7.857
D)	5 percent hydrogen peroxide	6.820
E)	Solution A in solution D	5.114
F)	Solution B in solution D	4.996
G)	Solution C in solution D	4.756

The enhancement of the sporicidal action of hydrogen peroxide is not due to the independent sporicidal action of the salts because solutions containing only the salts had no sporicidal effect. The hydroxyl radical is implicated as the agent which enhances sporicidal action because metal ions enhance the production of the hydroxyl radical from hydrogen peroxide (Barb et al., 1951) and because this radical is the most potent oxidant known to man (Neta and Dortman, 1968). Others may also implicated the hydroxyl radical as an agent of spore destruction (Powers and Cross, 1970; Powers et al., 1972).

When the concentration of either ferric chloride or cupric sulfate was greater than 0.1 mM the hydrogen peroxide decomposed rapidly and had no sporicidal effect. When the concentration of these salts in the hydrogen peroxide solution was below 0.01 mM enhancement of the sporicidal action of hydrogen peroxide was not observed. Variability in the sporicidal action of hydrogen peroxide may sometimes be due to contaminate ferric, cupric, or other metal ions. Addition of ferric or cupric ions to solutions of hydrogen peroxide may have special applications in sterilization procedures where the temperature or concentration or both of hydrogen peroxide needs to be minimized.

Effect of pH on the Sporicidal Action of Hydrogen Peroxide

The effect that pH has on the sporicidal action of hydrogen peroxide has been examined by others (Curran et al., 1940; Cerf and Hermier, 1972). However, their experimental techniques were subject to criticism and their results inconclusive.

Cerf and Hermier (1972) noticed that the time required to destroy a given number of spores decreased with a decrease in pH in 15 percent hydrogen peroxide held at 80°C; however, the correlation was not sufficiently good to determine precisely the influence of pH. Curran et al. (1940) observed that the rate of spore destruction by a 1 percent solution of hydrogen peroxide held at 50°C generally decreased as the pH increased. Under alkaline conditions hydrogen peroxide breaks down rapidly (Schumb et al., 1955), thus the decrease in sporicidal activity at pH 9 noticed by Curran et al. (1940) may have been due to a loss of the hydrogen peroxide.

In this study the effect that varying the pH level had on the sporicidal activity of hydrogen peroxide was examined using 2 different means of pH adjustment; hydrochloric acid and/or sodium hydroxide and McIlvaines buffer. The pH levels selected were 2.2, 3.2, 4.2, 5.2, 6.2, and 7.2. At pH levels above and below this range hydrogen perioxide decomposes rapidly (Schumb et al., 1955). The effect of pH on the sporicidal action of hydrogen peroxide was not significant regardless of the method of pH adjustment (Tables 72 and 73, Appendix), although hydrogen peroxide was most lethal at the lower pH levels (pH 2.2 and pH 3.2).

The breakdown of hydrogen peroxide caused by low concentrations of metal ions is pH dependent (Schumb et al., 1955). Thus the enhancement of the sporicidal action of hydrogen peroxide by metal ions may be pH dependent. If such is the case, the effect varying the pH has on the sporicidal action of hydrogen peroxide may be masked due to the presence of low concentrations of contaminating metal ions in the treatment

solutions.

Selection of Spores Resistant to Hydrogen Peroxide

Development of spore resistance to hydrogen peroxide could be a problem in food processes that use hydrogen peroxide as a bactericide. Therefore an attempt was made to determine whether or not resistance could be increased by subculturing of spores that survived exposure to hydrogen peroxide.

Subculturing of spores surviving an exposure to 5 percent hydrogen peroxide at 50°C for 10 successive generations did not result in either an increase in spore resistance to hydrogen peroxide or to an increase or decrease in catalase activity. In fact, resistance to hydrogen peroxide decreased.

Thacker (1975) observed that the frequency of yeast cell mutation relative to the number of cells surviving a given exposure to hydrogen peroxide, decreased drastically as the concentration of hydrogen peroxide was increased from 100 ppm to 10,000 ppm (1 percent). Above 10,000 ppm no mutations were recovered. If the mechanism of hydrogen peroxide induced mutation in yeast cells is similar to a probable mechanism of hydrogen peroxide-induced mutation in spores, the failure to recover hydrogen peroxide resistant mutants from spores surviving a 5 percent exposure to hydrogen peroxide may be due to the fact that the hydrogen peroxide concentration was too high.

Watson and Schubert (1969) increased the hydrogen peroxide resistance of Salmonella typhimurium by subculturing the wild type LT2 strain

in media in which the hydrogen peroxide concentration was increased as culture resistance increased. Perhaps subculturing of a Bacillus sp. in a like manner would result in an increase in the resistance of spores subsequently produced by such a subculture. In food processes where low concentrations of hydrogen peroxide are added to food to lower the bacteria count such as with milk (Food and Drug Administration, 1962) and eggs (USDA ARS Pub., 1969) development of resistance to hydrogen peroxide could be a problem.

SUMMARY

Factors affecting the destruction and recovery of spores of Bacillus subtilis ATCC 9372 were studied. The time required for colony formation and variability in the amount of time required to attain maximum colony counts increased as the time of exposure to hydrogen peroxide increased. Repair of spore injury may require time which in turn accounts for the delay in colony formation. To quantitate the number of spores surviving an exposure to hydrogen peroxide, particularly when hydrogen peroxide is being evaluated as a sterilant, it is recommended that plates be counted periodically until maximum counts are attained.

The effect various storage conditions have on maintaining spore resistance to hydrogen peroxide was investigated. Length of storage time had a significant quadratic effect on spore resistance; resistance decreased during the first 35 days of storage but regained prestorage resistance after 56 days. Spores stored at -29°C were significantly more resistant than spores stored at 4°C. Spores stored in 0.85 percent sodium chloride were significantly more resistant than spores stored in distilled water or Butterfield's buffer. The resistance of spores stored in 0.85 percent sodium chloride at 4°C varied less during storage and was generally higher than the resistance of spores stored under other conditions. The rate of appearance of colonies was little affected by spore storage treatment. From these findings a set of storage conditions that maintained spore resistance to hydrogen peroxide was found. Such a development allows for many experiments to be performed on a single batch

of spores without concern for changes in or loss of spore resistance to hydrogen peroxide.

By storing spores in 0.85 percent sodium chloride at 4°C, the influence 5 different media had on recovery was determined. Of the media examined the medium of Wang et al. (1964) enabled recovery of the most spores. A comparison of the formulae of the media indicated that glucose and yeast extract are particularly important for recovery. The medium of Wang et al. (1964) contained both glucose and yeast extract which probably explains its superiority to the other media.

The influence that varying the method of hydrogen peroxide removal had on recovery was evaluated with 5 different media. Recovery in the medium of Wang et al. (1964) was greater than recovery in any of the other media despite changes made in the method of hydrogen peroxide removal.

Reducing the temperature of the catalase solution for neutralization of the hydrogen peroxide from 22°C to 1°C had a significant positive effect on recovery. The lethal effect of hydrogen peroxide may be halted more quickly at the low temperature because the sporicidal effect of hydrogen peroxide decreases as the temperature decreases. Holding the catalase solution at 1°C for removal of hydrogen peroxide from spores treated with hydrogen peroxide is recommended particularly when the sporicidal effect of different concentrations of hydrogen peroxide have is being compared. Addition of glucose to the catalase solution appeared to benefit recovery if the plating medium used for recovery did not contain added glucose. Suspending the catalase in different solutions such as distilled water, Butterfield's buffer, and 0.05 M phosphate

buffer did not have a significant effect on recovery. Addition of DL-methionine, or yeast extract, or glucose and yeast extract, or glucose and DL-methionine to the catalase solutions did not improve recovery after 1 week of storage at 4°C before plating.

The effect that each constituent of the medium of Wang et al. (1964) had on recovery was examined. Increasing the concentration of yeast extract had a positive exponential effect on recovery. Increasing the concentration of glucose from 0.0 g/l to 2.5 g/l resulted in a 1000-fold increase in recovery; but increasing the glucose concentration from 2.5 g/l to 5.0 g/l had a negative effect on recovery of spores exposed to hydrogen peroxide for 10 min and a zero effect on the recovery of spores exposed to hydrogen peroxide for 12 min.

Increasing the concentration of vitamin-free casamino acids from 0.0 g/l to 9.0 g/l and increasing the concentration of ferrous sulfate from 0.0 g/l to 0.1 g/l both had a positive and significant linear effect on recovery. Increasing the manganous sulfate concentration from 0.0 g/l to 0.1 g/l had a positive effect on recovery. Increasing the concentrations of either ferrous sulfate or manganous sulfate above 0.1 g/l had a negative effect on recovery especially if the pH of the medium was adjusted to 6.8 before autoclaving. When the medium is autoclaved at a neutral pH, manganous sulfate and ferrous sulfate may be involved in the formation of toxic compounds or the destruction of nutrients essential for spore recovery. Spore recovery was significantly higher at pH levels of 6.8 and 7.0 than at lower or higher pH levels. Increasing the concentration of agar from 15.0 g/l to 30.0 g/l had a positive linear effect

on the recovery of spores exposed to hydrogen peroxide for 8 min; however, for spores exposed 10 min and 12 min increasing the agar concentration had a negative linear effect on recovery.

There was a significant yeast extract by vitamin-free casamino acids interaction with regard to recovery of spores exposed to hydrogen peroxide. Regardless of the length of time of exposure, the effect that increasing the yeast extract concentration had in the absence of vitamin-free casamino acids was negative and the effect that increasing the yeast extract concentration had when vitamin-free casamino acids were in the medium was positive. The positive effect that increasing the concentration of yeast extract had when vitamin-free casamino acids were added may be due to an improvement in the balance of amino acids attained when both ingredients are added; vitamin-free casamino acids contributes amino acids to the medium not found in yeast extract.

The medium that permitted recovery of the highest number of injured spores was a modified version of the medium of Wang et al. (1964) that contained increased concentrations of yeast extract, ferrous sulfate, and vitamin-free casamino acids. The composition of the improved medium was 7.5 g of yeast extract/l, 2.5 g of glucose/l, 6.0 g of vitamin-free casamino acids/l, 15.0 g of agar/l, 0.1 g of ferrous sulfate/l, and 0.1 g of manganous sulfate/l. In general, the nutrient requirements of spores exposed to hydrogen peroxide are both enhanced and more exacting.

The effect of various additives in the medium of Wang et al. (1964) was studied however no additive was shown to have a significant positive effect on recovery. Additives such as spore exudate, sodium chloride,

and L-alanine had no significant effect on recovery. Additives such as magnesium sulfate, DL-methionine and starch all had significant negative effects on recovery. The effect that addition of dipicolinic acid to 5 different media had on recovery could not be evaluated due to the formation of crystals in the media. These crystals made it difficult or impossible to count colonies. All of the additives added to the medium of Wang et al. (1964) were selected because a priori information indicated that they might improve recovery; however, none significantly improved recovery. Only one concentration of each of these additives was examined.

The effect pH has on the sporicidal action of hydrogen peroxide was examined and shown not to be significant. Addition of 0.1 mM ferric chloride or cupric sulfate to the hydrogen peroxide enhanced the sporicidal action of hydrogen peroxide. At higher concentrations, these salts decomposed the hydrogen peroxide rapidly, thereby reducing the lethal effect of hydrogen peroxide; with lower concentrations of these salts the sporicidal action of the hydrogen peroxide was not augmented. Because these salts had no lethal effect on the spore when used alone (in the absence of hydrogen peroxide), and because they catalyze the breakdown of hydrogen peroxide, they presumably enhance the sporicidal action of hydrogen peroxide by forming the hydroxyl radical (an intermediate formed during hydrogen peroxide breakdown) which is a very strong oxidant. Other salts such as ferric citrate, zinc sulfate, and chromium chloride did not enhance the sporicidal action of hydrogen peroxide.

Subculturing of spores surviving exposure to 5 percent hydrogen peroxide held at 50°C did not result in an increase in the resistance of

spores produced after 10 consecutive generations of subculturing. The development of spore resistance to hydrogen peroxide may be a practical concern in food processes that utilize hydrogen peroxide as a bactericide. If a strain of bacteria that produces spores resistant to hydrogen peroxide could be developed, comparisons between the resistant strain and the parent strain might elucidate the mechanism of the sporicidal action of hydrogen peroxide.

CONCLUSIONS

The following conclusions can be made in regard to procedures and techniques used for destruction and recovery of spores of Bacillus subtilis ATCC 9372 that have been exposed to hydrogen peroxides.

1. The time required for colony formation and variability in the amount of time required to attain maximum colony counts increase as the time of exposure to hydrogen peroxide increases.
2. Length of time of spore storage has a significant quadratic effect on spore resistance. Spore resistance decreases during the first 35 days but regains prestorage resistance after 56 days.
3. Spores stored at -29°C remain significantly more resistant than spores stored at 4°C.
4. Storage of spores in 0.85 percent sodium chloride at 4°C maintains spore resistance to hydrogen peroxide at a higher level and with less variability than storage in distilled water or Butterfield's buffer.
5. Recovery of spores using a catalase solution held at 1°C is significantly higher than recovery when the temperature of the catalase solution is 22°C.
6. The number of spores recovered in the medium of Wang et al. (1964) is significantly higher than the number recovered in the medium of Halvorson (1957), Plate Count Agar, Trypticase Soy Agar, and Nutrient Agar.
7. Increasing the concentration of yeast extract in the medium of Wang et al. (1964) from 0.0 g/l to 10.0 g/l has a positive exponential

effect on recovery.

8. Increasing the concentration of glucose in the medium of Wang et al. (1964) from 0.0 g/l to 2.5 g/l increases the number of spores recovered by 1000-fold.
9. Increasing the concentration of vitamin-free casamino acids in the medium of Wang et al. (1964) has a positive linear effect on recovery.
10. Increasing the concentration of ferrous sulfate in the medium of Wang et al. (1964) from 0.0 g/l to 0.1 g/l has a positive linear effect on recovery. Increasing the concentration of ferrous sulfate above 0.1 g/l has a negative effect on recovery when the pH of the medium is adjusted to 6.8 before autoclaving.
11. Increasing the concentration of manganous sulfate in the medium of Wang et al. (1964) from 0.0 g/l to 0.1 g/l has a positive effect on recovery. Increasing the concentration of manganous sulfate above 0.1 g/l has a negative effect on recovery when the pH of the medium is adjusted to 6.8 before autoclaving.
12. Increasing the concentration of agar in the medium of Wang et al. (1964) from 15.0 g/l to 30.0 g/l has a positive linear effect on the recovery of spores exposed to hydrogen peroxide for 8 min and a negative linear effect on the recovery of spores exposed 10 min and 12 min.
13. There is a significant positive interaction between yeast extract by vitamin-free casamino acids for recovery of spores exposed to hydrogen peroxide.
14. Modifying the medium of Wang et al. (1964) by increasing the

concentrations of yeast extract, ferrous sulfate, and vitamin-free casamino acids results in an improved recovery medium. Autoclaving of the manganous sulfate and ferrous sulfate apart from the rest of the medium results in improving recovery and allows for adjustment of the pH of the medium before autoclaving.

15. Addition of spore exudate, sodium chloride, or L-alanine does not significantly influence recovery of injured spores. Addition of magnesium sulfate, DL-methionine, and starch has a negative effect on recovery.
16. Higher numbers of spores are recovered at pH levels of 6.8 and 7.0 than at pH levels of 6.4, 7.3, 7.6, and 7.9.
17. Varying the pH of the hydrogen peroxide from 2.2 to 7.2 has no significant effect on sporocidal activity.
18. Addition of 0.1 mM ferric chloride or cupric sulfate to hydrogen peroxide enhances the sporicidal action of hydrogen peroxide.
19. Subculturing of spores surviving exposure to 5 percent hydrogen peroxide held at 50°C does not result in an increase in the resistance of spores produced after 10 consecutive generations of subculturing.

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APPENDIX

Table 35. Analysis of variance of the effect that storage of spores for 15, 35, or 56 days at 4°C or -29°C in either distilled water, Butterfield's buffer or 0.85 percent sodium chloride had on the log of the number of spores enumerated before exposure to hydrogen peroxide

Source of variation[a]	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	3.596	1.798	<.01
Storage time (Time)	2	0.120	0.060	.58
Storage temperature (Temp)	1	0.023	0.023	.57
Time*Temp interaction	2	0.030	0.015	.80
Storage solution (Sol)	2	0.298	0.149	.12
Time*Sol interaction	4	0.148	0.037	.70
Temp*Sol interaction	2	0.110	0.055	.55
Temp*Time*Sol interaction	4	0.229	0.057	.50
Residual	34	2.270	0.067	
Corrected total	53	6.824	0.129	

[a]Time = storage time; Temp = storage temperature; Sol = storage solution. These are used in the following tables.

Table 36. Analysis of variance of the effect that storage of spores for 15, 35, or 56 days at 4°C or -29°C in either distilled water, Butterfield's buffer or 0.85 percent sodium chloride had on the log of the number of spores recovered after a 1 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	2.522	1.261	<.01
Time	2	0.008	0.004	.73
Temp	1	0.014	0.014	.29
Time*Temp interaction	2	0.025	0.013	.37
Sol	2	0.034	0.017	.26
Time*Sol interaction	4	0.044	0.011	.53
Temp*Sol interaction	2	0.009	0.004	.70
Temp*Time*Sol interaction	4	0.060	0.015	.32
Residual	34	0.418	0.012	
Corrected total	53	3.135	0.059	

Table 37. Analysis of variance of the effect that storage of spores for 15, 35, or 56 days at 4°C or -29°C in either distilled water, Butterfield's buffer or 0.85 percent sodium chloride had on the log of the number of spores recovered after a 5 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	3.591	1.795	<.01
Time	2	0.299	0.149	.30
Temp	1	0.161	0.161	.26
Time*Temp interaction	2	0.132	0.066	.59
Sol	2	0.261	0.130	.35
Time*Sol interaction	4	0.176	0.044	.85
Temp*Sol interaction	2	0.639	0.320	.08
Temp*Time*Sol interaction	4	0.671	0.168	.26
Residual	34	4.121	0.121	
Corrected total	53	10.051	0.190	

Table 38. Analysis of variance of the effect that storage of spores for 15, 35, or 56 days at 4°C or -29°C in either distilled water, Butterfield's buffer, or 0.85 percent sodium chloride had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sum of squares	Mean square	Probability of greater F
Replicate	2	5.935	2.967	.03
Time	2	4.819	2.409	.05
Temp	1	3.884	3.884	.03
Time*Temp interaction	2	0.719	0.360	.63
Sol	2	11.012	5.506	<.01
Time*Sol interaction	4	2.230	0.558	.57
Temp*Sol interaction	2	1.190	0.595	.54
Temp*Time*Sol interaction	4	3.255	0.814	.38
Residual	34	25.444	0.748	
Corrected total	53	58.489	1.104	

Table 39. Analysis of variance of the effect that storage of spores for 15, 35, or 56 days at 4°C or -29°C in either distilled water, Butterfield's buffer, or 0.85 percent sodium chloride had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	7.509	3.755	<.01
Time	2	3.667	1.833	.04
Temp	1	2.867	2.867	.02
Time*Temp interaction	2	0.850	0.425	.55
Sol	2	8.288	4.144	<.01
Time*Sol interaction	4	2.996	0.749	.24
Temp*Sol interaction	2	3.250	1.625	.05
Temp*Time*Sol interaction	4	1.574	0.394	.56
Residual	34	17.618	0.518	
Corrected total	53	48.622	0.917	

Table 40. Single degree of freedom comparisons made within storage time and storage solution treatments on the log of the number of spores recovered after storage for 15, 35, or 56 days at 4°C or -29°C in either distilled water (solution 1), Butterfield's buffer (solution 2), or 0.85 percent sodium chloride (solution 3)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Storage time	2	0.120	0.060	.58
Linear	1	0.120	0.120	.18
Quadratic	1	<0.001	<0.001	.95
15 days vs 35 days	1	0.025	0.025	.55
15 days vs 56 days	1	0.120	0.120	.19
35 days vs 56 days	1	0.035	0.035	.52
15 days vs 35 days and 56 days	1	0.085	0.085	.26
35 days vs 15 days and 56 days	1	<0.001	<0.001	.95
56 days vs 15 days and 35 days	1	0.095	0.095	.24
Storage solution	2	0.298	0.149	.12
Solution 1 vs solution 2	1	0.050	0.050	.60
Solution 1 vs solution 3	1	0.295	0.295	.04
Solution 2 vs solution 3	1	0.102	0.102	.22
Solution 1 vs solutions 2 and 3	1	0.196	0.196	.09
Solution 2 vs solutions 1 and 3	1	0.003	0.003	.83
Solution 3 vs solutions 1 and 2	1	0.248	0.248	.06
Residual	34	2.270	0.067	
Corrected total	53	6.824	0.129	

Table 41. Single degree of freedom comparisons made within storage time and storage solution treatments on the log of the number of spores surviving a 1 min exposure to hydrogen peroxide after 15, 35, or 56 days of storage at 4°C or -29°C in either distilled water (solution 1), Butterfield's buffer (solution 2), or 0.85 percent sodium chloride (solution 3)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Storage time	2	0.008	0.004	.73
Linear	1	<0.001	<0.001	.87
Quadratic	1	0.008	0.008	.62
15 days vs 35 days	1	0.007	0.007	.54
15 days vs 56 days	1	<0.001	<0.001	.87
35 days vs 56 days	1	0.004	0.004	.56
15 days vs 35 days and 56 days	1	0.034	0.034	.61
35 days vs 15 days and 56 days	1	0.008	0.008	.56
56 days vs 15 days and 35 days	1	<0.001	<0.001	.79
Storage solution	2	0.034	0.017	.26
Solution 1 vs solution 2	1	0.028	0.028	.14
Solution 1 vs solution 3	1	0.023	0.023	.18
Solution 2 vs solution 3	1	<0.001	<0.001	.88
Solution 1 vs solutions 2 and 3	1	0.034	0.034	.10
Solution 3 bs solutions 1 and 2	1	0.006	0.006	.51
Residual	34	0.418	0.012	
Corrected total	53	3.135	0.059	

Table 42. Single degree of freedom comparisons made within storage time and storage solution treatments on the log of the number of spores surviving a 5 min exposure to hydrogen peroxide after 15, 35, or 56 days of storage at 4°C or -29°C in either distilled water (solution 1), Butterfield's buffer (solution 2), or 0.85 percent sodium chloride (solution 3)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Storage time	2	0.299	0.149	.30
Linear	1	0.007	0.007	.80
Quadratic	1	0.292	0.292	.13
15 days vs 35 days	1	0.260	0.260	.15
15 days vs 56 days	1	0.007	0.007	.80
35 days vs 56 days	1	0.181	0.181	.23
15 days vs 35 days and 56 days	1	0.118	0.118	.67
35 days vs 15 days and 56 days	1	0.292	0.292	.13
56 days vs 15 days and 35 days	1	0.039	0.039	.58
Storage solution	2	0.261	0.130	.35
Solution 1 vs solution 2	1	0.044	0.044	.56
Solution 1 vs solution 3	1	0.258	0.258	.15
Solution 2 vs solution 3	1	0.088	0.088	.60
Solution 1 vs solutions 2 and 3	1	0.172	0.172	.24
Solution 2 vs solutions 1 and 3	1	0.003	0.003	.88
Solution 3 vs solutions 1 and 2	1	0.216	0.216	.19
Residual	34	4.121	0.121	
Corrected total	53	10.051	0.190	

Table 43. Single degree of freedom comparisons made within storage time and storage solution treatments on the log of the number of spores surviving an 8 min exposure to hydrogen peroxide after 15, 35, or 56 days of storage at 4°C or -29°C in either distilled water (solution 1), Butterfield's buffer (solution 2), or 0.85 percent sodium chloride (solution 3)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Storage time	2	4.819	2.967	.05
Linear	1	0.397	0.397	.52
Quadratic	1	4.421	4.421	.02
15 days vs 35 days	1	2.268	2.268	.09
15 days vs 56 days	1	0.397	0.397	.52
35 days vs 56 days	1	4.563	4.563	.02
15 days vs 35 days and 56 days	1	0.256	0.256	.57
35 days vs 15 days and 56 days	1	4.421	4.421	.02
56 days vs 15 days and 35 days	1	2.551	2.551	.07
Storage solution	2	11.012	5.506	<.01
Solution 1 vs solution 2	1	0.735	0.735	.67
Solution 1 vs solution 3	1	10.272	10.272	<.01
Solution 2 vs solution 3	1	5.511	5.511	.01
Solution 1 vs solutions 2 and 3	1	5.501	5.501	.01
Solution 2 vs solutions 1 and 3	1	0.740	0.740	.67
Solution 3 vs solutions 1 and 2	1	10.277	10.277	<.01
Residual	34	25.444	0.748	
Corrected total	53	58.489	1.104	

Table 44. Single degree of freedom comparisons made within storage time and storage solution treatments on the log of the number of spores surviving a 10 min exposure to hydrogen peroxide after 15, 35, or 56 days of storage at 4°C or -29°C in either distilled water (solution 1), Butterfield's buffer (solution 2), or 0.85 percent sodium chloride (solution 3)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Storage time	2	3.667	1.833	.04
Linear	1	0.950	0.950	.18
Quadratic	1	2.716	2.716	.03
15 days vs 35 days	1	0.883	0.883	.20
15 days vs 56 days	1	0.951	0.951	.18
35 days vs 56 days	1	3.667	3.667	.01
15 days vs 35 days and 56 days	1	<0.001	<0.001	.98
35 days vs 15 days and 56 days	1	2.716	2.716	.03
56 days vs 15 days and 35 days	1	2.784	2.784	.03
Storage solution	2	8.288	4.144	<.01
Solution 1 vs solution 2	1	0.382	0.382	.60
Solution 1 vs solution 3	1	7.530	7.530	<.01
Solution 2 vs solution 3	1	4.521	4.521	<.01
Solution 1 vs solutions 2 and 3	1	3.767	3.767	.01
Solution 2 vs solutions 1 and 3	1	0.759	0.759	.23
Solution 3 vs solutions 1 and 2	1	7.907	7.907	<.01
Residual	34	17.618	0.518	
Corrected total	53	48.622	0.917	

Table 45. Analysis of variance of the effect media (medium W, plate count agar, medium H, trypticase soy agar and nutrient agar), catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 hr vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation[a]	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	1.530	1.530	<.01
Catalase solution temperature (Temp)	1	1.485	1.485	<.01
Catalase concentration (Conc)	1	0.470	0.470	.05
Temp*Conc	1	0.040	0.040	.57
Catalase contact time (Time)	1	0.009	0.009	.79
Temp*Time	1	0.311	0.311	.11
Conc*Time	1	0.086	0.086	.59
Temp*Conc*Time	1	0.119	0.119	.67
Glucose (Glu)	1	0.972	0.972	<.01
Temp*Glu	1	0.027	0.027	.65
Conc*Glu	1	0.483	0.483	.05
Temp*Conc*Glu	1	0.110	0.110	.65
Time*Glu	1	0.004	0.004	.85
Temp*Time*Glu	1	0.051	0.051	.53
Conc*Time*Glu	1	0.012	0.012	.75
Temp*Conc*Time*Glu	1	0.081	0.081	.58
Media (Med)	4	172.644	43.161	<.01
Temp*Med	4	4.600	1.150	<.01
Conc*Med	4	0.232	0.058	.76
Temp*Conc*Med	4	0.285	0.072	.67
Time*Med	4	0.900	0.225	.13
Temp*Time*Med	4	0.559	0.140	.34
Conc*Time*Med	4	0.067	0.017	.96
Temp*Conc*Time*Med	4	0.271	0.068	.70
Glu*Med	4	0.572	0.143	.33
Temp*Glu*Med	4	0.180	0.045	.83

[a] Temp = catalase solution temperature; Conc = catalase concentration; Time = catalase contact time; Glu = glucose; Med = media. These are used in the following tables.

Table 45 (Continued)

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater f
Conc*Glu*Med	4	0.659	0.165	.26
Temp*Conc*Glu*Med	4	0.460	0.115	.55
Time*Glu*Med	4	0.061	0.015	.97
Temp*Time*Glu*Med	4	0.158	0.040	.86
Conc*Time*Glu*Med	4	0.220	0.055	.77
Temp*Conc*Time*Glu	4	0.271	0.068	.70
Error	79	9.626	0.123	
Corrected total	159	197.556		

Table 46. Analysis of variance of the effect catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 hr vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the recovery of spores in medium W after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	0.065	0.065	.04
Temp	1	0.144	0.144	<.00
Conc	1	0.072	0.072	.03
Temp*Conc	1	0.006	0.006	.53
Time	1	0.067	0.067	.04
Temp*Time	1	0.024	0.024	.19
Conc*Time	1	0.081	0.081	.02
Temp*Conc*Time	1	0.004	0.004	.61
Glu	1	0.001	0.001	.79
Temp*Glu	1	0.029	0.029	.15
Conc*Glu	1	0.042	0.042	.09
Temp*Conc*Glu	1	0.023	0.023	.20
Time*Glu	1	0.012	0.012	.65
Temp*Time*Glu	1	0.004	0.004	.60
Conc*Time*Glu	1	0.027	0.027	.17
Temp*Conc*Time*Glu	1	0.0003	0.0003	.87
Error	15	0.195	0.013	
Corrected total	31	0.796		

Table 47. Analysis of variance of the effect catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 h vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the log of the number of spores recovered in medium H after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	2.308	2.308	<.01
Temp	1	0.618	0.618	.03
Conc	1	0.155	0.155	.25
Temp*Conc	1	0.009	0.009	.77
Time	1	0.512	0.512	.04
Temp*Time	1	0.770	0.770	.02
Conc*Time	1	0.067	0.067	.55
Temp*Conc*Time	1	0.128	0.128	.29
Glu	1	0.276	0.276	.13
Temp*Glu	1	0.150	0.150	.25
Conc*Glu	1	0.937	0.937	.01
Temp*Conc*Glu	1	0.529	0.529	.04
Time*Glu	1	0.010	0.010	.77
Temp*Time*Glu	1	0.081	0.081	.60
Conc*Time*Glu	1	0.110	0.110	.33
Temp*Conc*Time*Glu	1	0.208	0.208	.18
Error	15	1.614	0.108	
Corrected total	31	8.482		

Table 48. Analysis of variance of the effect catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 hr vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the log of the number of spores recovered in nutrient agar after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	2.330	2.330	<.01
Temp	1	4.264	4.264	<.01
Conc	1	<0.00	<0.00	.99
Temp*Conc	1	0.233	0.233	.14
Time	1	0.039	0.039	.54
Temp*Time	1	0.002	0.002	.88
Conc*Time	1	0.002	0.002	.90
Temp*Conc*Time	1	0.001	0.001	.93
Glu	1	0.810	0.810	.01
Temp*Glu	1	<0.00	<0.00	.95
Conc*Glu	1	0.002	0.002	.88
Temp*Conc*Glu	1	0.006	0.006	.80
Time*Glu	1	0.033	0.033	.58
Temp*Time*Glu	1	0.033	0.033	.57
Conc*Time*Glu	1	0.065	0.065	.56
Temp*Conc*Time*Glu	1	0.084	0.084	.63
Error	15	1.479	0.099	
Corrected total	31	9.384		

Table 49. Analysis of variance of the effect catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 hr vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the log of the number of spores recovered in trypticase soy agar after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	1.445	1.445	<.01
Temp	1	1.053	1.053	<.01
Conc	1	0.428	0.428	.03
Temp*Conc	1	0.075	0.075	.65
Time	1	0.284	0.284	.08
Temp*Time	1	0.075	0.075	.65
Conc*Time	1	0.001	0.001	.90
Temp*Conc*Time	1	0.255	0.255	.09
Glu	1	0.435	0.435	.03
Temp*Glu	1	0.027	0.027	.58
Conc*Glu	1	0.160	0.160	.18
Temp*Conc*Glu	1	0.012	0.012	.71
Time*Glu	1	0.008	0.008	.75
Temp*Time*Glu	1	0.089	0.089	.31
Conc*Time*Glu	1	<0.001	<0.001	.97
Temp*Conc*Time*Glu	1	0.050	0.050	.55
Error	15	1.198	0.080	
Corrected total	31	5.594		

Table 50. Analysis of variance of the effect catalase solution temperature (1°C vs 22°C), catalase concentration (10 mg/9 ml vs 20 mg/9 ml), catalase contact time (1 hr vs 5 hrs), and glucose in the catalase solution (0.0 g/9 ml vs 0.1 g/9 ml) had on the log of the number of spores recovered in plate count agar after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	0.446	0.446	<.01
Temp	1	0.002	0.002	.59
Conc	1	0.048	0.048	<.01
Temp*Conc	1	0.005	0.005	.65
Time	1	0.007	0.007	.24
Temp*Time	1	<0.001	<0.001	.90
Conc*Time	1	0.001	0.001	.60
Temp*Conc*Time	1	0.003	0.003	.57
Glu	1	0.022	0.022	.05
Temp*Glu	1	<0.001	<0.001	.90
Conc*Glu	1	0.001	0.001	.73
Temp*Conc*Glu	1	0.001	0.001	.74
Time*Glu	1	0.002	0.002	.56
Temp*Time*Glu	1	0.002	0.002	.56
Conc*Time*Glu	1	0.029	0.029	.03
Temp*Conc*Time*Glu	1	0.009	0.009	.19
Error	15	0.076	0.005	
Corrected total	31	0.654		

Table 51. Analysis of variance of the effect 1 week of storage in each of 7 solutions containing 1 mg of catalase/9 ml had on the log of the number of spores surviving a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	0.307	0.154	.15
Sol	6	0.280	0.047	.72
Time	1	1.232	1.232	<.01
Sol*Time interaction	6	0.483	0.080	.41
Error	26	1.979	0.076	
Corrected total	41	4.281		

Table 52. Analysis of variance of the effect varying the concentration of yeast extract (0.0, 2.5, 5.0, 7.5 and 10.0 g/l) in medium W had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.390	0.130	<.01
Yeast extract	4	35.021	8.755	<.01
Linear	1	22.857	22.857	<.01
Quadratic	1	10.547	10.547	<.01
Cubic	1	1.486	1.486	<.01
Quartic	1	0.131	0.137	<.01
Error	12	0.085	0.007	
Corrected total	19	35.497		

Table 53. Analysis of variance of the effect varying the concentration of glucose (0.0, 2.5, 5.0, and 7.5 g/l) in medium W had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	1.074	0.358	.02
Glucose	3	25.934	8.645	<.01
Error	9	0.530	0.059	
Corrected total	15	27.537		

Table 54. Analysis of variance of the effect varying the concentration of vitamin-free casamino acids (0, 1, 2 and 3 g/l) in medium W had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.419	0.140	.01
Vitamin-free casamino acids	3	0.015	0.005	.14
Linear	1	0.014	0.014	.03
Quadratic	1	<0.001	<0.001	.70
Cubic	1	0.001	0.001	.52
Error	9	0.020	0.002	
Corrected total	15	0.454		

Table 55. Analysis of variance of the effect varying the concentration of vitamin-free casamino acids (0, 3, 6 and 9 g/l) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.127	0.042	<.01
Vitamin-free casamino acids	3	0.024	0.008	.04
Linear	1	0.020	0.020	.01
Quadratic	1	0.004	0.004	.18
Cubic	1	<0.001	<0.001	.67
Error	9	0.017	0.002	
Corrected total	15	0.169		

Table 56. Analysis of variance of the effect varying the concentration of vitamin-free casamino acids (4, 5, 6 and 7 g/l) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.003	0.001	.24
Vitamin-free casamino acids	3	0.014	0.005	<.01
Error	9	0.006	0.006	
Corrected total	15	0.023		

Table 57. Analysis of variance of the effect varying the concentration of agar (15, 20, 25 and 30 g/1) in medium W had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.437	0.146	<.01
Agar	3	0.145	0.048	<.01
Linear	1	0.140	0.140	<.01
Quadratic	1	0.004	0.004	.59
Cubic	1	0.001	0.001	.68
Error	9	0.046	0.005	
Corrected total	15	0.628		

Table 58. Analysis of variance of the effect varying the concentration of agar (25, 30 and 35 g/1) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.094	0.031	<.01
Agar	2	0.001	<0.001	.12
Linear	1	0.001	0.001	.05
Quadratic	1	<0.001	<0.001	.56
Error	6			
Corrected total	11			

Table 59. Analysis of variance of the effect varying the concentration of manganous sulfate (0.0, 0.1 and 1.0 g/l) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of freedom	Mean square	Probability of greater F
Replicate	3	0.118	0.039	.04
Manganous sulfate	2	0.383	0.128	<.01
Error	6	0.046	0.008	
Corrected total	11	0.547		

Table 60. Analysis of variance of the effect varying the concentration of manganous sulfate (0.1, 0.3, 0.5 and 0.7 g/l) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	5	0.506	0.101	<.01
Manganous sulfate	3	12.411	4.137	<.01
Error	15	0.130	0.009	
Corrected total	23	13.048		

Table 61. Analysis of variance of the effect varying the concentration of ferrous sulfate (0.0, 0.001, 0.01 and 0.1 g/l) in medium W had on the log of the number of spores recovered after an 8 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.389	0.130	<.01
Ferrous sulfate	3	0.080	0.027	<.01
Linear	1	0.080	0.080	<.01
Quadratic	1	<0.001	<0.001	.96
Cubic	1	<0.001	<0.001	.85
Error	9	0.028	0.003	
Corrected total	15	0.496		

Table 62. Analysis of variance of the effect varying the concentration of ferrous sulfate (0.0, 0.1 and 1.0 g/l) in the medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	3	0.133	0.044	<.01
Ferrous sulfate	2	0.063	0.032	<.01
Error	6	0.009	0.002	
Corrected total	11	0.206		

Table 63. Analysis of variance of the effect varying the concentration of ferrous sulfate (0.1, 0.3 and 0.7 g/l) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	5	0.067	0.013	.25
Ferrous sulfate	2	1.764	0.882	<.01
Error	10	0.085	0.008	
Corrected total	17	1.916		

Table 64. Analysis of variance of the effect varying the pH level (6.4, 6.8, 7.0, 7.3, 7.6 and 7.9) of medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	0.040	0.040	<.01
pH	5	1.458	0.292	<.01
Error	5	0.001	<0.001	
Corrected total	11	1.500		

Table 65. Analysis of variance of the effect ferrous sulfate (0.0 g/l vs 0.1 g/l) and vitamin-free casamino acids (0 g/l vs 6 g/l) in medium W had on the log of the number of spores recovered after a 12 min exposure to hydrogen peroxide

Source of variation[a]	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	0.420	0.210	<.01
Ferrous sulfate	1	0.004	0.004	.50
Vitamin-free casamino acids (VFCA)	1	0.696	0.696	.00
Ferrous sulfate*VFCA interaction	1	0.032	0.032	.07
Error	6	0.040	0.007	
Corrected total	11	1.191		

[a]VFCA = vitamin-free casamino acids; this will be used in the following tables.

Table 66. Analysis of variance of the effect glucose (2.5 g/1 vs 5.0 g/1), yeast extract (5.0 g/1 vs 7.5 g/1), agar (15 g/1 vs 30 g/1) and vitamin-free casamino acids (0 g/1 vs 6 g/1) in medium W had on the log of the number of spores recovered after a 10 min exposure to hydrogen peroxide

Source of variation[a]	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	0.483	0.483	<.01
Glucose (Glu)	1	0.062	0.062	<.01
Yeast extract (YE)	1	0.005	0.005	.27
Glu*YE	1	0.008	0.008	.15
Agar (Ag)	1	0.037	0.037	<.01
Glu*Ag	1	<0.001	<0.001	.83
YE*Ag	1	<0.001	<0.001	.98
Glu*YE*Ag	1	0.002	0.002	.50
VFCA	1	0.162	0.162	<.01
Glu*VFCA	1	<0.001	<0.001	.94
YE*VFCA	1	0.015	0.015	.06
Glu*YE*VFCA	1	0.004	0.004	.28
Ag*VFCA	1	0.001	0.001	.68
Glu*Ag*VFCA	1	0.009	0.009	.14
YE*Ag*VFCA	1	0.006	0.006	.20
Glu*YE*Ag*VFCA	1	0.003	0.003	.64
Error	15	0.054	0.004	
Corrected total	31	0.852		

[a]YE = yeast extract; Ag = Agar. These are used in the following table.

Table 67. Analysis of variance of the effect glucose (2.5 g/l vs 5.0 g/l), yeast extract (5.0 g/l vs 7.5 g/l), agar (15 g/l vs 30 g/l) and vitamin-free casamino acids (0 g/l vs 6 g/l) in medium W had on the log of the number of spores recovered after a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	0.050	0.050	<.01
Glu	1	0.004	0.004	.20
YE	1	0.010	0.010	.03
Glu*YE	1	<0.001	<0.001	.65
Ag	1	0.027	0.027	<.01
Glu*Ag	1	<0.001	<0.001	.90
YE*Ag	1	0.001	0.001	.57
Glu*YE*Ag	1	0.005	0.005	.13
VFCA	1	1.119	1.119	<.01
Glu*VFCA	1	0.022	0.022	<.01
YE*VFCA	1	0.018	0.018	<.01
Glu*YE*VFCA	1	<0.001	<0.001	.74
Ag*VFCA	1	0.018	0.018	<.01
Glu*Ag*VFCA	1	0.015	0.015	.02
YE*Ag*VFCA	1	0.015	0.015	.01
Glu*YE*Ag*VFCA	1	<0.001	<0.001	.68
Error	15	0.030	0.002	
Corrected total	31			

Table 68. Analysis of variance of the effect that addition of spore exudate (10 ml/l), sodium chloride (2.5 g/l), or L-alanine (0.1 g/l) to medium W had on the log of the number of spores recovered after a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	12.443	12.443	<.01
Additive	3	0.489	0.163	.36
Error	19	2.742	0.144	
Corrected total	23	15.652		

Table 69. Analysis of variance of the effect that addition of magnesium sulfate (0.01 g/l), DL-methionine (0.1 g/l), or starch (1 g/l) had on the log of the number of spores recovered after a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	0.506	0.253	.01
Additive	3	1.632	0.816	.01
Error	6	0.014	0.002	
Corrected total	11	2.152		

Table 70. Analysis of variance of the effect that addition of ferric citrate (0.1 mM), zinc sulfate (0.1 mM), or chromium chloride (0.1 mM) to hydrogen peroxide solution had on the log of the number of spores surviving a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Hydrogen peroxide additive	3	1.238	0.413	.18
Error	8	1.600	0.200	
Corrected total	11	2.837		

Table 71. Analysis of variance of the effect that addition of ferric chloride (0.1 mM), cupric sulfate (0.1 mM), or both (0.05 mM of each) had on the log of the number of spores surviving a 10 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Hydrogen peroxide additive	2	0.188	0.094	.69
Error	6	1.396	0.233	
Corrected total	8	1.584		

Table 72. Analysis of variance of the effect varying the pH of the hydrogen peroxide solution using hydrochloric acid and/or sodium hydroxide had on the log of the number of spores surviving a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	2	0.063	0.032	.86
pH	5	1.032	0.206	.45
Error	10	2.011	0.201	
Corrected total	17			

Table 73. Analysis of variance of the effect varying the pH of the hydrogen peroxide solution using McIlvaines' buffer had on the log of the numbers of spores surviving at 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	2.218	2.218	.04
pH	5	0.822	0.164	.86
Error	17	7.451	0.507	
Corrected total	23	10.491		

Table 74. Analysis of variance of the effect of the ferrous sulfate by manganous sulfate interaction in medium W on the log of the number of spores recovered after a 12 min exposure to hydrogen peroxide

Source of variation	Degrees of freedom	Sums of squares	Mean square	Probability of greater F
Replicate	1	2.672	2.672	.06
Ferrous sulfate (Fe)	1	0.504	0.504	.29
Managanous sulfate (Mn)	1	0.035	0.035	.75
Fe*Mn interaction	1	1.412	1.412	.12
Error	3	0.928	0.309	
Corrected total	7	5.549		