PHYSIOLOGY OF AND THE INFLUENCE OF SELECTED STRESS FACTORS ON THE GROWTH AND SURVIVAL OF THERMOPHILIC *BACILLUS* SPECIES ASSOCIATED WITH VACUUM PACKED MEAT PRODUCTS

BY

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CONTENTS

1. INTRODUCTION

During the past few years studies on the water activity (a_w) of aqueous solutions, in connection with the formulation of intermediate moisture foods (IMF) have been done (Labuza et al., 1972; Leistner and Rodel, 1975; Labuza et al., 1976; Troller, 1979; Benmerqui et al., 1979; Chirife, et al., 1980; Chirife and Ferro Fontan, 1980; Alzamora et al., 1981).

Water activity is an important factor in the manufacture of food systems and formulations (Labuza et al., 1976). Most chemical reactions and microbiological activity are controlled directly by the water activity of the food system (Labuza et al., 1972; Leistner and Rodel, 1975).

Bacteria and molds associated with meat and meat products frequently cause spoilage and sometimes food poisoning or alternatively may be essential for the fermentation of certain processed meats such as salami. Microbial spoilage and food poisoning take place if the a_w of the substrate is favourable for the multiplication of the organisms involved (Leistner and Rodel, 1975).

The growth of micro-organisms as a function of a_w has been an subject of great interest among microbiologists. The a_w influences the exponential growth and stationary phase, as

well as the death rate of a culture (Troller and Christian, 1978). Most organisms occurring in meat and meat products proliferate at a high a_w, whereas only a few require a reduced a_w for growth. Leistner and Rodel (1975) concluded that there is a limiting a_w below which certain organisms will not grow.

A factor frequently overlooked yet of crucial importance to safety and shelf-stability is the degree of contamination of the raw product with bacterial spores (Hauschild and Simonsen, 1986). Equally important is the load of bacterial spore contamination of non-meat ingredients, i.e. spices, starch and sugar (Jackson et al., 1982). Spices are commonly decontaminated by fumigation and irradiation, and manufacturers of meat products should have no difficulty in selecting spices with viable bacterial counts of $<10,4\ g^{-1}$ or mesophilic spore counts of $<5 \times 10,3\ g^{-1}$ (Hauschild and Simonsen, 1986).

The microbiological stability of several types of foods and heat processed products in particular, is determined by the number of bacterial spores in the product. Murrel and Scott (1966) stated that the limits for several physical environmental factors, including water activity, were usually narrower for spore formation than for growth. Jakobsen and Murrel (1977) showed that glycerol was less inhibitory to bacterial spores than sugars and salts.

Shelf-stable, intermediate moisture meat products are the result of the interaction of a number of factors. The most important of these factors are, (i) the use of ingredients with low initial levels of micro-organisms, (ii) heat treatment to lower the level of vegetative cells present in the finished product, (iii) the addition of anti-fungal agents, (iv) the addition of humectants (i.e. glycerol) to bind free water in the product, thus lowering the a_w of the product and thus preventing any micro-organisms from growing, (v) the pH of the product adjusted to a level at which anti-microbial activity is enhanced and (vi) the product is vacuum-sealed in retort pouches to retain optimal conditions for shelf-stability for as long as possible.

One of the main criteria for the stability of shelf stable products, is the ability to eliminate the outgrowth of spores in the product. The ability to eliminate or control spore germination is closely linked with the a_w of the product (Leistner et al., 1981; Jakobsen and Murrel, 1977). The a_w affects the metabolic activities of the organisms as well as their resistance to heat and survival. Most bacterial spores germinate at an a_w of 0,99-0,98, but with a decrease in a_w there is a decrease in germination. Thus, a combination of heat treatment and a low a_w would effectively control the sporeformers in the product. But sometimes there is an exception to the rule (Leistner et al., 1981).

Bacterial counts of experimental shelf stable frankfurter type sausages, manufactured at NORM FOODS, Rustenburg (NF/26-2) increased to 1,3 x 10^6 colony forming units (cfu) at 37°C as well as 50°C. The a_w of the sausages was 0,93 to 0,94. All isolates were thermophilic sporeforming bacteria. The question that arose was that if these bacteria survived the manufacturing process, could they germinate and grow at such high temperatures and low a_w values? As the microbiological stability of heat processed products are determined by the number of surviving bacterial spores, it is of utmost importance to define the factors determining growth and survival of these organisms, especially at low a_w values and in the presence of other controlling factors such as pH, nitrite and sorbate.

The effect of a_w on spore germination has been studied by Baird-Parker and Fraeme (1967), Jakobsen et al. (1972), Jakobsen and Murrel (1977) and Anagnostopoulos and Sidhu (1981). However, little information on the germination of thermophilic bacterial spores at low a_w values and higher temperatures could be gleaned from existing literature. Furthermore, little information on the influence of additives like sorbate and nitrite on germination of thermophilic bacterial spores at low a_w values could be found.

Furthermore, very little information on the physiology of thermophilic Bacillus species other than B.stearothermophilus could be found.

Since it was necessary to produce an intermediate moisture food, shelf stable product, it was imperative to eliminate or control thermophilic bacterial endospores. In order to achieve this goal a thorough knowledge of the physiology of thermophilic Bacillus species was required.

This report contains an investigation into the taxonomy and physiology of thermophilic Bacillus species and their growth and survival under stress conditions.

2. TAXONOMY AND PHYSIOLOGY OF THERMOPHILIC *BACILLUS* SPECIES

2.1 Literature review

2.1.1 Taxonomy and historical aspects

The occurrence in microorganisms of uniquely heat resistant spores was demonstrated by Ferdinand Cohn in 1876 in the mesophile *Bacillus subtilis*. This discovery was followed by the isolation of a thermophilic aerobic spore-former by Miquel in 1888, which grew at 73°C. During the next 50 years an interest developed in aerobic sporeforming *Bacillus* strains capable of growth at temperatures at which denaturation of proteins, inactivation of enzymes and destruction of microorganisms readily occur (Wolf and Sharp, 1981).

This early period is well summarized in the review of Allen (1953). The early reports record the ubiquitous occurrence of the thermophiles and the ease of their isolation from virtually any sample of water, soil, or mud and even freshly fallen snow. Thermophilic members of the genus *Bacillus* predominated in these isolations, whereas non-sporeforming bacteria were less common. The activity of these strains at

high temperature led to the early demonstration of their thermostable enzymes (Wolf and Sharp, 1981).

The lack of interest in thermophiles during the past 50 years and in problems in enzymology and molecular biology which they pose is at least being rapidly redressed. Thus, the last few years have seen several publications dealing with these bacteria, and it is clear that their heat stable and rapidly acting enzymes, active at temperatures (60^{o}C) at which the effect of mesophilic contaminants can be virtually ignored, are likely to prove valuable agents of commercial exploitations, e.g. in the hydrolysis of polysaccharides and proteins (Brock, 1978; Fields, 1970; Sharp et al., 1980; Srivastava and Baruah, 1986; Williams, 1975).

Taxonomic studies and descriptions of Bacillus thermophiles until about 1949 are rather inadequate. The exceptions were B. coagulans and B. stearothermophilus (Donk, 1920). Data and taxonomic studies on the thermophiles are almost entirely due to the patient and persistent efforts of Ruth Gordon and her colleagues over 25 years (1949 to 1974) (Gordon, 1981).

During the two decades 1926-1948 progress in the physiology and systematics of Bacillus thermophiles was very limited. The period started with the second edition of "Bergey's Manual of Determinative Bacteriology" with the listing of

some sporing thermophiles and with B. coagulans appearing amongst the mesophiles (Wolf and Sharp, 1981).

In the sixth edition of "Bergey's Manual of Determinative Bacteriology" Smith (1948) listed descriptions of 19 mesophilic species of Bacillus and of 20 thermophiles capable of growth at 55°C. The thermophiles were classified largely on sporangial morphology and several species were recognized. Smith emphasized the difficulty of presenting a more rational scheme given the data available on the thermophiles. This inadequate state of affairs must have acted as a stimulus for the more detailed study presented shortly thereafter by Gordon and Smith (1949).

In 1946 Smith, Gordon and Clark published the first monograph on the mesophilic species of Bacillus, but included an amended description of B. coagulans based on the study of eighth strains. A fundamental study of Bacillus thermophiles (Gordon and Smith, 1949) followed the publication of the original monograph and the work reported in this paper formed the basis of subsequent entries in the seventh and eight edition of "Bergey's Manual of Determinative Bacteriology" (Breed et al., 1957; Gibson and Gordon, 1974). The classical paper of Gordon and Smith (1949) has also served as the stimulus and mine of information for all subsequent studies on Bacillus thermophiles. In their study they examined 216 thermophilic

cultures derived from a large variety of sources. These test strains were subjected to a large number of physiological, cultural and microscopic tests. A detailed examination of the results indicated some fundamental differences between B. coagulans and B. stearothermophilus, and suggested that all previously described Bacillus thermophiles including new isolates from natural sources, conveniently fell into one or other of these two species.

The definition of Bacillus thermophiles is limited to the better known thermophilic Bacillus species and types, for which considerable information is scattered in the literature. Much of the available data are on B. stearothermophilus and a variety of Bacillus isolates which have been identified with it (Farrel and Cambell, 1969; Heinen and Heinen, 1972; Sharp et al., 1980).

In an extensive study by Allen (1953) on thermophiles of the genus Bacillus, she finally obtained a collection of 105 cultures all of which grew at 65°C and were classified into four groups as follows:

Group 1: Organisms with distinct oval, bulging sporangia, morphologically and biochemically similar to B. circulans. These organisms bear strong resemblence to a number of B. stearothermophilus strains which Walker and Wolf (1971) identified in their group 3.

Group 2: These organisms showed slight bulging of the sporangium, grew in acidified media, and closely resembled B. coagulans.

Group 3: These organisms resembled B. subtilis on biochemical and morphological grounds, their sporangia were totally non-swollen, some reduced nitrate to N_2 and might be closely related to B. licheniformis.

Group 4: The isolates in this group produced distinctly swollen sporangia and spherical terminal spores. They resembled B. sphaericus.

Walker and Wolf (1971) reported the results of an extensive study on the biochemical, physiological and serological properties of 230 strains into three distinct major groups, two of which were further divided into minor subgroups.

Following the isolation of the Gram-negative non-sporulating thermophile Thermus aquaticus (Brock and Freeze, 1969) from a superheated pool in Yellowstone National Park, a sporulating thermophile was isolated from the same pool (Heinen, 1971). The temperature at the sampling site was a constant 86^{o}C and the pH 8,2. The new strain, designated YT-G, was reported to be a Gram-negative rod, which produced short filaments distinctively composed of a number of single

cells and produced dull yellowish grey colonies. Two more thermophilic strains, YT-F and YT-P, were subsequently isolated from the Yellowstone National Park and compared with the original strain YT-G (Heinen and Heinen, 1972). All strains were found to be Gram- variable although young cells of strains YT-F and YT-P appeared to be Gram-negative.

Heinen and Heinen (1972) differentiated all three caldo-active strains from B. stearothermophilus on the basis of their temperature optima, fatty acid pattern and submicroscopical structure. The three strains can be distinguished by readiness to sporulate, their different temperature optima and the morphological differences in their cell walls and membranes. Heinen and Heinen (1972) proposed the names B. caldotenax. B. caldolyticus and B. caldovelox for strains YT-G, YT-P and YT-F, respectively. The term caldo-active describes extremely thermophilic bacteria which show no active metabolism at lower temperatures.

Sharpe et al. (1980) compared the biochemical properties, DNA base composition, bacteriophage and bacteriocin sensitivities, esterases and antibiotic resistance of seven strains of B. stearothermophilus and the three caldo-active strains. The antibiotic sensitivity patterns of the three caldo-active strains did not show any significant differences from those of B. stearothermophilus strains

while sensitivity to selected bacteriophages indicated that the former were more sensitive to infection than the latter. Phenotypically the caldo-active bacteria appear to closely resemble other strains classifiable with B. stearothermophilus, a taxon which embraces a wide variety of different strains.

In the preceding review of the literature, attention has been drawn to the description of strict thermophiles giving rise to cylindrical spores within non swollen sporangia and spherical spores contained in distinctively swollen sporangia (Schenk and Aragno, 1979).

It seems appropiate to distinguish between strict and moderate thermophilic Bacillus species. Recent criteria such as determination of G + C base content and especially DNA homologies have hardly been applied to members of this group of thermophiles, which clearly deserves more detailed study (Wolf and Sharp, 1981).

The obligate thermophile, B. schlegelii (Schenk and Aragno, 1979) is the first chemolithoautotroph to be comprehensively described. It is strictly aerobic, oxidizes hydrogen in the presence of O_2 and CO_2, produces a distinctly swollen sporangium and spherical spores. Its optimum temperature is $70^{o}C$ and its % G+C content 67-68%.

Although moderate thermophiles are obviously adapted to growth at high temperatures, they are frequently found where high temperatures do not occur. Aerobic, sporeforming thermophiles have been isolated from almost every kind of environment, including desert sands, tropical and temperate soils, air, fresh snow, cold and warm waters, both salt and fresh and foods of all varieties (Norris et al., 1981; Farrel and Campbell, 1969; Harris and Fields, 1972; Heinen and Heinen, 1972). Several theories have been advanced to explain the occurrence of moderate thermophiles in low temperature environments. Some authors considered the habitat of the thermophiles to be the tropics, where they constitute an important part of the soil microorganisms. It cannot be assumed, however, that all thermophiles found in cold and temperature regions are of tropical origin. Other authors considered that the termophilic bacteria survived from early geological periods and that mesophiles resulted from adaptation as the earth's crust cooled. Some believe that thermophiles are variants of mesophilic bacteria and that progressive adaptation occurred right up to the obligate thermophilic stage (Norris et al., 1981).

2.1.2 **Physiology**

2.1.2.1 Morphological and biochemical characteristics

The aerobic, sporeforming bacilli that constitute the genus Bacillus were among the earliest of the bacteria to be described, and the genus has played a major role in the development of microbiology (Berkley et al.,1984). Most members of the genus are not difficult to isolate or to cultivate. The heat and drought resistance of the spores assist both isolation and maintenance of the cultures in the laboratory.

Colony morphology and other such cultural characteristics are of limited use for the classification and identification of Bacillus species (Berkeley et al., 1984), though to the experienced worker they may be valuable. For the experienced and inexperienced workers alike, observations of the morphology of vegetative cells and sporangia are more useful. Whatever the identification system employed, it must be established that any strain suspected of being a Bacillus species is indeed an aerobic endospore forming rod. This is most conveniently done by the examination of smears of live cultures, young and sporulated cells, by phase contrast microscopy.

The prime authority on cellular morphology and biochemical characteristics of Bacillus strains is Gordon, who published a simplified key to typical strains of many species in the genus (Gordon, 1981). As a result of an international inter-laboratory reproducibility trail a modified version of this key has been published (Norris et al., 1981). These methods and this key should enable the tentative identification of typical strains of common species. Provided careful attention is paid to details, especially of medium composition and test procedure, this identification method of Norris et al. (1981) should result in the proper identification of many unknown strains. But difficulty may still be encountered with what proved to be typical or intermediate strains.

In "Bergey's Manual of Systematic Bacteriology" (Sneath et al., 1986) Bacillus cells are described as rodshaped, straight or nearly so. They form endospores and are very resistant to many adverse conditions. Gram-positive, or positive only in the early stages of growth. Aerobic or facultatively anaerobic. Exhibit a wide diversity of physiological ability, psychrophilic to thermophilic, acidophilic to alkalophilic. Catalase formed by most species. Oxidase-positive or -negative. Chemoorganothrophs; one species a facultative chemolithotroph. The cell wall peptidoglycan of most

species belongs to the direct cross-linked meso-diaminopimelic acid type.

The cells of Bacillus may occur singly or in chains of considerable length. The rods may have rounded or squared ends and may be quite small (0,5 x 1,2 µm) or rather large (2,5 x 10 µm). The form of the endospore and the shape of the spore-bearing mother cell, the sporangium, is a characteristic feature of Bacillus species. Endospores are usually cylindrical, or ellipsoidal, or oval or round. The spore may be located in a central, subterminal, terminal or lateral position within the sporangium. Gordon (1981) arranged the strains she studied into three groups, based on the shape of the spore and swelling of the sporangium. Group 1 has ellipsoidal spores which do not cause the sporangium to swell, group 2 has ellipsoidal spores swelling the sporangium, and group 3, spherical spores with swollen sporangium.

Several Bacillus species produce carbohydrate capsules and most species are motile by means of peritrichous flagella.

Bacillus stearothermophilus is an obligate thermophile with optimum and maximum temperatures of growth of about 55°C-65°C. At the other extreme there are the acidophilic thermophilic strains which fall into the species B. coagulans. It is a facultative thermophile which grows

optimally at 55°C and at a pH of 5,0-6,5 with a maximum growth temperature of 60°C to 65°C. Rods are often in chains.

Gibson and Gordon, in the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) listed six species with strains capable of growth at 55°C: B. subtilis, B. licheniformis, B. stearothermophilus, B. coagulans, B. brevis and B. acidocaldarius. Most B. stearothermophilus and B. acidocaldarius strains will grow at 65-75°C but not below 40°C, and may described as obligate thermophiles. Members of the other species, which will grow at room temperature but not above 65°C, are described as facultative thermophiles (Norris et al., 1981).

Singleton and Amelunxen (1973) considered three distinct mechanisms to account for the activity of thermophilic microorganisms:

1. Thermophiles may contain factors which increase the thermal stability of enzymes.
2. Conversely, mesophiles also may contain factors which increase the thermostability of their enzymes.
3. Cellular components of thermophiles may possess inherent stability independent of exogenous factors.

Amelunxen and Murdock (1978) pointed out that thermal stability persists even after extensive purification and

recrystallization of certain enzymes. Also, since molecular weights of thermostable enzymes and their homologous enzymes from mesophiles are essentially identical, any stabilizing factors present would be of very low molecular weight.

The only thermostable enzyme reported to differ considerably from its mesophilic counterpart was an α - amylase from the strain of B. stearothermophilus. (Amelunxen and Murdock, 1978). Almost all in vitro studies, on the thermal stability of proteins, suggested some inherent stability of the protein structure which is dependent on the amino acid sequence and composition. Several proteins from thermophiles and mesophiles proved similar in molecular mass and in structure and differences were associated with a very small number of amino acids.

There is little evidence that the cell wall plays any significant role in thermal stability. Novitsky et al. (1974) compared the amino acid and amino sugar composition of the cell wall peptidoglycan of a facultative thermophilic strain of B. coagulans grown at 37^{o}C and 55^{o}C. At 55^{o}C, all of the wall components except alanine were present in a higher proportion and contained less peptide cross bridging.

2.1.2.2 Sporulation and germination

The primary function of all spores appears to be the survival of the species. The comparative biology and physiology of these resting forms have been reviewed by Sudo and Dworkin (1973) and by various investigators (Tipper and Gaunthier, 1972; Gerhardt et al., 1975; Gould and Dring, 1975; Gould, 1977; Moat, 1979; Szulmajster, 1979). Bacterial endospores are extremely resistant to heat and other environmental factors and may survive the process used in the preservation of foods. Outgrowth of spores into the vegetative phase may result in food spoilage or the production of toxins that may give rise to serious illness (Jay, 1978; Norris et al., 1981).

Endospores are resistant stages in the life cycle of several Gram-positive bacteria. They are formed inside the vegetative cell and enable the organism to survive without metabolism (cryptobiosis). They differ from the vegetative cells in many respects like optical refractility, ultrastructure, chemical composition and resistance to chemical and physical stress.

Endospore formation in bacteria involves a number of morphological, cytological and metabolic changes (Norris et al., 1981). These changes can be grouped into 7 distinct stages, as follows.

Stage 1: The axial filament stage. There is a marked reduction in the growth rate. Characteristic changes take place in the appearance and the distribution of the DNA of the cell. The axial filament does not present an irreversible stage in the sporulation process (Keynan, 1978).

Stage 2 and 3: Septum formation and forespore engulfment. The development of the forespore septum is the first definitive sign of a cell beginning with sporulation. A septum develops, usually at one end of the cell, and at least one nuclear equivalent of the chromatin material is enclosed within the segment. This is followed by the unbalanced growth of the membranes of the two resulting protoplasts such that the larger one completely surrounds and engulfs the smaller one (Fitz-James and Young, 1969). During the engulfment part of the membrane of the larger protoplast becomes inverted in such a way that the developing spore (forespore) contains two membranes, the outer one of which is back-to-front, and the whole forespore is still surrounded by the mother cell membrane (Gould, 1977). There is considerable evidence that the functional polarity of the outer forespore membrane is indeed reversed. For instance, peptidoglycan is laid down, in the cortex, between the two forespore membranes (Gould, 1977), and peptidoglycan is normally laid down on the outer surface of

the bacterial cell. It therefore seems that the "outer" surfaces of both forespore membranes are involved in synthesis of peptidoglycan (see Fig. 2.1).

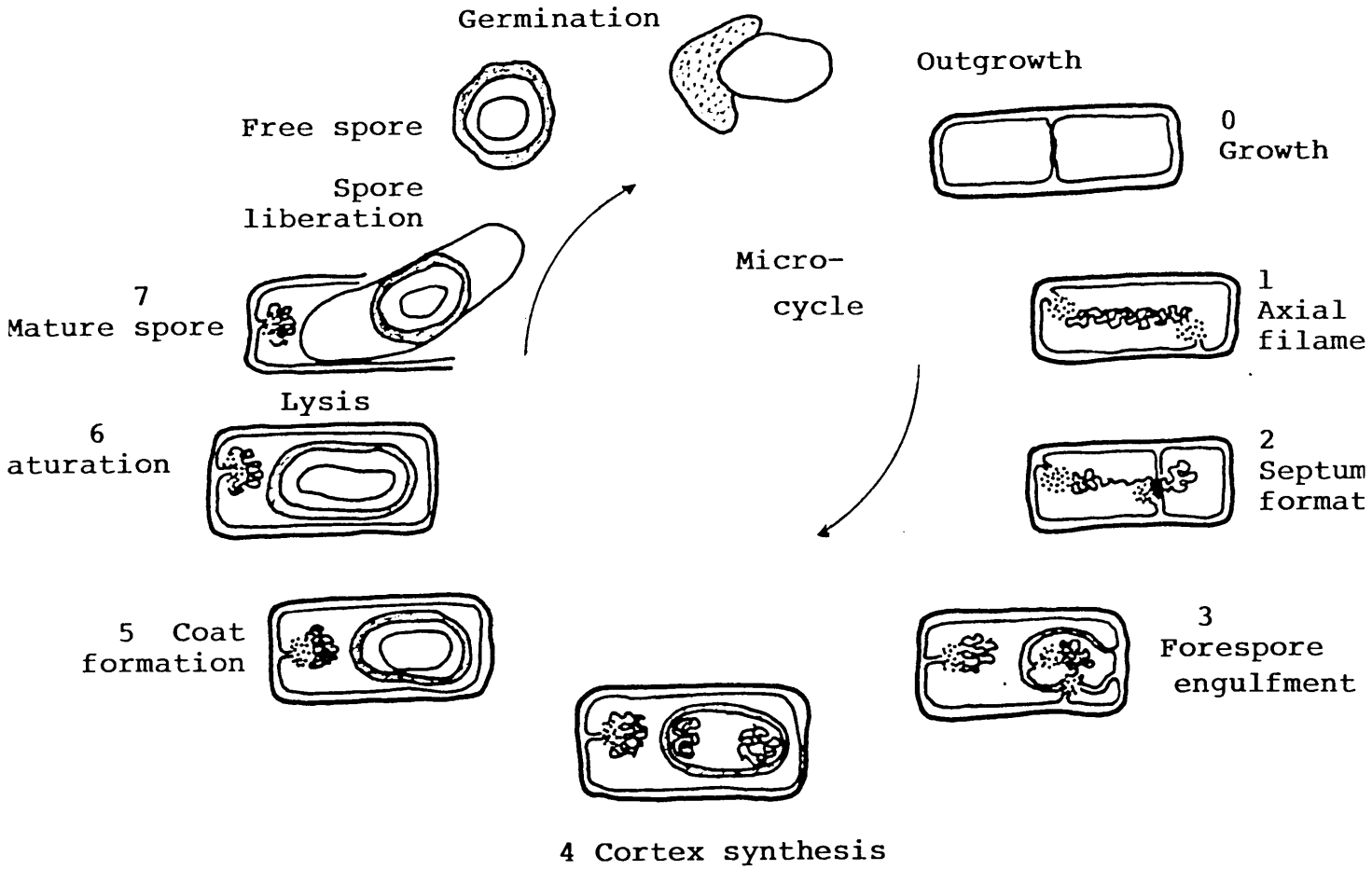

Fig. 2.1 Formation of the bacterial endospore, in particular regarding the disposition of membranes and the formation of the cortex.

Stage 4: Cortex formation. As mentioned before (stage 3), the cortical layers are formed between the inner and the outer membranes of the forespore. The same peptidoglycan precursors which are synthesized during vegetative growth for cell wall synthesis are utilized during the specific cortex peptidoglycans (Keynan, 1978).

Stage 5: Coat formation. Finally during spore formation the spore coat is laid down, consisting of protein that is relatively resistant to attack by enzymes and chemical reagents (Aronson and Fitz-James, 1976). The coat is laid down around the outer forespore, membrane, and therefore within the mother cell, although, when the spore is later released, the coat becomes the outer surface. Coats probably do not play a direct role in the maintenance of dormancy and heat resistance, but they do protect spores from some enzymes and chemical reagents (Keynan, 1978).

Stage 6: Maturation. Following the deposition of the cortical layers and development of the spore coat, other less well defined changes occur which represent a final stage of maturation of the spore, such as the development of refractility and heat resistance. This process is associated with the synthesis of dipicolinic acid and calcium uptake into the maturing spore (Pelczar *et al.*, 1977).

Stage 7: Lysis. Lysis of the sporangium and liberation of the mature spore. The diagrammatic representation of sporulation in Bacillus species can be seen in Figure 2.2.

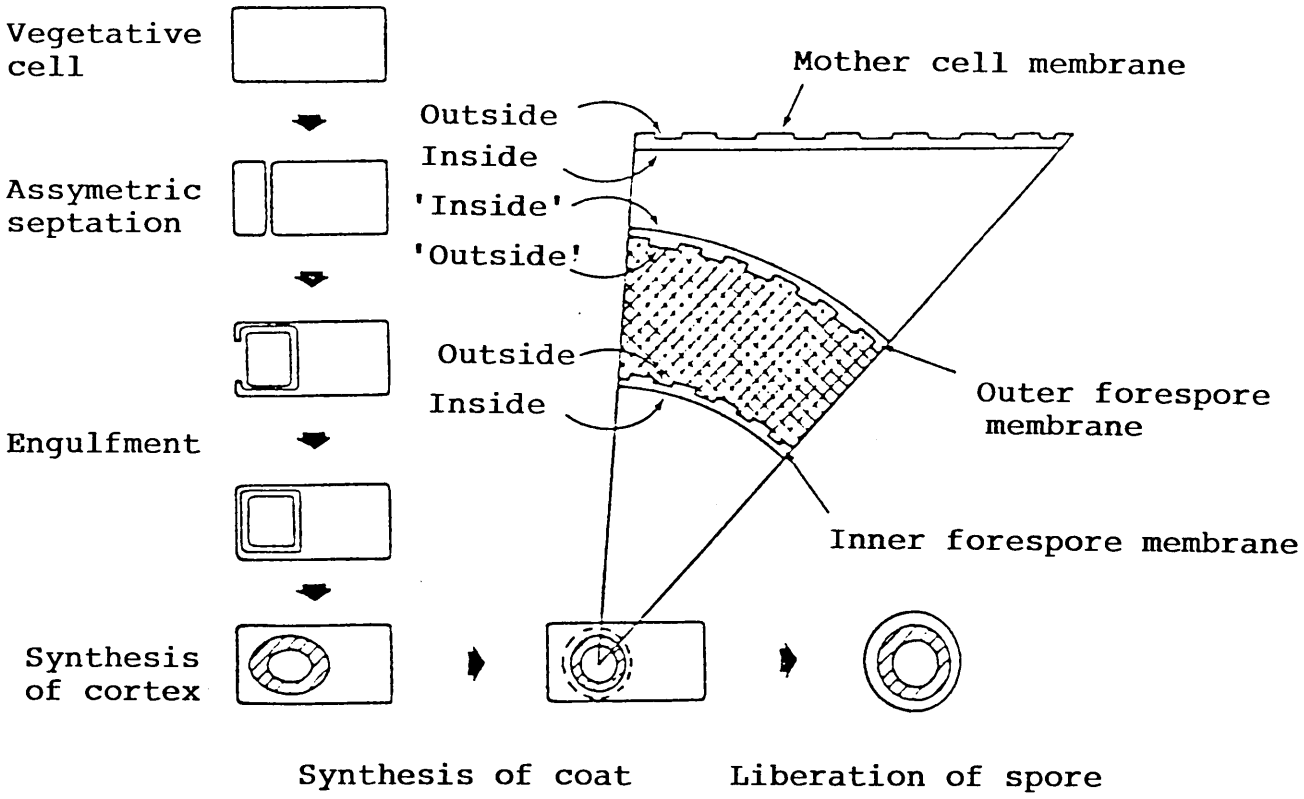

Fig. 2.2 Diagrammatic representation of the sporulation cycle in Bacillus (from Gould, 1977).

A large number of genetic loci have been implicated as playing a role in the sporulating process (Piggot and Coote, 1976). A considerable body of evidence has been accumulated which indicates that there are many structural and regulatory genes associated with sporulation. The genetics of the sporulation process has been studied extensively only in B. subtilis by Piggot and Coote (1976). For B. subtilis, more than 30 sporulation loci have been indentified as specifically affecting sporulation but not vegetative growth.

The transition from the dormant bacterial spore to the actively growing vegetative cell is conveniently divided into the two processes of germination and outgrowth. Germination, once initiated, is irreversible and is essentially a degradative process whereby the spore loses its characteristic properties. It is accompanied by rehydration and swelling of the spore protoplast and this, coupled with a loss of refractility and fragmentation of the spore peptidoglycan, promotes a drop in optical density of the spore suspension. Germination can be initiated by certain amino acids, ribosides and sugars (Keynan, 1978).

Outgrowth follows germination and involves the metabolic changes necessary to promote active cell division. A new germ cell emerges from the spore, elongates and divides.

2.1.2.3 Heat resistance

Bacterial spores properly gains its increased heat resistance in comparison with the vegetative cell through a partial dehydration of the spore protoplast (Marshall and Murrel, 1970). Various theories have been proposed for the physiological mechanism for heat resistance, including several mechanisms based on partial dehydration, hydrostatic pressure applied to the protoplast by the contractile cortex and an expansive cortex (Gerhardt and Murrel, 1978; Gould and Dring, 1975). Algie (1980) presented a theory for dehydration to be produced by reverse osmosis, with the pressure being applied by the cortex as it is growing centripetally.

2.2 **Procedures**

2.2.1 **Isolation of thermophilic Bacillus species**

Isolations were made from shelf stable frankfurter type sausages (NF/26-2), at the process plant of NORM FOODS at Rustenburg, four to five months after manufacture. Isolations were also made from the non-meat products used in the manufacture of these sausages. The a_w of the product was 0,93 and the pH 5,25.

The isolates were incubated at 45°C and maintained in tryptone soy agar or broth (TSB) (BIOLAB).

2.2.2 Determination of characteristics

The isolates were numbered and these microorganisms were subjected to morphological, physiological, biochemical and antibiotic tests. Bacillus coagulans (DSM 2312) and B. stearothermophilus (DSM 494) were used as control microorganisms during all the tests. Incubation was at 45°C unless otherwise stated.

i. **Morphology.** The morphology of the isolates was studied by Gram staining (Pelczar et al., 1977).

ii. **Gram reaction.** Huckler's modification of the Gram stain was used (Bartholomew, 1962). Bacteria were cultured on Nutrient Agar (Biolab) and stained after growth for 24 h (Gerhardt et al., 1981).

iii. **Catalase and benzidine reactions.** Cultures were incubated aerobically on Nutrient Agar for 48 h. The plates were flooded with equal volumes of benzidine base reagent and 5% (v/v) H_2O_2 (Deibel and Evans, 1959).

iv. **Anaerobic growth.** Cultures were grown for 2 d in anaerobic flasks with Anaerocult (Merck).

v. **Growth in 5% and 7% NaCl.** Cultures were inoculated into Nutrient broth tubes (MERCK) containing 5% and 7% NaCl respectively. The cultures were incubated for 48 to 72 h.

vi. **Acid and gas from carbohydrates.** Carbohydrate fermentations were done by adding 0,5% carbohydrate to a basal medium containing no carbohydrate with 0,004% chlorophenol red as the indicator. The production of CO_2 from mannitol, glucose, arabinose and xylose were done by including Durham tubes in the tubes. Cultures were incubated for 48 h (Gerhardt et al., 1981).

vii. **Reduction of nitrate to nitrite.** The reduction of nitrate to nitrite was done according to Gerhardt et al. (1981).

viii. **Hydrolysis of starch.** Cultures were streaked onto Nutrient Agar plates with 0,2% soluble starch and incubated for 48 h. The plates were then flooded with iodine solution (Gerhardt et al., 1981).

ix. **Hydrolysis of gelatin.** Nutrient Agar plates supplemented with 0,4% gelatin were inoculated and incubated at optimum temperature for 48 h. The plates were flooded with 15% $HgCl_2$ in 20% HCl (Gerhardt et al., 1981).

x. **Indole production.** Carbohydrate and nitrate free liquid medium containing a source of tryptophan (15 tryptone broth) were inoculated. Results were recorded after 24 h of incubation, using Kovac's indole reagent (Gerhardt et al., 1981).

xi. **Voges-Proskauer reaction.** Tubes of MRVP-broth were inoculated and after incubation of 2 d, 1 ml of culture was removed and 0,6 ml of 5% α-naphthol added. Then 0,2 ml of 40% aqueous KOH was added (Gerhardt et al., 1981).

xii. **Citrate utilization.** A single streak on a slant of Simmon's agar was made and incubated for 5 d. The entire surface of a slant of Christensen citrate agar was streaked out and incubated for 5 d.

xiii. **Lactate configuration.** The method of Bergmeyer as described by Gerber (1984) was used.

xiv. **Growth at 45°C, 55°C, 60°C and 65°C.** Visual growth in TSB-broth was recorded after 2 d.

xv. **API 50 CHB carbohydrate fermentation.** The API 50 CHB galleries, containing the following 49 different dehydrated carbohydrates, were used: Glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, galactose, glucose, fructose,

mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, ∝-methyl-mannoside, ∝-methyl-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, -gentobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate.

The microtubes were inoculated strictly according to the manufacturer's instructions. Carbohydrate fermentations was recorded after 24 h inoculation.

xvi. **Antibiotic sensitivity.** Cultures were grown in peptone water broth for 6 h. Plates were seeded with 0,5 ml culture and allowed to dry. Twenty different antibiotic discs were then placed on them and incubated for 24 h. The diameter of the zone of no growth was then measured.

2.3 Results and discussion

2.3.1 Isolation of thermophilic Bacillus species

Thermophilic bacteria could be isolated from all sausage samples. All isolates were facultative thermophiles that grew at 35°C to 55°C. With few exceptions, all grew well at 60°C, but none at 65°C.

A total of 16 isolates were made from different habitats. Of these only seven numbered (43, 44, 50, 57, 58, S1 and B8) showed the typical characteristics of thermophilic Bacillus species. Three (43, 57, 58) were isolated from typical frankfurter sausage (NF 26/2), kept on the roof of a building for shelf stability testing, two (50 and B8) from country sausage (NL 26/2), kept at 50°C for shelf stability testing, two (S1 and 44) from the spices used in the manufacture of NF 26/2 and NL 26/2 sausages.

2.3.2 Characteristics of thermophilic Bacillus species

The biochemical reactions of the Bacillus isolates are contained in Table 2.1. B. coagulans and B. licheniformis were the two thermophilic bacilli generally found in the products. There was no decrease in spore counts after a treatment at 95°C for 100 min. The results of carbohydrate fermentation, using the API 50 CHB, are given in Table 2.2 and showed that there were four dominating groups within the isolates.

All seven isolates were Gram-positive, endospore forming rods. Isolates 43, 44, 57, 58 and B8 were long thin rods with terminal spores, while isolates 50 and S1 were rods of medium length with central spores. Isolates 50 and S1 produced slimy colonies on agar plates incubated at 45°C.

According to the identification method of Norris *et al.* (1981), isolates 43, 44, 57 and 58 were identified as *Bacillus coagulans*, isolates 50 and S1 as *B. licheniformis*, while isolate B8 as *B. stearothermophilus*.

The production of large amounts of lactic acid as fermentation product is unusual in the genus *Bacillus*, although *B. coagulans* carries out a typical homo-lactic fermentation. It is interesting to note that all lactic acid produced was of the L(+) configuration. Thermophilic spore formers are among the principal causes of "flat-sour" spoilage, because of the high heat resistance of their spores. *B. coagulans* and *B. stearothermophilus* are the most common bacteria that produce acid without gas (Norris *et al.*, 1981). Spores of the other strains of *Bacillus* are not so heat resistant as those of the flat-sour organisms and do not, therefore, pose a significant spoilage problem (Norris *et al.*, 1981).

The thermophilic sporeformers were found in a variety of meat products and food ingredients. *B. coagulans* was the most common species isolated, followed, by *B. licheniformis* and *B. stearothermophilus*.

Reaction of isolates to different antibiotics are presented in Table 2.3 However characterisation of the isolates according to antibiotic sensitivity was not possible.

Table 2.1: Physiological and biochemical characteristics of thermophilic Bacillus-isolates

	Characteristics of isolates								
Tests	DSM 494	DSM 2312	43	44	50	S1	57	58	B8
Catalase	-	+	+	+	+	+	+	+	+
Benzidine	-	+	+	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+
Sabouraud agar	+	+	+	+	+	+	+	+	+
Growth in:									
5% NaCl	-	-	-	-	+	+	-	-	-
7% NaCl	-	-	-	-	+	+	-	-	-
Hydrolisis of:									
Starch	+	-	-	-	+	+	-	-	+
Gelatin	-	-	-	-	+	+	-	-	-
Citrate (Simmons)	-	-	-	-	+	+	-	-	-
(Christensen)	-	-	+	+	+	+	+	+	-
VP	+	-	+	+	+	+	+	+	+
Nitrate reduction	-	-	+	+	+	+	-	-	-
Growth at:									
45°C	+	+	+	+	+	+	+	+	+
55°C	+	+	+	+	+	+	+	+	+
60°C	+	+	+	+	-	-	+	+	+
65°C	-	-	-	-	-	-	-	-	-
Acid from:									
Glucose	+	+	+	+	+	+	+	+	+
Arabinose	-	-	+	+	+	+	+	+	-
Xylose	-	+	+	+	+	+	-	-	-
Mannitol	+	-	+	+	+	+	+	+	+
Gas from glucose	-	-	-	-	-	-	-	-	-
Lactic acid		L+	L+	L+			L+	L+	
Position of spore	T	T	T	T	C	C	T	T	T

T = terminal

C = central

+ = positive reaction

- = negative reaction

DSM 494 = Bacillus stearothermophilus

DSM 2312 = B. coagulans

Table 2.2: Fermentation of 49 different substrates by thermophilic Bacillus isolates using the API 50 CHB system

	Reaction of isolate								
Substrate	DSM 494	DSM 2312	43	44	50	S1	57	58	B
Glycerol	+	+	+	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-	-	-	-
D - Arabinose	-	-	-	-	-	-	-	-	-
L - Arabinose	-	-	+	+	+	+	+	+	-
Ribose	-	+	+	+	+	+	+	+	-
D - Xylose	-	+	+	+	+	-	-	-	-
L - Xylose	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-
β Methyl xyloside	-	-	-	-	-	-	-	-	-
Galactose	-	+	+	+	+	+	+	+	-
Glucose	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+
Rhamnose	-	+	-	-	-	-	+	+	-
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	+	-	-	+	+	-	-	+
Mannitol	+	-	+	+	+	+	+	+	+
Sorbitol	-	+	-	-	+	+	-	-	-
α-Methyl mannoside	-	-	-	+	-	-	+	+	-
α-Methyl glucoside	+	+	+	+	+	+	+	+	+
N Acetyl glucosamine	+	+	+	+	+	+	+	+	+
Amygdalin	-	-	+	+	+	+	+	+	-
Arbutin	-	-	+	+	+	+	+	+	-
Esculin	-	+	+	+	+	+	+	+	+
Salicin	-	-	+	+	+	+	+	+	-
Cellobiose	-	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+
Lactose	-	+	-	+	-	-	+	+	-
Melibiose	+	+	+	+	-	-	+	+	-
Saccharose	+	+	-	-	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	+
Inuline	-	-	-	-	-	-	-	-	-
Melezitose	+	-	-	-	-	-	-	-	-
Raffinose	+	-	-	-	-	-	-	-	-
Amidon	+	+	-	-	+	+	+	+	-
Glycogen	+	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	+	+	-
β-Gentiobiose	-	-	+	+	+	+	+	+	-
D - Turanose	+	-	-	-	+	+	-	-	-
D - Lyxose	-	-	-	-	-	-	-	-	-
D - Tagatose	-	-	-	-	+	+	-	-	-
D - Fucose	-	-	-	-	-	-	-	-	-
L - Fucose	-	-	-	-	-	-	-	-	-
D - Arabitol	-	+	+	+	-	-	+	+	-
L - Arabitol	-	-	-	-	-	-	-	-	-
Gluconate	-	-	+	+	+	+	-	-	-
2-Keto gluconate	-	-	-	-	-	-	-	-	-
5-Keto gluconate	-	-	+	+	-	-	-	-	-

+ = positive reaction

- = negative reaction

Table 2.3: Sensitivity of two authentic cultures and seven isolates of Bacillus to antibiotics

	Diameter of zone of no growth (mm) of isolate								
Antibiotic	DSM 494	DSM 2312	43	44	50	S1	57	58	B8
Oxytetracycline T30	55	49	50	30	23	25	28	31	28
Chlortetracycline CT25	51	43	46	24	11	17	29	30	29
Chloramphenicol C30	53	41	36	32	23	20	21	27	19
C50	51	51	40	32	22	25	25	28	17
Cephalexin CN10	40	50	31	35	19	23	35	47	16
Cephalothin KF30	59	53	43	47	--	--	38	41	21
Kanamycin K30	15	45	37	35	19	25	34	45	22
16	23	60	43	43	37	26	41	51	26
Steptomycin S10	--	29	27	27	15	--	31	38	15
S25	12	37	35	31	--	--	34	39	17
Penicilin G PG2	--	49	31	36	--	--	30	35	23
PG4	--	55	38	36	--	--	37	37	25
Ampicilin AP25	--	69	51	43	--	--	41	40	28
Fusidic acid FC10	--	57	27	23	--	--	23	23	18
Nalidix acid NA30	25	--	25	--	--	--	23	15	--
Neomycin NE30	11	28	53	31	21	--	31	34	18
Rifampicin RP5	58	37	29	25	19	20	24	25	23
Cloxalin CX5	--	39	23	29	24	--	24	23	26
Erythromycin E10	53	51	33	31	23	23	32	28	25
Sulphamethoxazole 25	49	04	--	--	--	--	--	--	--

-- = No inhibitory zones were observed

3. GROWTH AND SURVIVAL UNDER STRESS CONDITIONS

3.1 Literature review

3.1.1 Chemical preservatives

3.1.1.1 Sodium Nitrite

Preservation of meat with salt preceeded the international use of nitrate and nitrite by many centuries. The history and use of nitrate and nitrite in meat curing, indicated that cooked meats and fish were preserved in sesame oil in jars as early as 3 000 BC in Mesopotamia. It is believed that meat perservation was first practiced in the saline deserts of Asia. At 900 BC the perservation of meat with salt and smoke was well established, and this method was later adopted by the Romans. During the late Roman times it was noticed that red patches were formed on the surface of meats preserved with salt. The thought that the reddening effect noticed was due to nitrate impurities of the salt led to the deliberate addition of nitrate to the meat to achieve colour uniformity (Sofos et al., 1979).

The use of nitrate in meat products date back many years. Sodium nitrite have several functions during the production of cured meat products. The sodium nitrite aids the

production of colour, development of flavour, acts as anti-oxidants, has anti-microbial activities, improves texture of the product and has inhibitory action against Clostridium botulinum growth and toxin production (Lin and Sebranek, 1979).

Sodium nitrate ($NaNO_3$) and sodium nitrite ($NaNO_2$) as well as the potassium salts of these compounds are used in the curing formula for cured meats, since they stabilize red meat colours and also inhibit some spoilage and food poisoning microorganisms and contribute to product flavour development. In an acid environment, the nitrite ion ionizes to yield nitrous acid. The latter further decompose to yield nitric oxide (NO), which is the important product from the standpoint of colour fixation. Nitric oxide reacts with the myoglobin under reducing conditions to produce the desirable red pigment, nitroso - myoglobin (Jay, 1978).

In 1923 the Bureau of Animal Industry of the United States Department of Agriculture (USDA) gave permission for experimentation on the direct use of nitrite in meat products. A series of experiments were undertaken by Kerr et al. (1926) to determine the practicability, as well as the advantages and disadvantages, of the direct use of nitrite in meat curing. The aspect of human safety was given primary consideration. The extensive and pioneering work led to the following conclusions:

(a) Sodium or potassium nitrate could be successfully replaced by sodium nitrite in the curing of meat.

(b) A quantity of 156-625 μ g of sodium nitrite g^{-1} meat was sufficient for colour fixation, depending on the meat and the curing process employed.

(c) The levels of sodium nitrite necessary for meat curing were not higher than the nitrite levels found in meats cured with nitrites, and unconverted nitrate was avoided.

(d) The curing period could be shortened by the direct use of nitrite.

(e) The quality and wholesomeness of meats cured with sodium nitrite were not inferior compared with meat cured with nitrates.

As a result of these findings, the USDA authorized the use of sodium nitrite in meat curing under the condition that the finished product will not contain sodium nitrite in excess of 200 $\mu g.g^{-1}$ (Sofos et al., 1979).

In 1954 the hepatotoxic properties of nitrosoamines, and in 1956 the carcinogenic properties of nitrosoamines were discovered. Extensive research, led to the conclusion that sodium nitrite used at approved levels, reduced the risk of botulinal toxicity in cured meat products and nitrosoamines

were mostly found in crisp-fried bacon at parts per billion levels (Gray and Randall, 1979).

As previously stated, the effect of nitrate and subsequently nitrite on cured meat colour development was noticed many years ago. It has been shown that nitrite concentrations considerably lower than those used in practice, will provide the characteristic cured meat product, only about 25 $\mu g.g^{-1}$ is needed for development of the colour (McDougall et al., 1975).

The antimicrobial activity of nitrite is closely linked with pH, temperature, a_w and NaCl concentrations. The inhibition is stronger at lower pH, lower temperature and lower a_w. When nitrite is used together with NaCl in meat products the inhibition of nitrite becomes stronger (Bell and De Lacy, 1984).

The role of nitrate may be: (a) to enhance destruction of spores by heat, (b) to increase spore germination during thermal processing with subsequent destruction of the germinated spores by heat, (c) to prevent germination of outgrowth of the spores and (d) to react with some type of meat components to form a more inhibitory compound (Lechowich et al., 1978).

Bacterial spores are more sensitive to heat in the presence of curing ingredients. Roberts and Ingram (1966) examined the ability of aerobic (Bacillus) and anaerobic (Clostridium) spores to grow in media containing different concentrations of curing salts (NaCl, KNO_3, $NaNO_2$) after various degrees of heating. The data indicated that heating at realistic temperatures and in the presence of acceptable nitrite concentrations had no effect on subsequent development of the spores. The nitrite effect was found to be pH-dependent, increasing tenfold from pH 7,0 to 6,0.

In another study (Perigo et al., 1967; Perigo and Roberts, 1968) it was concluded that the heating of the medium with nitrite produced an unknown substance or agent ten times more inhibitory than nitrite alone. This agent is referred to as the Perigo Factor. The existence of this factor or effect has been confirmed by many other workers. The factor does not develop in all culture media, and heating to at least 100°C is necessary for its development, although some activity develops in meats when heated to as low as 70°C (Jay, 1978).

Roberts and Garcia (1973) screened a range of Bacillus species and feacal streptococci and two strains of Salmonella typhimurium for their resistance to the Perigo Inhibitor. At pH 6,0 it inhibited nine of the 14 strains of Bacillus species.

Of all the possible mechanisms presented through the years and the vast amount of work reported, no single mechanism seems to explain entirely the nitrite effect on the safety of cured meat products and to apply in culture media and all the types of meat products. It is not safe to conclude that what applies to culture media can be assumed as taking place in meat systems, or that all the cured meat products are the same.

Duncan and Foster (1968) reported that sodium nitrate had no apparent effect on germination and outgrowth on Bacillus spores at concentrations up to 2%.

Nitrite concentration effect on total bacterial growth in vacuum-packaged bacon has shown that 200 $\mu g.g^{-1}$ delayed bacterial growth for 4 to 5 weeks. In non vacuum-packed bacon, the nitrite level showed very little effect on bacterial growth (Sofos et al., 1979). The role of curing salts in preventing spoilage of pasteurized meat products by Bacillus species therefore remains uncertain.

3.1.1.2 Potassium sorbate

Sorbic acid was first discovered in 1859 in London by the German chemist A.W. Hofmann. It was formed by the reaction of rowan berry oil with strong alkali. The antimicrobial

properties of sorbic acid were first discovered in Germany in 1939, and its use as a food preservative increased gradually after being permitted in most countries (Sofos and Busta, 1981).

Sorbic acid is the trans-trans form of hexa-2,4 dienoic acid and has this structure:

$$CH_3 - CH = CH - CH = CH - COOH$$

The solubility of sorbic acid in water at room temperature is relatively low (0,16%). The major advantages of the alkali salt of sorbic acid is its good water solubility. Potassium sorbate, the salt most widely used in food applications, can be made into solutions of more than 50% in cold water without difficulty (Sofos et al., 1979).

Sorbic acid and its potassium salt are the most widely used forms of the compound and are collectively known as sorbates. Practical applications of sorbate include preservation of human foods, animal feed, pharmaceuticals, cosmetic products and packaging materials. The practical applications of sorbate as a food preservative include dairy products, bakery products, fruit and vegetable products and

other food products (Sofos and Busta, 1981).

Sorbic acid is more effective in acid foods than in neutral foods, i.e. sorbic acid works best below pH 6,0 and is generally ineffective at pH 7,0 (Jay, 1978).

The undissociated molecule is essential to antimicrobial growth by inhibiting cellular uptake of substrate molecules such as amino acids, phosphate and organic acids. It is primarily effective against moulds and yeasts although some activity is displayed against certain bacteria. The inhibition of moulds by sorbic acid has been reported due to the inhibition of dehydrogenase enzyme systems in moulds. The lactic acid bacteria are not affected by sorbates at pH of 4,5 and above. These compounds have been used as mould inhibitors on hard cheeses and cucumber fermentations where undesirable yeasts are suppressed without affecting the desirable lactic acid bacteria (Jay, 1978).

Several reports have suggested that sorbic acid exerts a selective inhibition against all types of catalase-positive microorganisms, and it could be used as a selective agent for catalase-negative lactic acid bacteria and clostridia. A concentration of 0,1% sorbic acid in liver infusion agar exerted a marked inhibitory effect on most catalase-positive microorganisms. It was concluded that the effectiveness of sorbic acid was dependent upon the concentration used, the

growth medium and the pH of the medium.

At the present time the only approved use of sorbate in meats, is that of dipping the casings of sausages in a 2,5% potassium sorbate solution to prevent mould growth on the surface. The reported lack of inhibition of clostridia in laboratory medium and the observations that its activity against bacteria was selective must have contributed as major reasons for the limited use of sorbate in meat products (Sofos and Busta, 1981).

Sorbic acid is utilized in the body in a way similar to other fatty acid. Only the first step of β-oxidation is omitted, α-, β-dehydrogenation, since sorbic acid already has a double bond. The half life of sorbic acid in the body is 40 to 110 min, depending on the dose. No sorbic acid residues were found in the muscular tissues of domestic animals that were given food containing sorbic acid. It was reported that sorbic acid was harmless to rats and dogs when incorporated into their diets to the extent of 5%. Its toxicity was lower than that of sodium benzoate which must be detoxified in the liver (Sofos and Busta, 1981).

Knowing that sorbate is an antimicrobial preservative that prevents or delays growth through a static or cidal effect either on the spore or the cell, it is of importance to understand the mechanism through which it exerts these

effects. An understanding of these mechanisms will enable us to improve its applications. It may be helpful in the search for new, safe and effective preservatives that the world needs to assure its continuous supply of safe, wholesome, nutritious and adequate food (Sofos et al., 1979).

Some work implicated that the inhibition of various enzyme systems and their reactions as the mechanism of microbial growth inhibition by sorbate. Sulphydryl-containing enzymes have been implicated in relation to sorbate inhibition. Fumarase was reported as the site of inhibition of oxidative metabolism of catalase-positive bacteria, yeasts and moulds in the presence of sorbate. As a mechanism of inhibition, it was suggested that sorbate reacts slowly with the cysteine through an addition reaction with the thiol group of cysteine and that this is the mechanism of inhibition of the sulphydryl enzymes. Another enzyme reported as being inhibited by sorbate is proteinase, while it was suggested that sorbate inhibited respiration through its competitive action with acetate on the site of acetyl-CoA formation. Sorbate would competitively combine with coenzyme A and acetate and would consequently inhibit the enzyme reaction relating coenzyme A (Sofos and Busta, 1981).

Freese et al. (1973) indicated that fatty acids also can act as growth inhibitors. The amount of fatty acid necessary to

produce inhibition of Gram-positive B. subtilis decreases with increasing chain length and unsaturated and saturated fatty acids are equally effective. It has been suggested that the difference in fatty acid senstitivities between Gram-positive and Gram-negative bacteria may result from the outer membrane of Gram-negative bacteria preventing fatty acids from reaching the inner fatty acid-sensitive cytoplasmic membrane.

The antimicrobial action of sorbate against six Bacillus species shows that at sorbate concentrations of 0,015 to 0,05% and pH 6,0, germination occurred and spore walls were lysed and vegetative cells emerged and elongated but failed to multiply (Sofos et al., 1979).

To summarize, sorbate was found to be an effective inhibitor of many microorganisms, including yeast, moulds and many bacteria. It is used in the preservation of a wide variety of products through the world. As with other food preservatives, it has advantages as well as limitations, but generally, when used with proper planning and under the correct conditions, the advantages outweigh the disadvantages. Its antimicrobial effect appears to be on spore germination as well as outgrowth, but the mechanisms of action are not well understood.

3.1.1.3 Nitrite and sorbate

Ivey et al. (1978), in an effort to reduce the initial nitrite levels in curing of bacon and still assure botulinum safety, tested low nitrite (40 $\mu g.g^{-1}$) and potassium sorbate (0,26%) concentrations in products inoculated with 1100 spores g^{-1} and incubated for 110 days. The results indicated that potassium sorbate reduced the number of toxin and swollen packages, and lengthened the time before toxicity was observed. The presence or absence of 40 μ g of nitrite g^{-1} had no significant effect on the sorbate inhibition.

The effects of nitrite and sorbate on the bacterial populations in frankfurters were examined by Hallerbach and Potter (1981) and Leistner et al. (1981). Sorbate-nitrite concentrations have been shown to greatly improve the botulinum safety of cured meat products, even more than nitrite and sorbate alone. One explanation of the effectiveness of such combinations, would be that nitrite and sorbate form a more potent inhibitor.

Sorbic acid can be considered as a unique food additive since it is a metabolizable fatty acid and its use would represent one food protecting another. Usage of nitrite-sorbate mixtures to protect cured meats against botulinum toxicity is an attractive alternative due to the following

factors (Sofos et al., 1979):

(a) With the lower nitrite levels the nitrosamine formation potential would be minimized.
(b) C. botulinum would be inhibited at least as well or even better compared with present formulations.
(c) The low nitrite level used would give the characteristic cured meat colour and flavour.
(d) The shelf life of the products would increase.
(e) Sorbate would not cause health problems, as being a metabolizable substance (generally recognized as safe).
(f) The current processing procedures would not have to be changed.

3.1.2 Water activity (a_w)

In the past few years studies on the a_w of aqueous solutions, in connection with the formulation of intermediate moisture foods (IMF) have been undertaken (Labuza et al., 1972; Leistner and Rodel, 1975; Labuza et al., 1976; Benmerqui et al., 1979; Chirife et al., 1980; Chirife and Ferro Fontan, 1980; Alzamore et al., 1981; Leistner et al., 1981; Broughall et al., 1983; Chirife and Resnik, 1984).

Water activity is an important property in the manufacture of food systems and formulations. Most chemical reactions

and microbiological activity are controlled directly by the water activity of the food system.

According to Pirt (1975) water in the living cells has four basic functions: (i) As a chemical reactant in the cell, it enters into hydrolysis and condensations. (ii) It acts as a solvent for the cell pool of metabolites such as amino acids and their concentrations could be critical in metabolic regulation. (iii) It has a mechanical role in maintaining the cell's turgidity due to the hydrostatic pressure which develops in the cell. (iv) It has a structural function in hydration of protein and other cell components. Microorganisms grow in any substrate, liquid or solid, which contains a certain amount of water in a form available to them. This water is more or less available to the microorganisms by virtue of the effect of various solutes which are dissolved in it and a measure of this availability is the water activity. The water activity of the solution is expressed as the ratio of the water vapour pressure of the solution or food (P) to that of pure water (P_w) at the same temperature:

$$a_w = P/P_w$$

It follows that a_w is equivalent to the relative humidity of the atmosphere in equilibrium with the solution (Pirt, 1975; Jay, 1978):

$$R.H. = 100 \times a_w$$

The relation between water activity and osmotic pressure derived from thermodynamics is:

$$= \frac{-RT}{V} \times \ln a_w$$

V = the volume of 1 mole water.

Bacteria and moulds associated with meat and meat products frequently cause spoilage and sometimes food-poisoning or alternatively may be essential for the fermentation of certain meats. Microbial spoilage and food-poisoning take place if the a_w of the substrate is favourable for the multiplication of the organisms involved. The growth of microorganisms as a function of a_w has been an area of interest among microbiologists. Leistner and Rodel (1975) summarized the work on the basis that there is a limiting a_w below which certain organisms will not grow.

The a_w influences exponential and stationary growth phases, as well as the death rate of a culture (Troller and Christian, 1978). Most organisms associated with foods grow best at a relatively high a_w, with only a few requiring a low a_w for growth. If the a_w decreases, then fewer genera of microorganisms are able to multiply on or in a food.

The minimum requirements of microorganisms may vary somewhat depending upon the solute used to adjust the a_w of the

substrate (Jakobsen et al., 1972; Jakobsen and Murrell, 1977). Most data listed (Leistner and Rodel, 1975) were obtained by adjusting the a_w with NaCl and therefore they apply to meat products. If, instead of NaCl as a humectant, sucrose is used, then the differences in the limiting a_w values for growth are generally small. However, in the presence of glycerol some organisms, such as Clostridium botulinum (Baird-Parker and Fraeme, 1967) grow, sporulate and germinate at a lower a_w than if NaCl and sucrose are the humectants. This has to be born in mind if glycerol is used as humectant in the preparation of intermediate moisture foods.

Certain relationhips have been shown to exist between a_w, temperature an nutrition. First, at any temperature, the ability of microorganisms to grow is reduced as the a_w is lowered. Secondly, the range of a_w over which growth occurs is greatest at the optimum temperature for growth, and thirdly, the presence of nutrients increases the range of a_w over which the organisms can survive (Jay, 1978).

The effect of a_w on spore germination has received comparatively little attention (Baird-Parker and Fraeme, 1967; Jakobsen et al., 1972; Jakobsen and Murrell, 1977; Anagnostopoulos and Sidhu, 1981) and practically no information has been made available on the spore germination of thermophilic bacteria.

Generally the sporulation, germination and vegetative cell growth of Bacillaceae are inhibited at a_w <0,95. The limiting a_w for the formation of spores of B. cereus proved to be 0,95 for NaCl, glucose and sorbitol, whereas it was about 0,91 for glycerol (Jakobsen and Murrel, 1977). Apparently somewhat higher a_w is required for sporulation than for growth of Bacillaceae. On the other hand, there is general agreement that spores of Bacillaceae may initiate germination at a_w levels appreciably lower than those which will permit vegetative cell growth. Germination of Bacillus does occur at lower a_w levels with glycerol than with NaCl. Of concern for shelf stable products (SSP) is the vegetative cell growth of Bacillaceae, that surviving spores might develop into cultures that might cause food spoilage. The growth of Bacillus sp. is generally inhibited at a_w <0,95. Only few exceptions to this rule are known, and occurred when high incubation temperatures were used. Certain foods adjusted to a lower a_w with glycerol need special precautions since the limiting a_w values for growth of bacilli are in the range a_w = 0,93 - 0,91 (Leistner and Rodel, 1975).

The inhibition of microorganisms, also in intermediate moisture foods, does not solely depend on the a_w, but also on the pH, Eh, heat (F value), chilling (t value), preservatives and competitive microorganisms. Traditional

and new IMF should be protected against spoilage organisms by a combination of these hurdles that ensure the necessary microbial stability of the products (Leistner et al., 1981).

3.1.3 pH

Resistance of bacterial spores to moist heat is influenced by various factors including pH and water activity. Bartsch and Walker (1982) showed that when temperature and pH deviated from those favouring optimal growth the a_w tolerances also shifted to higher levels, and the lag phase became extended.

It is well known that meat from fatigued animals spoils faster than that from rested animals, and that this is a direct consequence of final pH attained upon completion of rigor mortis (Jay, 1979).

3.2 Procedures

The following Bacillus species were used as control organisms:

Bacillus stearothermophilus (DSM 494)

Bacillus coagulans (DSM 2312)

All the strains isolated (2.3.1) were used in the experiments. For the preparation of spores, 250 ml Tryptone

soy broth (TSB) with 420 μ g.1^{-1} $MnSO_4.4H_20$ was inoculated with vegetative cells and incubated at 45°C for 24 h. This was then centrifuged at 8 000 rpm for 15 min in a Sorvall RC-5B, the supernatant discarded and the pellet resuspended in distilled water. The spore suspension was centrifuged for a second time at 8 000 rpm for 15 min. The washing procedure was repeated 3 times. Concentrations were approximately 10^7 mature phase bright endospores ml^{-1} of sterile distilled water. Suspensions were checked by phase-contrast microscopy for absence of vegetative cells, and were stored at 4°C.

3.2.1 The influence of sodium nitrite on germination and growth

Incubation temperature was 45°C for 24 h throughout. TSB was used as growth medium and was supplemented with sodium nitrite at concentrations ranging from 100 mg.1^{-1} to 2000 mg.1^{-1} (pH 6,0) for detecting sodium nitrite resistance. After moist heat sterilization of all media at 121°C for 15 min, the media were inoculated with 0,1 ml of the stock spore suspension of each strain. All the tubes inoculated with spore suspension were heat shocked for 10 min at 80°C.

Outgrowth of endospores resulting in vegetative growth after 24 h was determined in terms of absorbance (λ = 540 nm) with a Bausch & Lomb Spectronic 20 spectrophotometer.

Results were calculated in terms of percentage outgrowth, relative to maximal outgrowth in control tubes without any addition of sodium nitrite. All experiments were done in duplicate and repeated once in time.

3.2.2 The influence of potassium sorbate on germination on growth

The incubation temperature and time were 45°C and 24 h throughout. TSB was used as growth medium and was supplemented with potassium sorbate at concentrations ranging from 500 $mg.l^{-1}$ to 5000 $mg.l^{-1}$ (pH 6,0) for detecting potassium sorbate resistance. After moist heat sterilization of all media at 121°C, the media were inoculated with 0,1 ml of the stock spore suspension of each strain. All the tubes inoculated with spore suspension were heat shocked for 10 min at 80°C.

Outgrowth of endospores resulting in vegetative growth was determined in terms of absorbance (λ = 540 nm) with a Bausch & Lomb Spectronic 20 spectrophotometer. Results were calculated in terms of percentage outgrowth, relative to maximal outgrowth in control tubes without any addition of potassium sorbate. All experiments were done in duplicate and repeated once in time.

3.2.3 Growth of thermophilic Bacillus species at reduced water activity

TSB was used as growth medium and was supplemented with different concentrations NaCl and glycerol resulting in different a_w-values (Jakobsen et al., 1972; Winer, 1984). The a_w-values of all solutions were checked with a Nova-Sina a_w meter after moist sterilization at 121°C for 15 min. The Nova-Sina was calibrated in advance using saturated salt solutions as reference values. Two series of the media with different a_w-values were inoculated with either 0,1 ml of a growing culture or 0,1 ml of the stock sporesuspension of each strain. All tubes inoculated with a sporesuspension were heat shocked for 10 min at 80°C.

Vegetative growth and outgrowth of endospores resulting in vegetative growth and were determined in terms of absorbance (λ = 540 nm) with a Bausch & Lomb Spectronic 20 spectrophotometer. Results were calculated in terms of percentage growth/outgrowth, relative to maximal growth/outgrowth in control tubes without adjusting the a_w. All experiments were done in duplicate and repeated once in time.

3.2.4 The effect of pH

Incubation temperature was 45°C throughout and the incubation time was 48 h. TSB was used as growth medium and the pH adjusted ranging from 4,5 to 7,0. After moist heat sterilization of the media at 121°C, the media were inoculated with 0,1 ml of an active growing culture of each strain.

The growth was determined in terms of absorbance (λ = 540 nm) with a Bausch & Lomb Spectronic 20 spectrophotometer. Results were calculated in terms of percentage growth, relative to maximal growth in control tubes at pH 7,0. All experiments were done in replicate and repeated once in time.

3.3 Results and discussion

3.3.1 Influence of sodium nitrite on germination and growth

The results obtained are given in Table 3.1 and graphically presented in Fig. 3.1 and 3.2. All the Bacillus coagulans isolates are grouped together with the authentic strain DSM 2312 and all B. stearothermophils and B. licheniformis with authentic strain DSM 494. The thermophilic Bacillus isolates showed higher resistance to sodium nitrite than the authentic strains (DSM 494 and 2312).

Isolate B8 was totally inhibited at a concentration of 600 $mg.1^{-1}$, but none of the other isolates was totally inhibited at 2000 $mg.1^{-1}$, where outgrowth of endospores was still detected. Above a concentration of 100 $mg.1^{-1}$ there was almost no decrease in the percentage outgrowth of the isolates, and at this concentration the percentage outgrowth ranged between 40% (DSM 494) Fig. 3.2 and 74% (57) Fig. 3.1.

The germination and outgrowth of isolates 43 and 44 were apparently more inhibited by a sodium nitrite concentration of 400 to 600 $mg.1^{-1}$ (Fig. 3.1), but at higher concentrations, it was inhibited in the same way as the other isolates. This phenomenon was observed in replicates as well as repetitions. It may be that at these low

concentrations some germination might have occurred, but that no outgrowth followed. However, this phenomenon was not controlled by microscopic observation and therefore constitutes a weak point in this study. It appeared that isolates 57 and 58 were the most resistant to sodium nitrite (Fig. 3.1).

Table 3.1: Percentage germination and outgrowth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at various $NaNO_2$ concentrations, expressed in terms of outgrowth at 0 $mg.l^{-1}$ $NaNO_2$

$NaNO_2$ ($mg.l^{-1}$)	% Germination and outgrowth of isolate								
	43	44	57	58	50	S1	B8	DSM 494	DSM 2312
0	100	100	100	100	100	100	100	100	100
100	53	46	89	80	74	77	69	48	66
200	40	54	89	79	66	75	25	42	40
400	40	75	88	79	61	74	21	42	66
600	66	66	84	79	60	69	18	40	63
1000	56	54	74	64	44	63	24	40	58
1500	54	50	65	65	49	63	24	35	48
2000	54	50	65	60	40	63	25	35	40

3.3.2 Influence of potassium sorbate on germination and growth

The results obtained are contained in Table 3.2 and graphically presented in Fig. 3.3 and 3.4. All B. coagulans

isolates were grouped together with authentic strain DSM 2312 in Fig. 3.3, and all B. licheniformis and B. stearothermophilus isolates together with authentic strain DSM 494 in Fig. 3.4. DSM 494 and isolate B8 were totally inhibited at a concentration of 2 000 mg.1-1, while isolate 50 was totally inhibited at a concentration of 5 000 mg.1-1. At a concentration of 5 000 mg.1-1 the percentage outgrowth of isolates 43, 44, S1 and DSM 2312 was 20%, while that of isolate 57 was 56% and isolate 58, 70%. Most of the isolates showed a higher resistance to potassium sorbate than DSM 494.

Table 3.2: Percentage germination and outgrowth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at various potassium sorbate concentrations, expressed in terms of outgrowth at 0 $mg.1^{-1}$ potassium sorbate.

Sorbate $mg.1^{-1}$	% Germination and outgrowth of isolate							DSM 494	DSM 2312
	43	44	57	58	50	S1	B8		
0	100	100	100	100	100	100	100	100	100
500	98	87	90	99	92	80	25	63	100
1000	98	71	90	99	70	64	25	46	94
1500	60	49	79	94	68	58	13	45	56
2000	60	55	73	84	56	48	0	2	42
3000	54	46	66	80	40	46	0	0	35
4000	49	45	62	73	20	44	0	0	29
5000	25	16	56	70	0	20	0	0	22

3.3.3 Growth of thermophilic Bacillus isolates at reduced water activity

Growth of vegetative cells and germination of spores of thermophilic bacilli were strongly inhibited by reduced water activities, irrespective of the humectant used.

NaCl as humectant:

Growth and germination of spores of DSM 494 and 2312 were totally inhibited at a_w = 0,97 (Fig. 3.5 and 3.6). Vegetative growth as well as germination and growth of isolates 44 and 43 were strongly inhibited at a_w = 0.947. Vegetative growth of cells of isolates B8, 57 and 58 were strongly inhibited at a_w = 0,95, but total inhibition took place at an a_w lower than 0,93 (Fig. 3.5). Isolates S1 and 50 proved to be resistant to low a_w values and at an a_w = 0,93, 82% growth was obtained.

There was very little difference between the effects of low a_w values on vegetative growth and germination of spores (Fig. 3.5 and 3.6). It therefore seems that the toxic effect of NaCl was the main cause of inhibition and not the reduced water activity.

Table 3.3: Percentage growth of vegetative cells of stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with NaCl used as humectant, expressed in terms of growth at a_w 0,99

	% Growth of isolate								
a_w	43	44	57	58	50	S1	B8	DSM 494	DSM 2312
0,99	100	100	100	100	100	100	100	100	100
0,97	98	100	27	28	119	123	81	0	0
0,95	10	8	11	14	99	103	27	0	0
0,93	0	0	11	11	84	91	13	0	0

Table 3.4: Percentage germination and outgrowth of spores of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with NaCl used as humectant, expressed in terms of outgrowth at a_w 0,99

	% Germination and outgrowth of isolate								
a_w	43	44	% 57	58	50	S1	B8	DSM 494	DSM 2312
0,99	100	100	100	100	100	100	100	100	100
0,97	86	89	21	22	108	104	80	0	0
0,95	4	2	7	2	84	96	24	0	0
0,93	0	0	0	0	76	84	7	0	0

Glycerol as humectant:

Results of glycerol as humectant on growth of vegetative cells of thermophilic endospore forming isolates are contained in Table 3.5, and graphically represented in Fig. 3.7.

The growth of the thermophilic bacilli showed that growth occurred at lower a_w levels than when NaCl was used as humectant (Fig. 3.7). Total inhibition of growth of DSM 494 took place at a_w = 0,953 while total inhibition of germination and growth at a_w = 0,957 (Fig. 3.8). With DSM 2312 total inhibition of growth took place at a_w= 0,925 and germination and outgrowth at a_w = 0,934. This was much lower, than when NaCl was used as humectant.

Growth of isolates S1 and 50 at a_w = 0,93 was respectively 72% and 68%, whereas germination and growth at the same a_w were 46% and 64% respectively. Inhibition of growth, and germination and growth of isolate B8 coincided (40% at a_w = 0,93). The growth of isolates 43, 57 and 58 was strongly inhibited at a_w = 0,93, but total inhibition of isolate 44 took place at a_w = 0,945 (Fig. 3.7). The percentage inhibition of germination and growth of isolates 57 and 58 at a_w = 0,93 was 20%, while total inhibition of isolates 43 and 44 took place at a_w = 0,929 (Fig. 3.8). This was contrary to all other observations.

From these results it is clear that the effect of reduced a_w was much greater on DSM 494 and DSM 2312 than on the thermophilic isolates.

Comparison of the results of NaCl and glycerol as humectant, clearly shows that these thermophilic Bacillus isolates can germinate and grow at an a_w <0,94. As mentioned earlier, NaCl was more effective than glycerol possibly due to the toxicity of NaCl.

Table 3.5: Percentage growth of vegetative cells of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with glycerol used as humectant, expressed in terms of growth at a_w 0,99

	% Growth of isolate								
a_w	43	44	57	58	50	S1	B8	DSM 494	DSM 2312
0,99	100	100	100	100	100	100	100	100	100
0,97	69	91	83	79	133	127	76	46	84
0,95	53	14	71	67	97	80	64	0	49
0,93	17	3	16	13	69	72	43	0	7

Table 3.6: Percentage germination and outgrowth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with glycerol used as humectant, expressed in terms of outgrowth at a_w 0,99

	% Germination and outgrowth of isolate								
		W						DSM	DSM
a_w	43	44	57	58	50	S1	B8	494	2312
0,99	100	100	100	100	100	100	100	100	100
0,97	88	95	71	72	108	98	70	37	83
0,95	70	60	57	52	85	77	56	0	53
0,93	6	7	20	12	65	46	40	0	0

3.3.4 **The effect of pH**

The results obtained are presented in Fig. 3.9 and 3.10. The thermophilic Bacillus isolates showed optimum pH at 6,00, with the exception of S1, which showed an optimum of pH 5,5. At pH 4,5 only isolates 43, 44, 58 and DSM 2312 grew reasonably well with the percentage growth of 56%, 60%, 36% and 22% respectively. No growth was measured with the other isolates at the same pH. The optimum of 43 could not be determined, but lay at or above pH7.

Table 3.7: Growth of thermophilic Bacillus isolates at various pH values, expressed as percentage growth at pH 7,0

	% Growth of isolate								
pH	43	44	57	58	50	S1	B8	DSM 494	DSM 2312
7,0	100	100	100	100	100	100	100	100	100
6,5	65	132	104	120	132	111	125	109	125
5,5	65	129	100	104	105	120	115	109	116
5,0	61	114	82	81	63	89	47	56	110
4,5	57	57	2	35	0	0	0	0	21

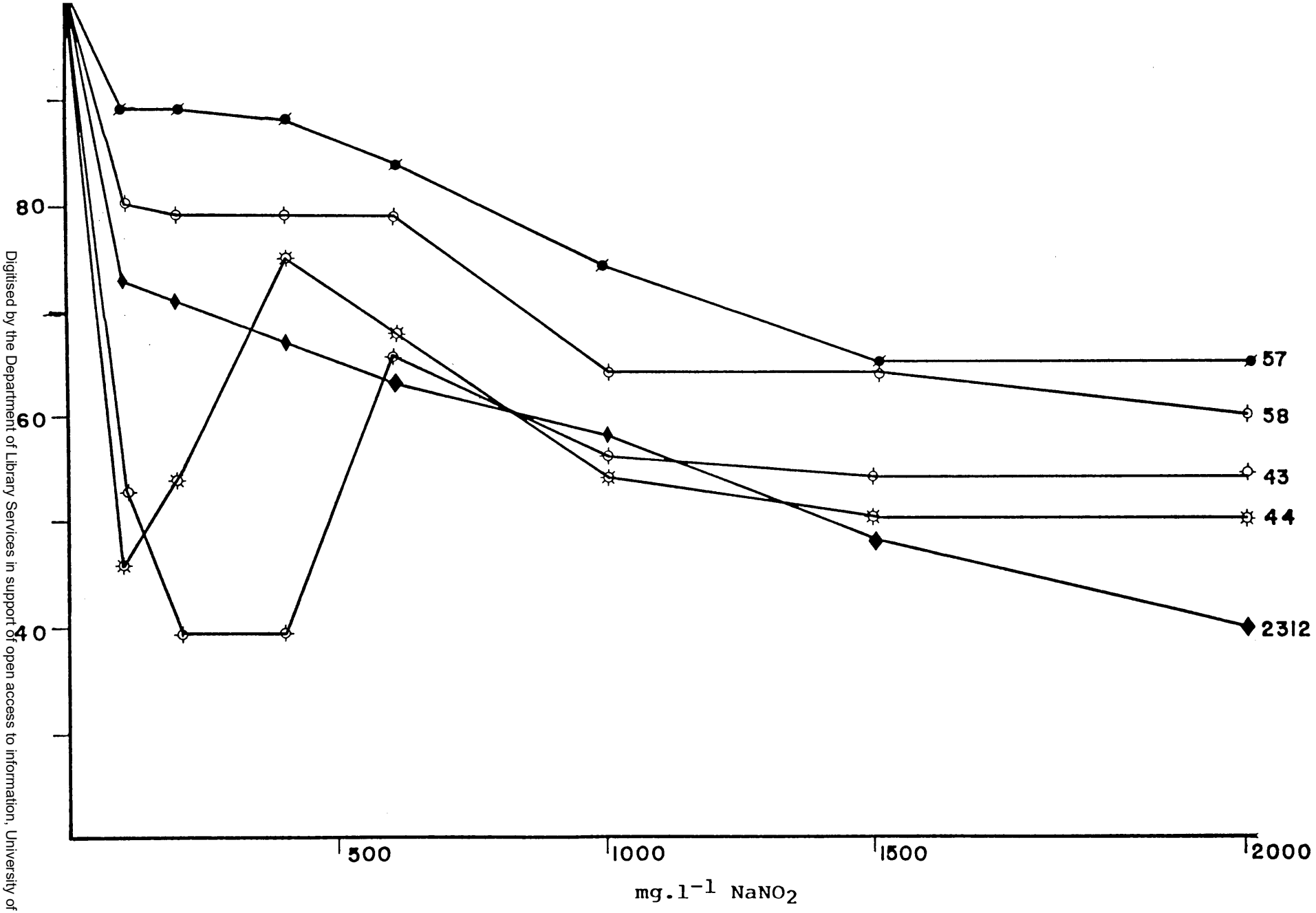

Fig. 3.1 The influence of different $NaNO_2$ concentrations on germination and growth of Bacillus coagulans DSM 2312 and thermophilic Bacillus isolates

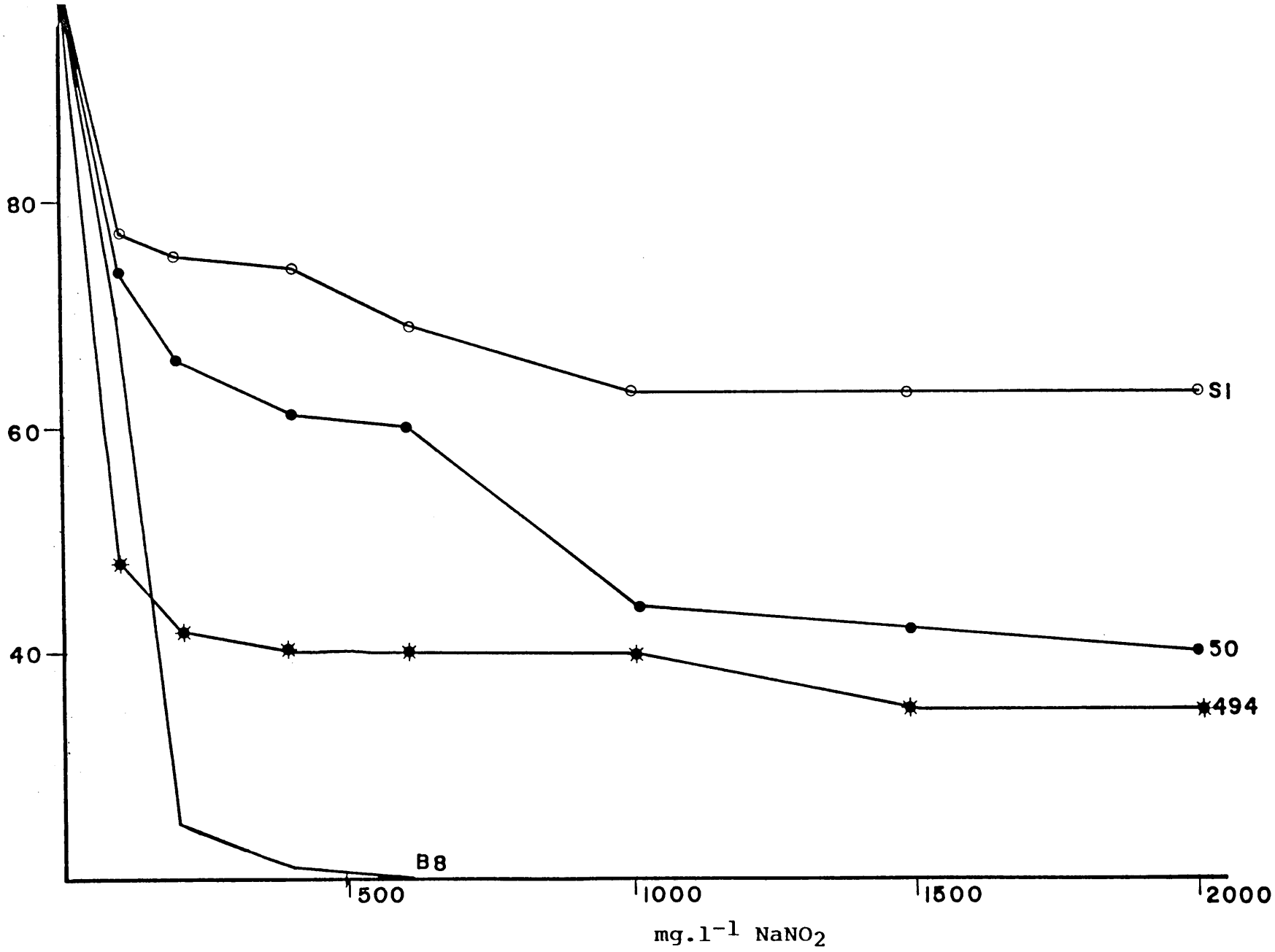

Fig. 3.2 The influence of different $NaNO_2$ concentrations on germination and growth of Bacillus stearothermophilus DSM 494 and thermophilic

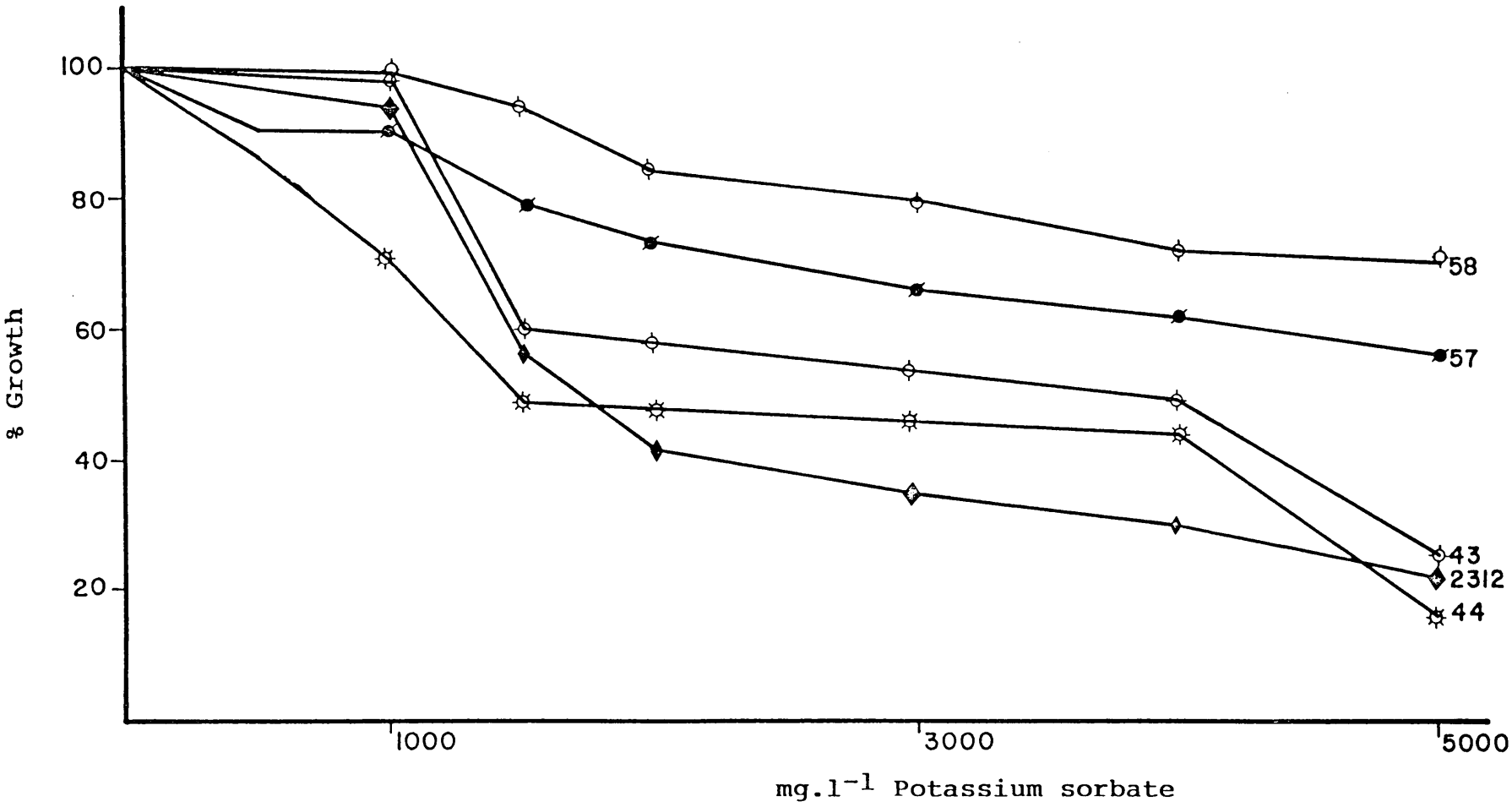

Fig. 3.3 The influence of different potassium sorbate concentrations on germination and growth of Bacillus coagulans DSM 2312 and thermophilic Bacillus isolates

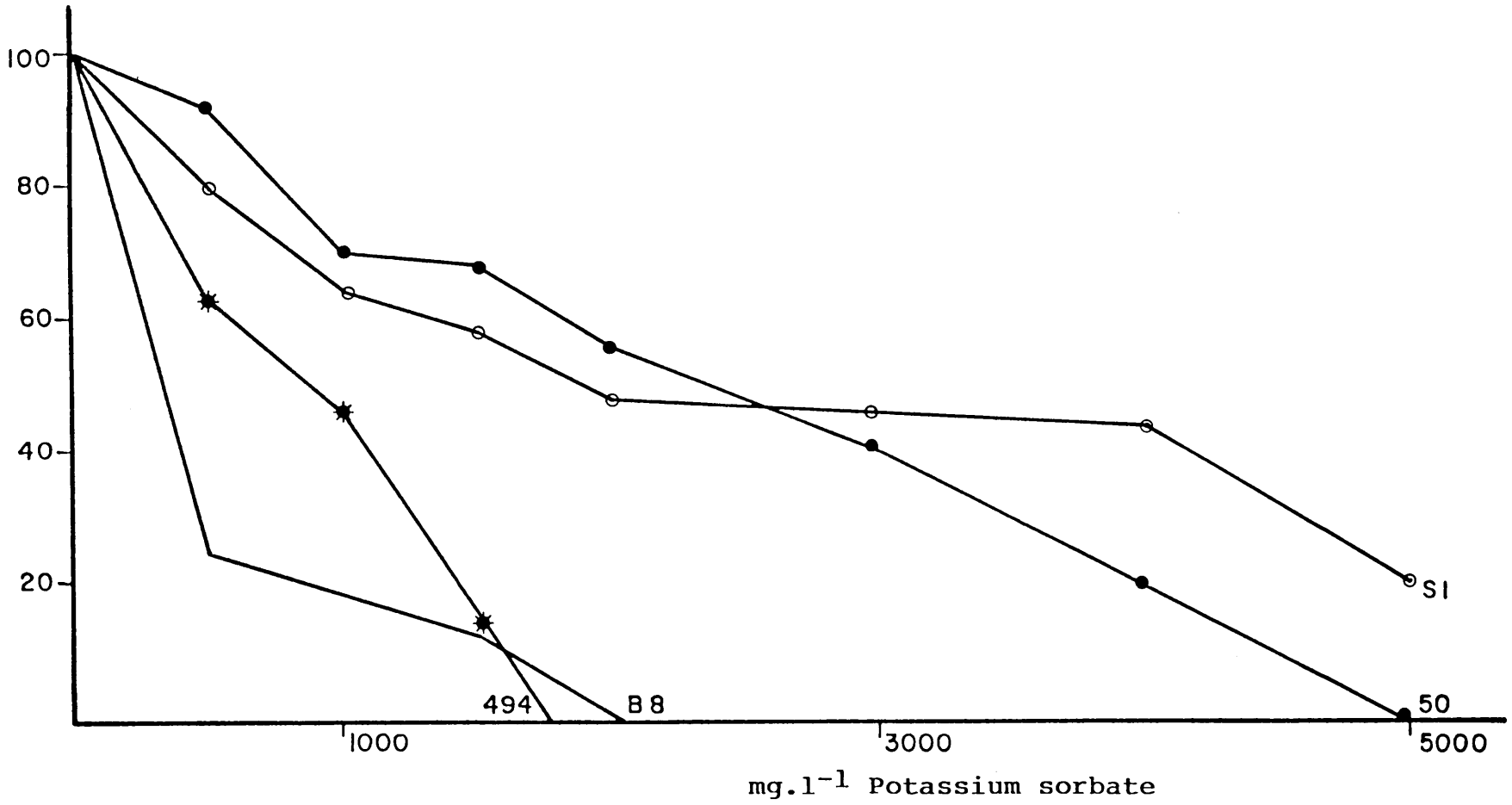

Fig. 3.4 The influence of different potassium sorbate concentrations on germination and growth of Bacillus stearothermophilus DSM 494 and thermophilic Bacillus isolates

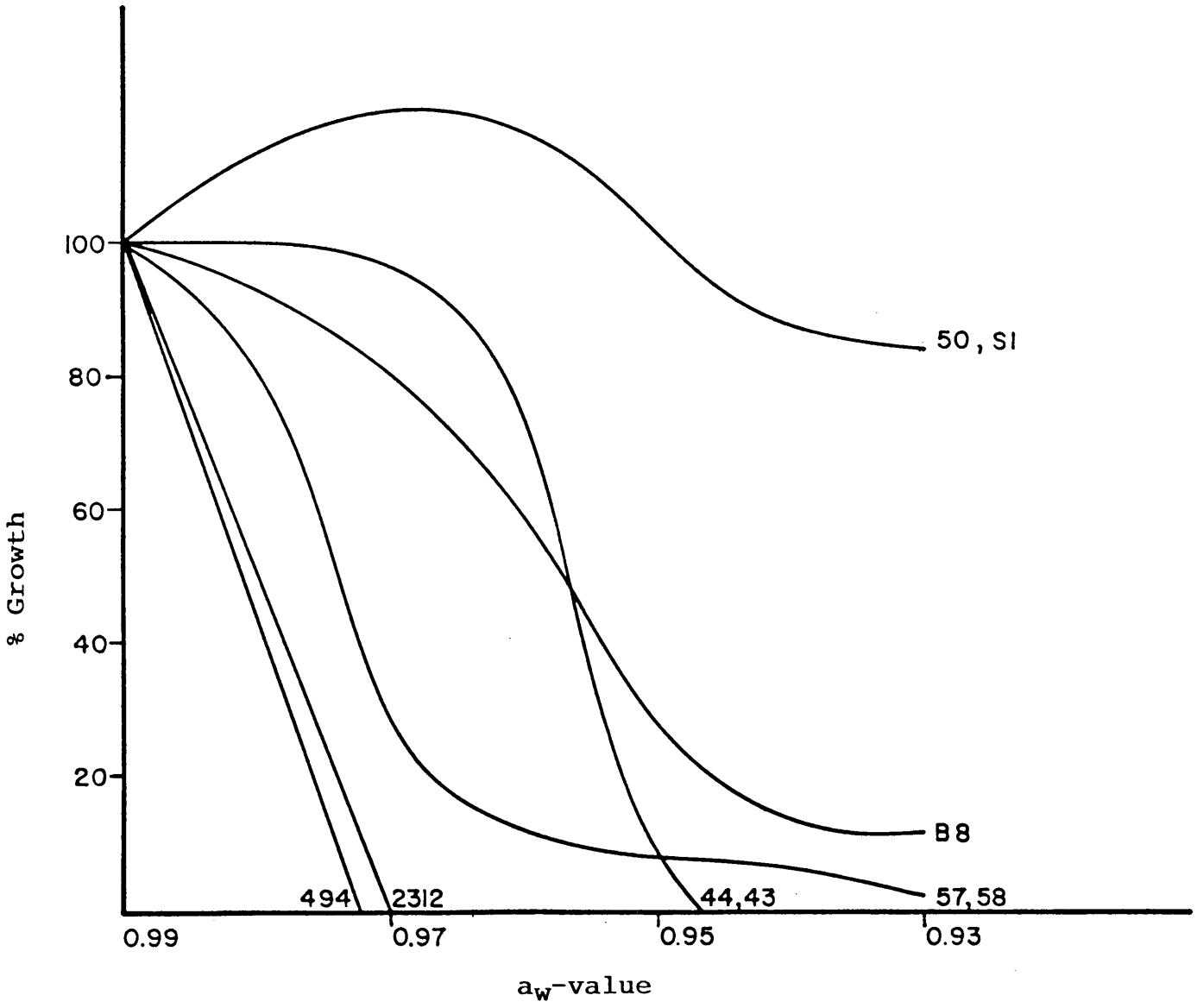

Fig. 3.5 Growth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w, with NaCl used as humectant.

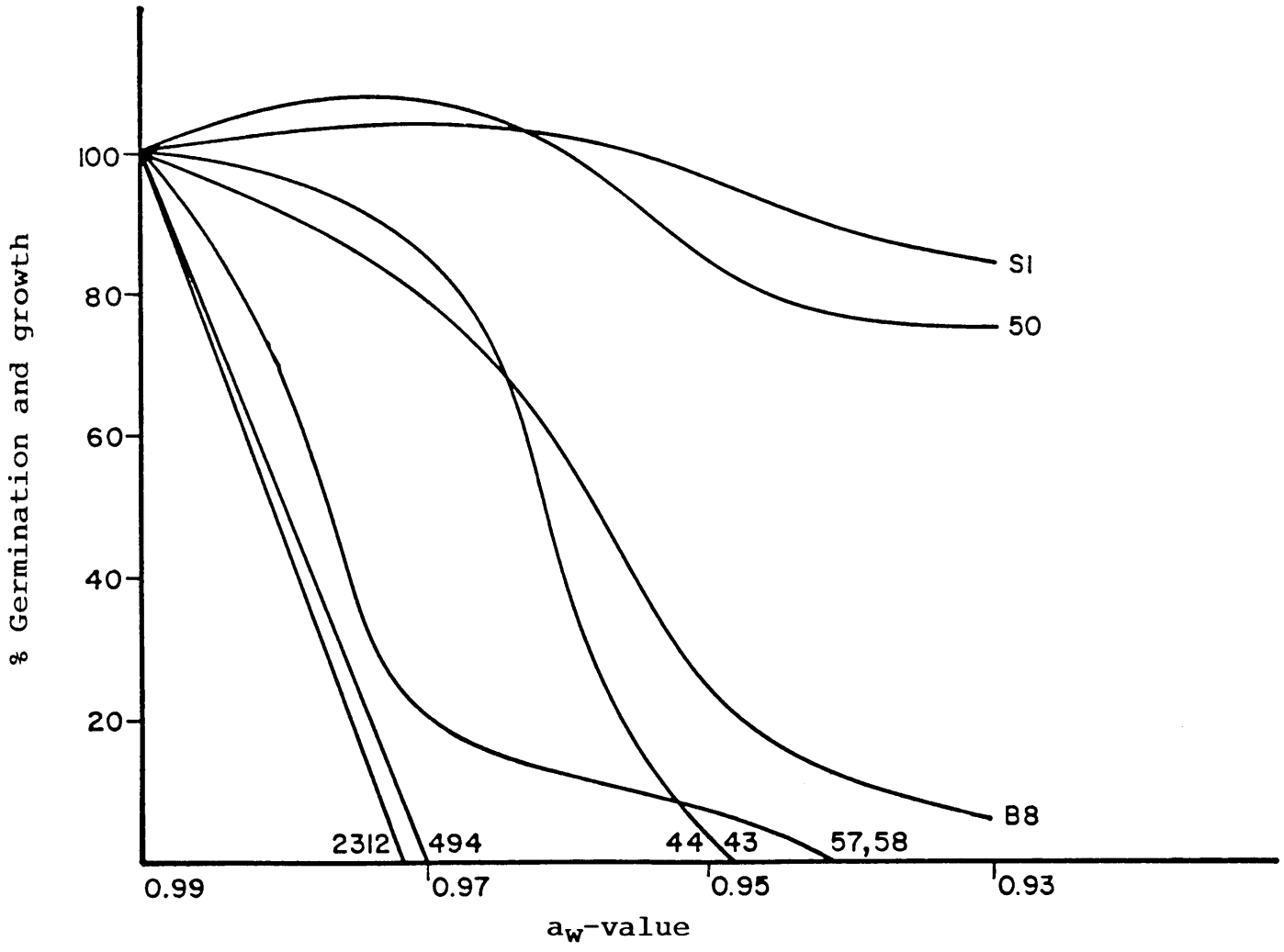

Fig. 3.6 Germination and outgrowth of Bacillus stearothermophilu DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with NaCl used as humectant.

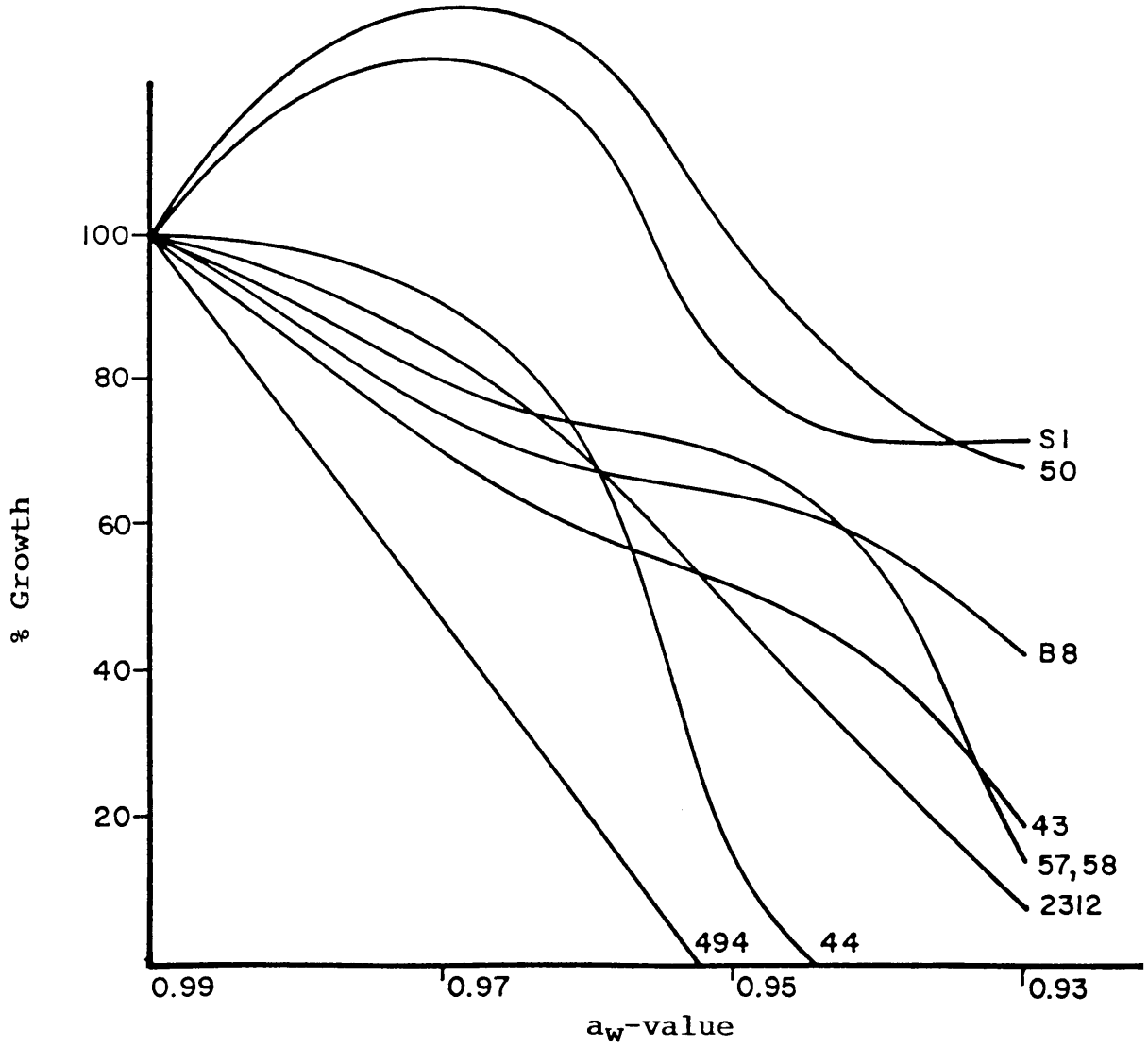

Fig. 3.7 Growth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates as reduced a_w, with glycerol used as

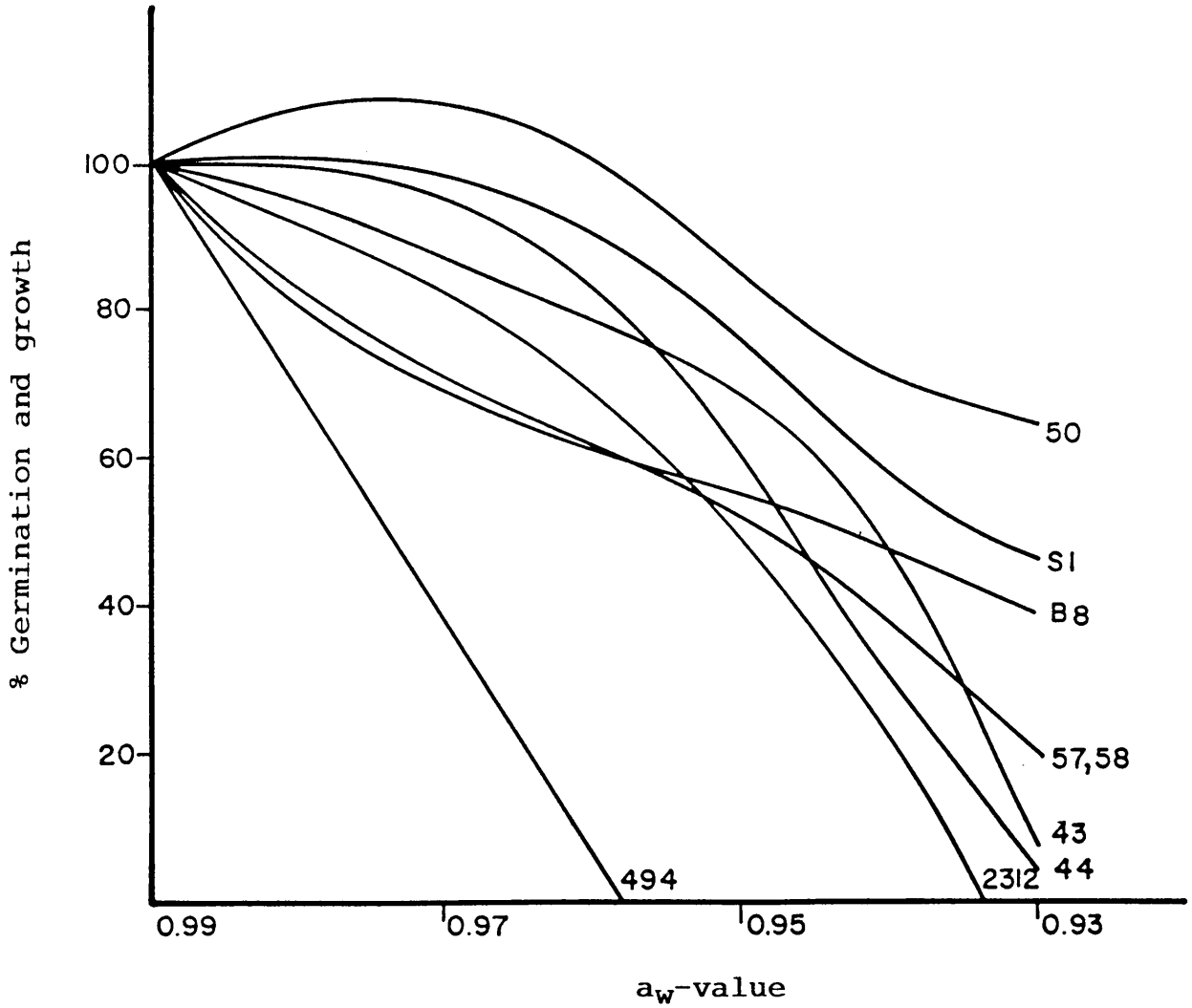

Fig. 3.8 Germination and outgrowth of Bacillus stearothermophilus DSM 494, B. coagulans DSM 2312 and thermophilic Bacillus isolates at reduced a_w with glycerol used as humectant.

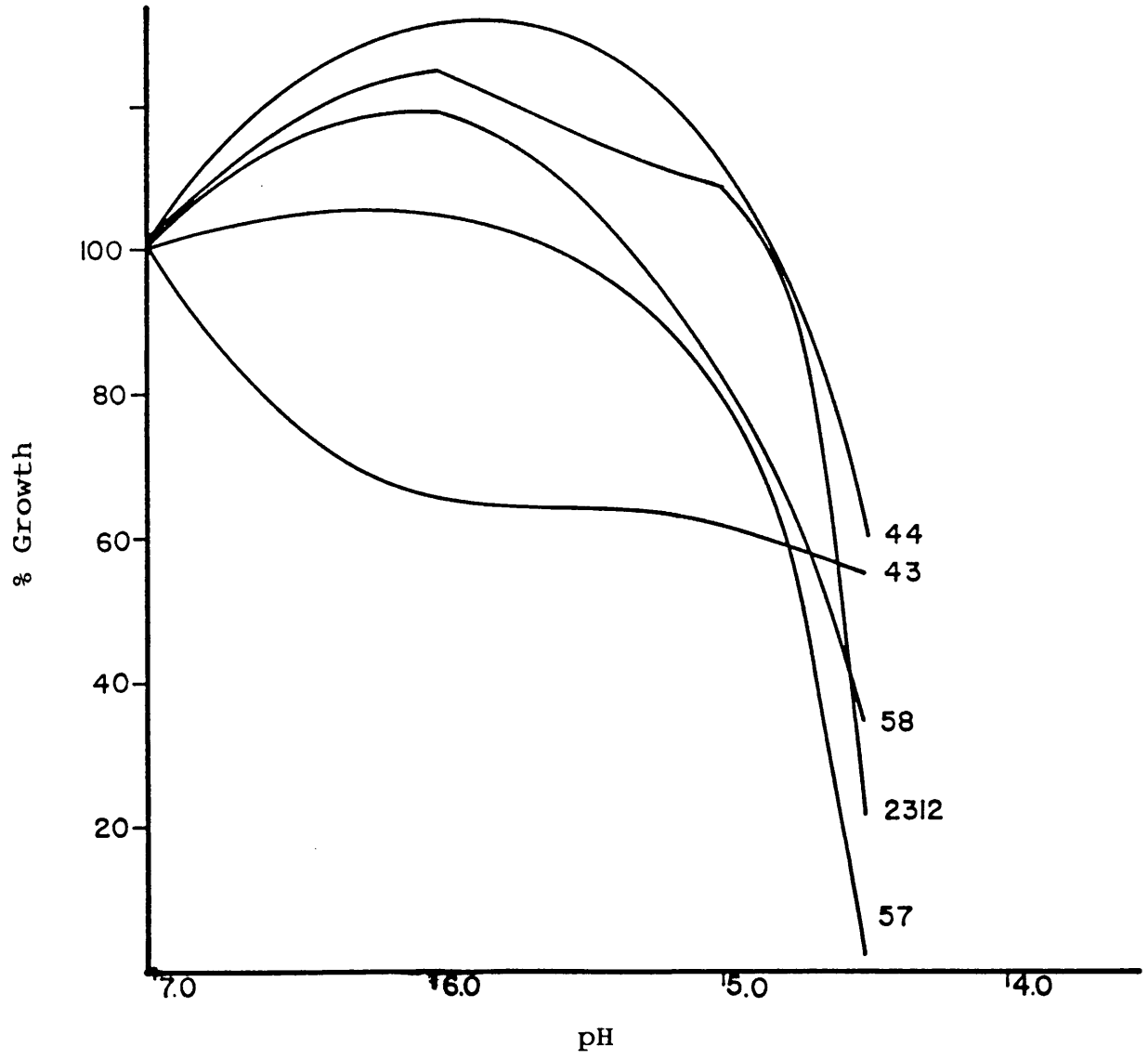

Fig. 3.9 The effect of pH on growth of Bacillus coagulans DSM 2312 and thermophilic Bacillus isolates.

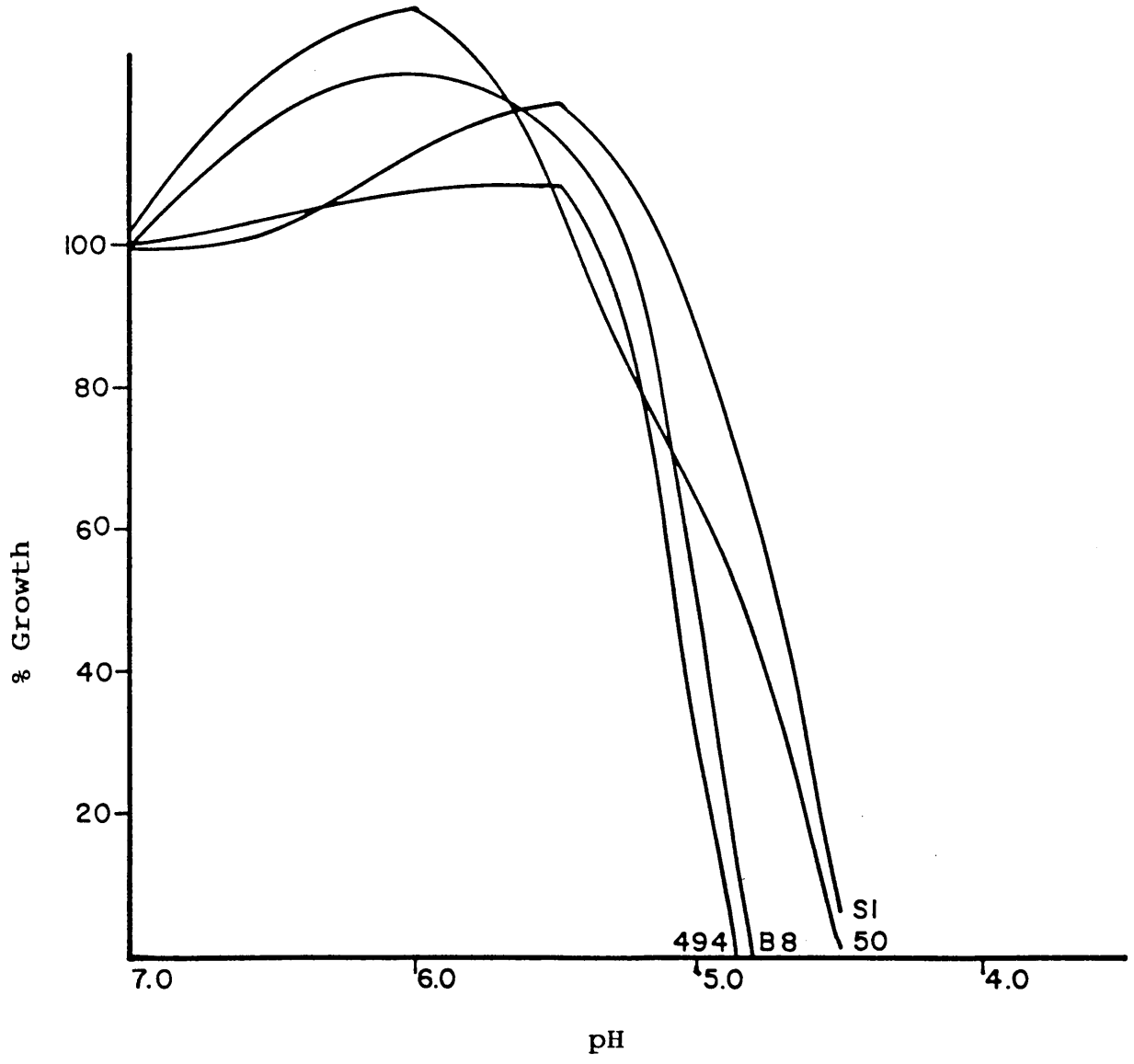

Fig. 3.10 The effect of pH on growth of Bacillus stearothermophilus DSM 494 and thermophilic Bacillus isolates.

4. SHELF STABLE PRODUCTS

4.1 Introduction

Shelf stable products (SSP) are heated in sealed containers sufficiently to inactivate all vegetative microorganisms. Surviving spores cannot spoil the food since the a_w is adjusted to <0,95. The a_w, preferably in combination with other hurdles, such as pH, Eh and preservatives should be low enough to inhibit the multiplication of species of Bacillus and Clostridium (Leistner and Rodel, 1975). Other organisms are of little concern since they are inactivated by heat and contamination after heat processing is prevented by the sealed container. Relatively little energy is required for processing and refrigeration is not required for storage.

It has to be borne in mind that commercial meat products empirically based on the SSP concept are already available. An example is genuine Italian Mortadella. This product has an a_w = 0,96 to 0,93, is sufficiently heated in a closed casing to inactivate vegetative organisms, and thus can be stored without refrigeration (Leistner and Rodel, 1975). Recently Stiebing (1986) reported that the shelf life of Frankfurter type sausages can be extended by applying more intensive heat treatment (F-value) in a sealed container or

by drying them more thoroughly (a_w), or by a combination of these methods.

Since SSP are more promising than IMF, their further development should be pursued. SSP are the result of the interaction of a number of factors. The most important of these factors are, (i) the use of ingredients with low initial levels of microorganisms, (ii) heat treatment to lower the level of vegetative cells present in the finished product, (iii) the addition of anti-fungal agents, (iv) the addition of humectants to bind free water in the product, lowering the a_w of the product and so preventing any microorganisms from growing, (v) the pH of the product adjusted to a level at which antimicrobial activity is enhanced, (iv) the product is vacuum-sealed in retort pouches to retain optimal conditions for shelf-stability for as long as possible (Leistner and Rodel, 1975; Leistner et al., 1981; Hauschild and Simonsen, 1981).

The a_w is an important property in the manufacture of food systems and formulations. Most chemical reactions and microbiological activity are controlled directly by the a_w of the food system (Labuza et al., 1972; Leistner and Rodel, 1975; Troller and Christian, 1978; Troller, 1979; Leistner et al., 1981). Most organisms occurring in meat and meat products proliferate at a high a_w. Only a few require a reduced a_w for growth.

A factor frequently overlooked, yet of crucial importance to safety and stability, is the degree of contamination of the raw product with microbial spores (Hauschild and Simonsen, 1986). Equally important is bacterial spore contamination of nonmeat ingredients, i.e., spices (Draughon et al., 1980; Jackson et al., 1982). Spices are commonly decontaminated by fumigation and irradiation and manufacturers should have no difficulty in selecting spices with low bacterial counts. The microbiological stability of several types of foods and heat processed products in particular is determined by the amount of bacterial spores.

Generally, the sporulation, germination, and vegetative cell growth of Bacillaceae are inhibited at a_w <0,95. The limiting a_w for the formation of mature spores of B. cereus proved to be about 0,95 for NaCl, glucose and sorbitol, whereas it was about 0,91 for glycerol (Jakobsen and Murrel, 1977). Of most concern for SSP is the vegetative cell growth of the surviving spores that might cause food poisoning or spoilage. The significant problem with most of the studies on the limiting a_w for growth of Bacillus and Clostridium are the relatively small number of strains that have been examined, and the question of whether the properties of these strains are representative of those strains occurring naturally.

In SSP all vegetative microorganisms must be inactivated by heat. The heat process should be mild but effective. The heat resistance of microorganisms increases with decreasing a_w (Leistner et al., 1981). Thus food products with an a_w <0,95 should be heated for a longer period than those with a higher a_w.

The ideal for this concept of SSP is to manufacture a product with a total count of not more than 10^4 cells $.g^{-1}$ and that it will remain the same for a certain minimum time. A total count of 10^5 cells $.g^{-1}$ is still acceptable, but a count of 10^6 cells $.g^{-1}$ and higher is totally unacceptable. It is further essential that the total counts will not increase during storage, specifically under certain storage conditions.

4.2 **Procedure**

A frankfurter type sausage was manufactured in our laboratory. The emulsion was prepared in a 40 l bowl cutter in the conventional manner. The emulsion was filled into sheep-casings (22 to 24 mm diameter) after which the product was dried, smoked, packaged and pasteurized. Samples of the meat were taken during the processing stages for microbiological examinations.

The products were dried at 70°C for 30 min. Smoking of the frankfurters took place at 70°C for 30 min. After drying and smoking the product was cooled at 4°C for 20 min. The sausages were then vacuum packed (3 x 25 g portions) and cooked at 76°C (core temperature) for 20 min.

The experimental products were made from choice cuts (topside). The topside contained an average of 72 to 75% moisture and 4 to 8% fat (between muscle and under skin fat). Then 10% water was added in the form of ice, and bacon with a moisture content of 8 to 11%. The final product contains 54 to 56% moisture with an a_w of 0,94 to 0,95.

4.2.1 **Shelf stability tests**

The manufactured products were subjected to different storage conditions for shelf stability testing:

(i) at 37°C: Most pathogenic microorganisms are able to germinate and multiply at this temperature. The products must be able to stay unchanged at this temperature for at least three months.

(ii) at 50°C: Products were exposed to this high temperature to examine any chemical and organoleptic changes. The growth of any thermophilic microorganisms was also established.

The routine sampling of the product after production and incubation under different conditions was designed to show any failure of the product in reaching optimum conditions for shelf stability. The products were tested for:

(i) The total number of colony forming units on STD 1 agar.
(ii) Spore counts on STD 1 agar.
(iii) Lactobacillus counts on MRS agar.
(iv) Damaged or blown packages.

The sausages were sampled at the time of production and after being placed at 37°C and 50°C for shelf stable testing. Samples were taken at 30 d intervals.

A total of 20 g of sausage was aseptically placed in 180 ml sterile Ringer solution and stomached in a stomacher for 2 min. A sample of this fluid was removed and a dilution series prepared. The rest of the stomached fluid in diluent was heated at 80°C for 10 min and a sample of the fluid used to prepare a dilution series. The dilution series was plated in tripticate on STD 1 agar and on MRS agar in the case of the unheated series. The plates were incubated at 37°C for 2 to 3 d, and the number of colonies counted and the counts given as the number of bacteria or spores $.g^{-1}$ of sausage.

The pH and a_w were also determined. Since the SSP concept is based on the lowered a_w of the product, it was essential that a proper a_w determination was done on the product. The instrument used was a Novo Sina a_w-meter.

4.2.3 The reaction of thermophilic Bacillus isolates in experimental shelf stable products

Frankfurter type sausages were manufactured as described in 4.2.1. The emulsion was divided into three batches. The first batch was used as control (EF/A), while the second batch (EF/B) was inoculated with a vegetative cell suspension of the thermophilic isolates (43, 44, 57, 58, 50, S1, B8). The third batch (EF/C) was inoculated with a spore suspension of the thermophilic isolates.

The reaction of these thermophilic Bacillus isolates in the products was tested with intervals of 30 d as described in 4.2.1.

4.3 Results and Discussion

4.3.1 Shelf stability tests

The results of the microbiological and other tests are presented in Table 4.1 and 4.2.

Table 4.1: Changes in microbial populations of experimental frankfurter (EF/A) during storage at 37°C

Storage time (d)	Total counts (g^{-1})	Spore counts (g^{-1})	Lactobacilli counts (g^{-1})	a_w	pH
Emulsion	2×10^4	$<10^2$	3×10^3	-	-
0	1×10^2	$<10^2$	$<10^1$	0,941	5,50
30	5×10^2	$<10^2$	$<10^2$	0,932	5,10
60	1×10^2	$<10^2$	$<10^1$	0,932	5,00
90	1×10^2	5×10^2	$<10^1$	0,930	5,05
120	1×10^2	$<10^2$	$<10^1$	0,930	5,05

Table 4.2: Changes in microbial populations of experimental frankfurters (EF/A) during storage at 50°C

Storage time (d)	Total counts (g^{-1})	Spore counts (g^{-1})	Lactobacilli counts (g^{-1})	a_w	pH
Emulsion	2×10^4	$<10^2$	3×10^2	-	-
0	1×10^2	1×10^2	$<10^1$	0,941	5,50
30	1×10^2	1×10^3	$<10^2$	0,941	5,20
60	1×10^2	2×10^2	$<10^2$	0,929	5,12
90	1×10^2	8×10^2	$<10^2$	0,933	5,10
120	1×10^2	1×10^2	$<10^2$	0,932	5,10

The a_w of the product (EF/A) was more or less constant during the storage time of 120 d, which was a good indication of the shelf stability. There was only a slight

difference of the a_w at 37°C and 50°C (Fig. 4.1). At first there was a decrease in the pH of the product, but after 30 d it remained constant during the whole period of testing.

If the number of colony forming units on STD 1 agar remained low and constant for samples at the time of production and after a long time interval, it indicated a high probability that the product was shelf stable. If fungi were present on these agar plates, it was considered an indication that the concentration of the anti-fungal agents were ineffective. This meant that the product would not be shelf stable. If the number of spores were much lower than the total number of colony forming units on STD 1 agar, non-spore formers should be suspected. This product should be treated as if its shelf life was short.

Lactobacilli counts were used as indicator organisms for heat sensitive bacteria. Any packages showing swelling due to gas formation indicated a non SSP.

The change in microbiological counts for EF/A are given in Fig. 4.2. The total counts, as well as the spore counts, remained more or less constant over the 120 d period. The highest count was 8×10^2 g^{-1}, a good indication that the product was shelf stable.

These results emphasised that when raw materials with a low microbial content were used, the results of the end product were better, and ensured a high standard for a good shelf stable product.

4.3.2 The reaction of thermophilic Bacillus isolates in the experimental shelf stable products

The results of the batches inoculated with suspensions of vegetative cells and spores are presented in Tables 4.3, 4.4, 4.5 and 4.6 respectively.

Table 4.3: Changes in microbial populations of experimental frankfurter (EF/B), inoculated with vegetative cells of thermophilic Bacillus isolates, during storage at 37°C

Storage Time (d)	Total counts (g^{-1})	Spore counts (g^{-1})	Lactobacilli counts (g^{-1})	a_w	pH
Emulsion	$7,2x10^4$	$<10^1$	$2,2x10^4$	-	-
0	$2,0x10^4$	$<10^1$	$<10^1$	0,943	5,57
30	$1,3x10^5$	$<10^1$	$1,2x10^5$	0,928	5,15
60	$2,0x10^3$	$<10^3$	$1,0x10^3$	0,934	5,12
90	$2,5x10^2$	$1,0x10^3$	$1,0x10^2$	0,934	5,15
120	$1,0x10^2$	$3,2x10^2$	$<10^2$	0,933	5,14

Table 4.6: Changes in microbial population of experimental frankfurter (EF/C), inoculated with a spore suspension of thermophilic Bacillus isolates, during storage at 50°C

Storage time (d)	Total counts (g^{-1})	Spore counts (g^{-1})	Lactobacilli counts (g^{-1})	a_w	pH
Emulsion	$5,5x10^6$	$7,5x10^6$	$3,0x10^6$	-	-
0	$3,2x10^6$	$3,1x10^5$	$5,0x10^5$	0,945	5,55
30	$3,1x10^5$	$3,1x10^5$	$3,5x10^4$	0,931	5,10
60	$1,1x10^4$	$3,7x10^4$	$2,0x10^4$	0,940	5,05
90	$1,5x10^4$	$4,5x10^3$	$2,5x10^3$	0,937	5,10
120	$1,2x10^4$	$6,4x10^3$	$1,5x10^3$	0,939	5,10

The a_w of EF/B showed a decrease after 30 d, but remained constant from 60 d on. The a_w of EF/C was constant through the period of testing, but was almost 0,01 higher than EF/A and EF/B (Fig. 4.3 and 4.4). With EF/B and EF/C the pH decreased at first, but after 30 d of storage it remained constant.

The ideal for the concept of SSP is to manufacture a product with a total colony count of not more than 10^4 cells $.g^{-1}$, and that it will remain constant for a certain minimum time. A total count of 10^5 cells $.g^{-1}$ was still acceptable, but a count of 10^6 cells $.g^{-1}$ and higher was totally unacceptable. It is further essential that the total counts will not

Table 4.6: Changes in microbial population of experimental frankfurter (EF/C), inoculated with a spore suspension of thermophilic Bacillus isolates, during storage at 50°C

Storage time (d)	Total counts (g^{-1})	Spore counts (g^{-1})	Lactobacilli counts (g^{-1})	a_w	pH
Emulsion	$5,5x10^6$	$7,5x10^6$	$3,0x10^6$	-	-
0	$3,2x10^6$	$3,1x10^5$	$5,0x10^5$	0,945	5,55
30	$3,1x10^5$	$3,1x10^5$	$3,5x10^4$	0,931	5,10
60	$1,1x10^4$	$3,7x10^4$	$2,0x10^4$	0,940	5,05
90	$1,5x10^4$	$4,5x10^3$	$2,5x10^3$	0,937	5,10
120	$1,2x10^4$	$6,4x10^3$	$1,5x10^3$	0,939	5,10

The a_w of EF/B showed a decrease after 30 d, but remained constant from 60 d on. The a_w of EF/C was constant through the period of testing, but was almost 0,01 higher than EF/A and EF/B (Fig. 4.3 and 4.4). With EF/B and EF/C the pH decreased at first, but after 30 d of storage it remain constant.

The ideal for the concept of SSP is to manufacture a product with a total colony count of not more than 10^4 cells $.g^{-1}$, and that will remain constant for a certain minimum time. A total count of 10^5 cells $.g^{-1}$ were still acceptable, but a count of 10^6 cells $.g^{-1}$ and higher were totally unacceptable. It is further essential that the total counts

increase during storage, specifically under certain storage conditions.

The experimental frankfurter (EF/B) which was inoculated with vegetative cells of thermophilic Bacillus isolates showed a decrease in total colony counts, but concomitant with the decrease, an increase in spore counts over the 120 d period at both storage temperatures. It seemed that when the conditions became unfavorable for vegetative growth, the thermophilic bacilli began to sporulate (Fig. 4.5).

With the experimental frankfurter (EF/C) which was inoculated with a spore suspension of thermophilic bacilli, total counts as well as in spore counts at a storage temperature of 50°C decreased, but were still in the region of 10^5 cells $.g^{-1}$ over the period of 120 d. At a storage temperature of 37°C, the spore as well as the total counts decreased after 30 d, but then started to increase to almost the original count (Fig. 4.6).

It is clear that the manufacturing process was not efficient in lowering the numbers of both vegetative cells as well as spores of thermophilic Bacillus isolates. However, the low a_w and pH of the product prevented the spores from germinating and causing spoilage.

Upon further examination of the colonies on MRS plates for lactobacilli counts, they were found to be the same thermophilic bacilli found on the total count plates. There was no Lactobacillus found on the MRS plates.

A factor frequently overlooked and yet of crucial importance to the safety and shelf stability is the degree of contamination of the raw materials with microbial spores.

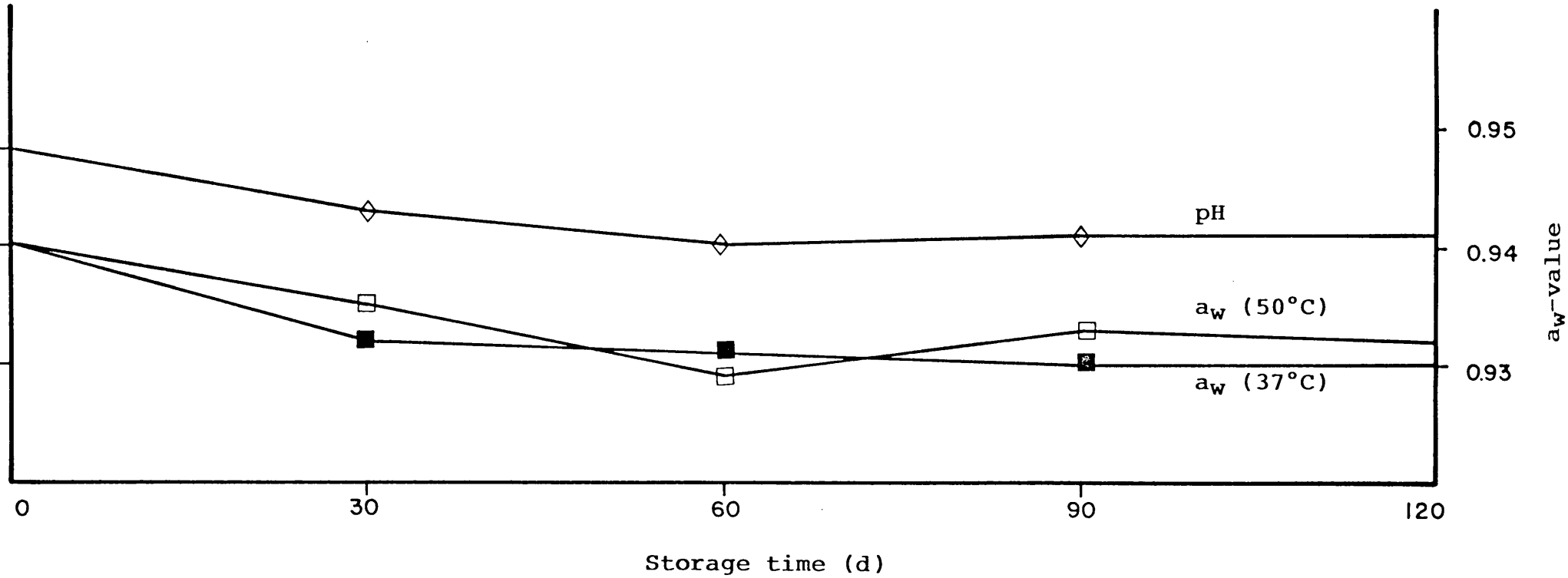

Fig. 4.1 The effect of storage on pH- and a_w-values of experimental frankfurter (EF/A).

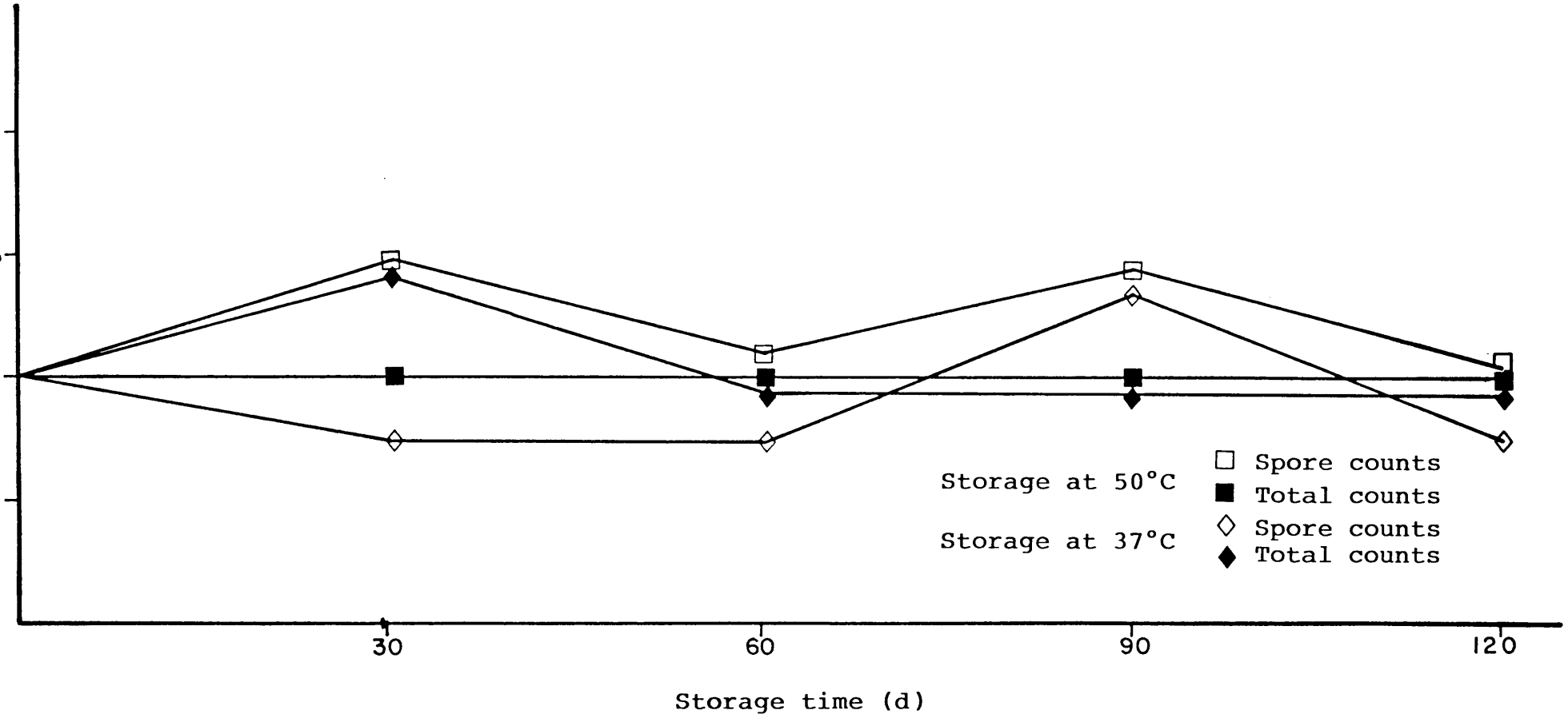

Fig. 4.2 The effect of storage at two different temperatures on microbial counts of experimental frankfurter (EF/A).

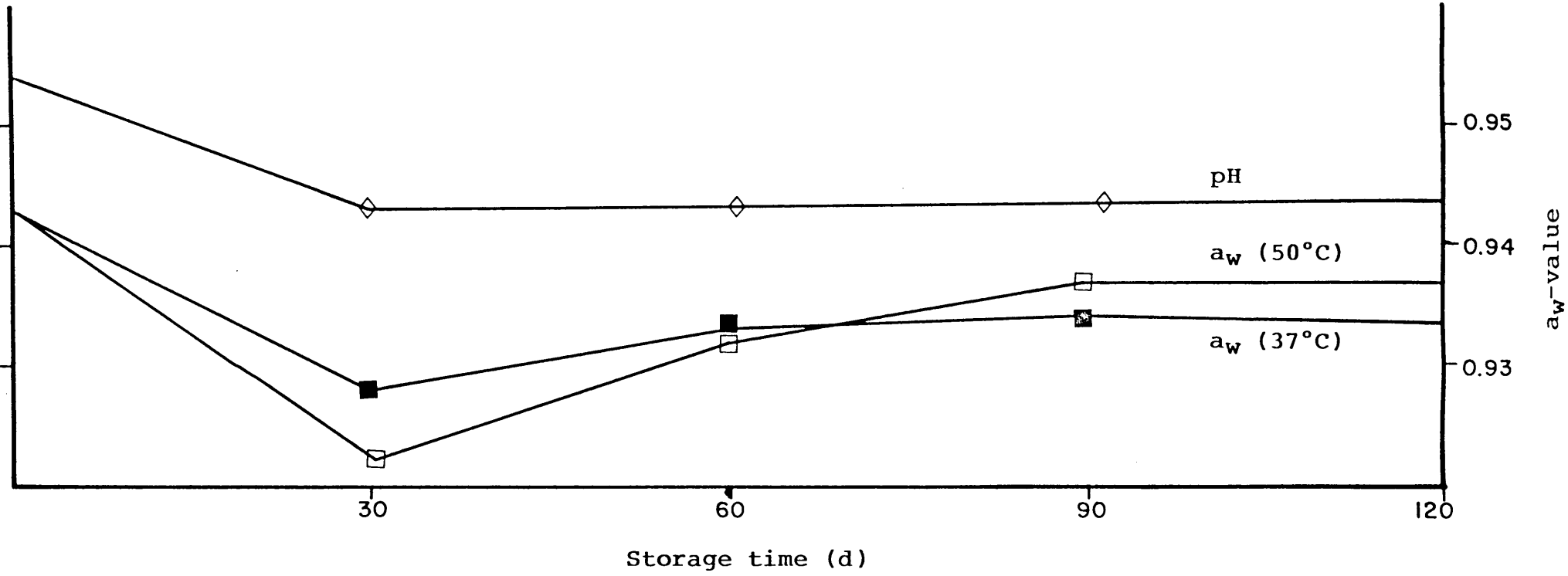

Fig. 4.3 The effect of storage on pH- and a_w-values of experimental frankfurter (EF/B), inoculated with vegetative cells of thermophilic Bacillus isolates.

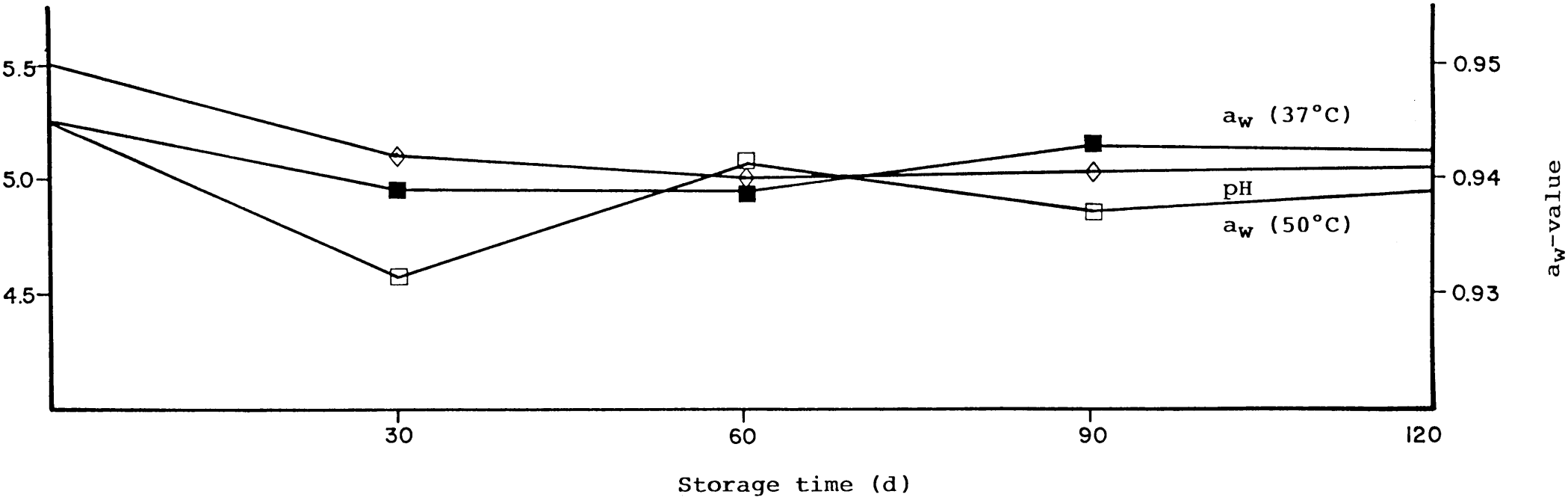

Fig. 4.4 The effect of storage on pH- and a_w-values of experimental frankfurter (EF/C), inoculated with a spore suspension of thermophilic Bacillus isolates.

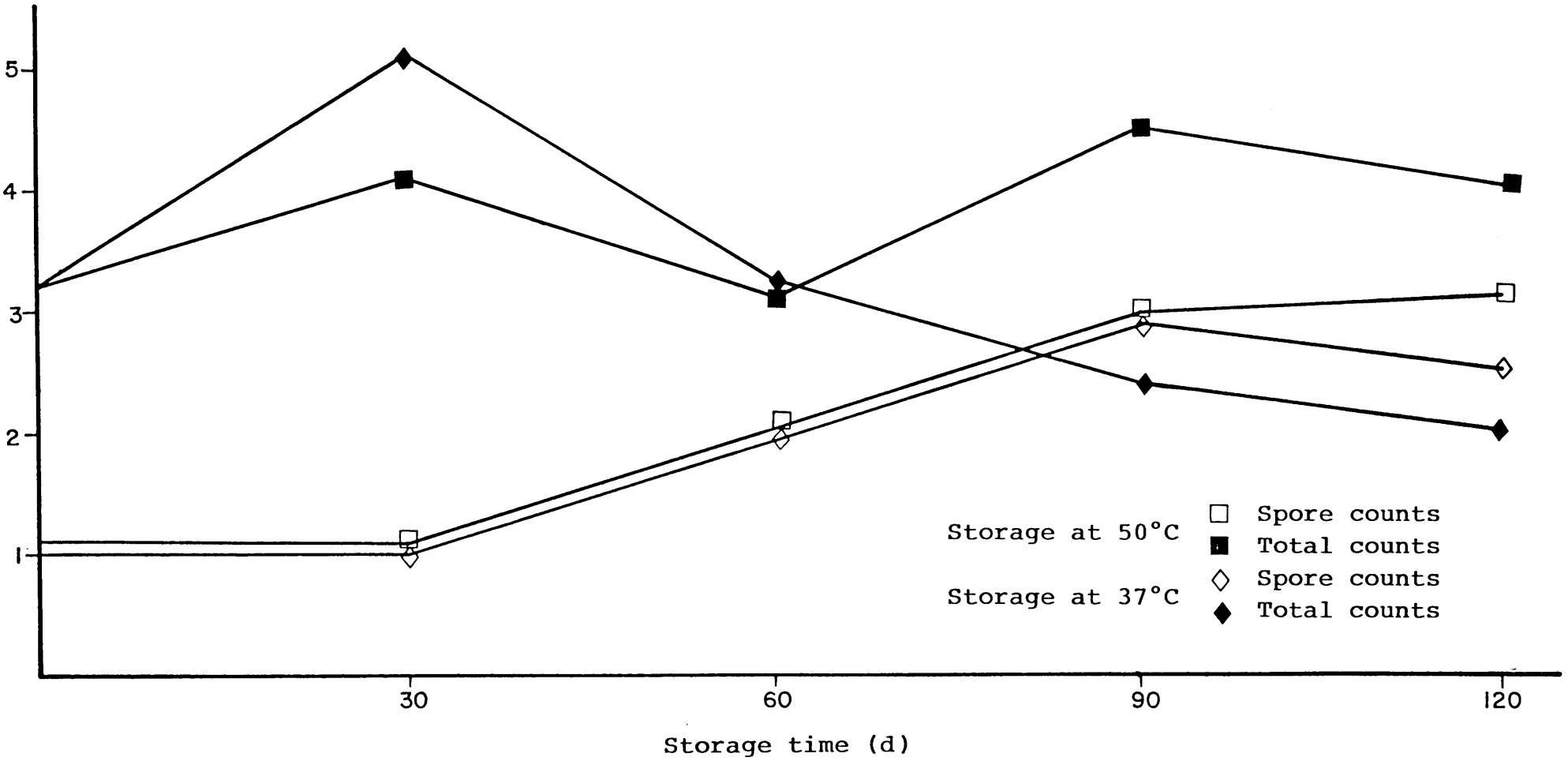

Fig. 4.5 The effect of storage at two different temperatures on microbial counts of experimental frankfurter (EF/B), inoculated with vegetative cells of thermophilic Bacilus isolates.

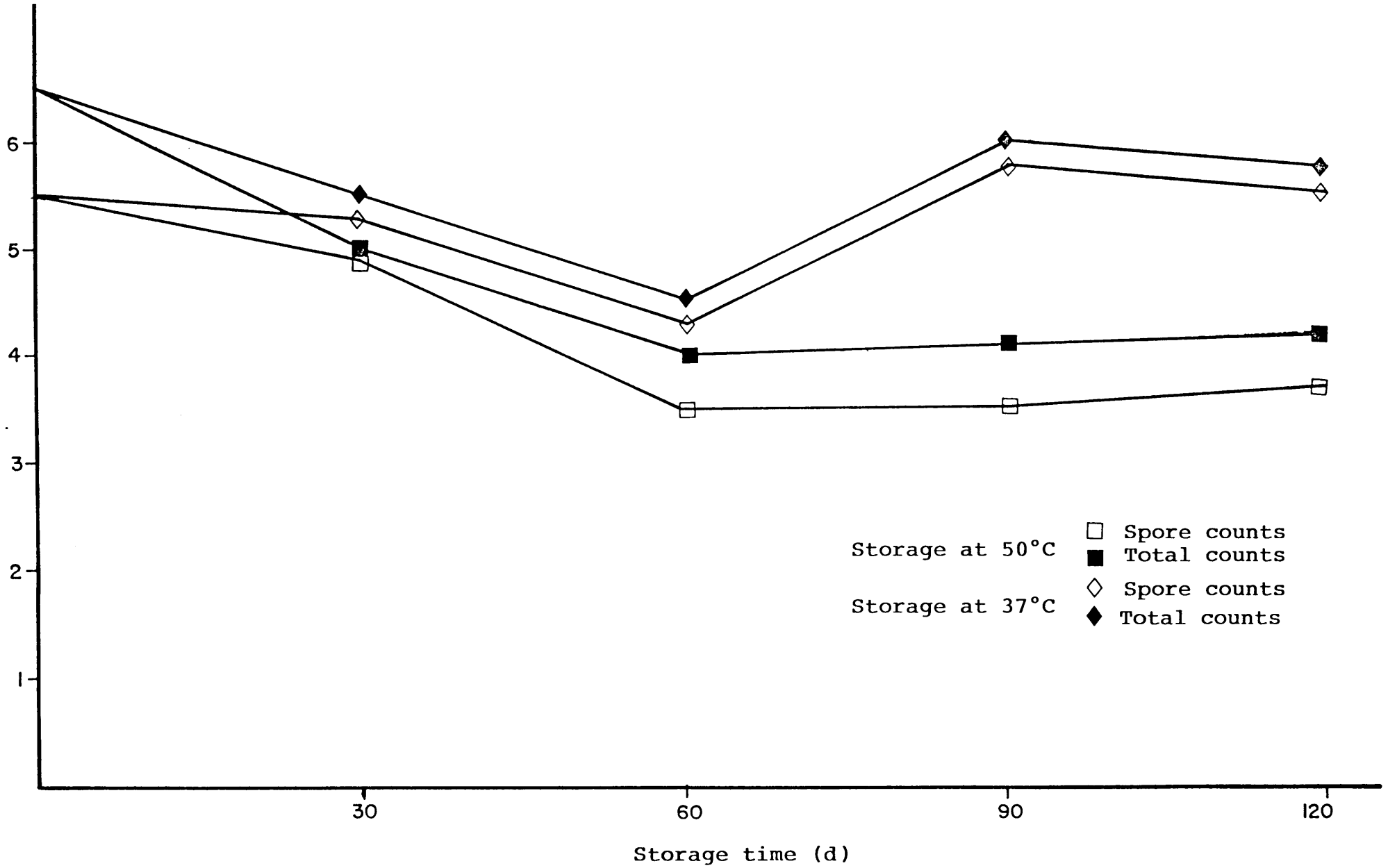

Fig. 4.6 The effect of storage at two different temperatures on microbial counts of experimental frankfurter (EF/C), inoculated with a spore suspension of thermophilic Bacillus isolates.

5. SUMMARY AND CONCLUSIONS

From all the 16 isolates obtained from different types of sausages and spices only 7 showed typical characteristics of thermophilic Bacillus species.

The results showed that the thermophilic bacteria frequently occurred in the products. All the isolates could be related to the bacteria found in spices and non-meat ingredients used in the manufacturing of the products.

API 50 CHB showed that there were four dominating groups of thermophiles within the isolates. All the isolates were Gram-positive rods producing endospores. B. coagulans (43, 44, 57 and 58) was the most common species isolated, followed by B. licheniformis (50 and S1) and B. stearothermophilus (B8).

The production of lactic acid as a fermentation product is unusual in the genus Bacillus, although B. coagulans (43, 44, 57, 58, DSM 2312) carries out a typical homolactic fermentation.

The thermophilic Bacillus isolates obtained in this study showed higher tolerance to sodium nitrite than the authentic strains (DSM 2312 and 494). Isolate B8 was totally inhibited at a concentration of 600 $mg.l^{-1}$ $NaNO_2$, but none of the other isolates was totally inhibited at a concentration of 2 000 $mg.l^{-1}$ $NaNO_2$.

DSM 494 and B8 were totally inhibited at a potassium sorbate concentration of 2 000 $mg.l^{-1}$, while isolate 50 was totally inhibited at a concentration of 5 000 $mg.l^{-1}$. None of the other isolates were inhibited at a concentration of 5 000 $mg.l^{-1}$ potassium sorbate.

When NaCl was used as a humectant to reduce a_w, germination and growth of DSM 494 and 2312 were totally inhibited at a_w = 0,971. The growth, germination and growth of 44 and 43 was strongly inhibited at a_w = 0,947. The vegetative growth of B8, 57 and 58 was strongly inhibited at a_w = 0,95, but total inhibition took place at a_w <0,93. Isolates S1 and 50 were highly tolerant to the low a_w values and at an a_w = 0,93 a 82% growth was obtained.

Due to the fact of the minimal difference observed between growth of vegetative cells and germination of spores, it seemed that the toxic effect of NaCl was the main cause of inhibition and not the reduced a_w.

A study of the thermophilic bacilli with glycerol as humectant indicated that growth occurred at lower a_w levels than when NaCl was used as humectant.

The effect of glycerol on DSM 494 and 2312 was more drastic than on the thermophilic isolates.

When NaCl and glycerol were used as the humectant, it was clear that these thermophilic isolates could germinate and grow at an a_w <0,94. The authentic strains DSM 494 and DSM 2312 were less tolerant to lower a_w values.

The optimum pH of the thermophilic bacilli was between 5,5 and 6,0.

The experimental frankfurter (EF/B) which was inoculated with vegetative cells of thermophilic bacilli showed a decrease in total colony counts, but concominant with the decrease, an increase in spore counts over the 120 d period of storage was observed.

The experimental frankfurter (EF/C) which was inoculated with a spore suspension of thermophilic bacilli, showed a decrease in total counts as well as in spore counts, but after 30 d spore counts started to increase to almost the original number.

It was evident that the manufacturing process was not efficient in the elimination of both the vegetative cells of the thermophilic bacilli, as well as their spores. It was just the low a_w and low pH of the product that prevented the spores from germinating and causing spoilage of the product.

This study showed that thermophilic isolates were more tolerant to low a_w and pH as well as more resistant to high nitrite and sorbate concentrations than the two authentic cultures, DSM 494 and DSM 2312.

It is therefore imperative to include local isolates when determining limiting factors for SSP.

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