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ATTENUATION OF *BACILLUS CEREUS* SPORES BY DIFFERENT FACTORS

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إضعاف جراثيم الباسيلس سيرس باستخدام عوامل مختلفة

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وجد أن جراثيم السلالة المختارة من الباسيلس سيرس قادرة على النمو في محلول كلوريد الصوديوم حتى تركيز ١٠٪ بينما كان نموها ضعيفاً في محلول ٤٪ من نترات الصوديوم . أما نيتريت الصوديوم فقد اختزل العد الحيوي للباسيلس سيرس بمقدار ١,٦٧ دورة لوغاريتمية عند تركيز ٠,١٪ كذلك وجد أن جراثيم الباسيلس سبرس كانت قادرة على النمو في درجات مختلفة من تركيز الأيون الهيدروجيني تتراوح ما بين ٤ إلى ٩,٨ . أما تركيز ١٠٪ من مستخلص الثوم فقد أختزل العد الحيوي حوالي ١,١٦ دورة لوغاريتمية . بتعريض جراثيم الباسيلس سيرس لدرجات حرارة ٩٠° ، ٩٥° ، ١٠٠° درجة مئوية وجد أن الوقت اللازم لاختزال العد الحيوي بمقدار دورة لوغاريتمية بالنسبة لدرجة حرارة ٩٠° كانت ١٧,٥ دقيقة وبالنسبة لدرجة حرارة ٩٥° كانت ١٠ دقائق . وكذلك عند درجة ١٠٠° مئوية كانت ١,٧ دقيقة .

Key Words: Bacillus cereus, curing agents, spore germination, temperature.

ABSTRACT

The spore suspension of *Bacillus cereus* K_{11S} was exposed to different concentrations of NaCl. $NaNo_3$ and $NaNO_2$ as curing agents. The spores were able to tolerate 10% NaCl. Up to 4%, $NaNO_3$ had little or minor effects on germination and growth of *B. Cereus* spores. $NaNO_2$ reduced the viable count of *B. Cereus* by 1.87 log cycle when administered at 0.1% concentration. Up to 1% it slightly affected the basic response of germination and growth of the bacterium. The spores could grow at pH ranging from 4.0 to 9.8. 10% garlic extract (v/v) reduced germination and growth of *B. cereus* spores by 1.16 log cycle.

The effect of different temperatures on spore suspension revealed that the D - values were 17.5, 10 and 1.4 minutes 95°, 95° and 100° C temperature respectively.

INTRODUCTION

One of the characteristics of bacterial spores in their resistance to adverse physical and chemical agents. This property has been extensively studied with the aim of establishing reliable sterilization procedures for the food and pharmaceutical industries. Originally, heat was the sterilizing agent of choice and the thermal destruction of spores has been reviewed in the pharmaceutical field by Sykes (1965), in food poisoning by Brown and Melling (1971), Stumbo (1973) Mossel (1975) and Silliker *et al.*, (1980). Banks *et al.*, (1988) reported that synergistic growth inhibition for heated *B. cereus* spores (65° C for one hour) was only noted for mixtures of organic acids; sodium benzoate and potassium sorbate inducing a pH close to the minimum (pH 4.2) for growth. Wong and Chen (1988) found that salts of organic acids (acetate, formate and lactate) and inhibitory effect on growth and germination of cells and spores of *B. cereus*. At 0.1 M, these three acids completely inactivated multiplication of *B. cereus* at pH 6.1, 6.0 and 5.6 respectively. They caused 50% inhibition of spore germination at pH 4.4, 4.3 and 4.2 respectively.

Common salt lowers the water activity of a system and thus renders the conditions less favourable to microbial life (Leuck, 1980). Sodium nitrate and sodium nitrite are used for curing meats since they stabilise red meat colour, inhibit some spoilage food poisoning organisms and contribute to flavour development. Several bacteria are capable of utilising nitrate as an electron acceptor thus reducing it to nitrite, which is highly reactive and capable of serving as a reducing and oxidising agent. In acid medium, it yields nitric acid (Jay, 1986); which attaches to the amino groups of the dehydrogenase systems of the microbial cell and thus causes an inhibitory action (Leuck, 1980; Silliker *et al.*, 1980).

Garlic exhibits antibacterial (Al-Delaimy and Ali, 1970; De Wit *et al.*, 1979; Mandis *et al.*, 1978) and antifungal (Barone and Tansey, 1977; Moore and Alkins, 1977), larvicidal (Amonkar and Banergi, 1971) and inhibitory to enzyme activities (Wills, 1956). Al-Delaimy and Ali (1970) reported that 4% fresh garlic extract inhibited the growth of *E. coli, Shigella dysenteriae, Salmonella typhi* and *Staphylococcus aureus*. Fresh garlic, ground with meat, prolongs the shelf - life of the latter (Al-Delaimy and Barakat, 1971).

In this investigation it was tried to find out the possible methods for attenuation or arresting spore gemination and growth of *B. cereus* in foods, for this purpose the most common methods of thermal treatment, pH, and some curing salts were used.

MATERIAL AND METHODS

In a previous investigation (Saleh *et al.*, 1993) *Bacillus cereus*, strain K_{11S} was isolated from different Egyptian foods. This strain was chosen for the detailed studies in this work. All methods and media, used for morphological and physiological studies were those reported in the international keys (Cowan and Steel, 1974; Compendium of Methods for Microbiological Examination of Foods, 1976; A Colour Atlas of *Bacillus* species and Bergey's Manual of Systematic Bacteriology, 1986).

The spores were cultured on the medium described by Parry *et al.*, (1983) and incubated, at 30° C, for 48 hours. The spores were then harvested by centrifugation for one hour at 4000 ppm. Spores were heat - shocked for 10 minutes at 60° C before any treatment for activation (Gilbert *et al.*, 1974; Yousten, 1975). The shock spores was kept in buffer solution at pH 8.0 (10^7 spores/ml) and stored at 4° C till needed.

According to Johnson *et al.*, (1983, 1984), 5 ml of *B. cereus* spore suspension were mixed with 0.85% preheated saline solution in a thermostatically controlled water bath, adjusted to either 90°, 95° or 100° C. Samples of the heated suspensions were then removed at intervals and rapidly cooled. 1 ml of treated and control tubes was inoculated into nutrient agar, using the pour plate technique. The viable count was recorded after 48 hours incubation at 30° C. The D_{90}, D_{95} and D_{100} values were calculated from the exponential portion of the graph in a semilogarithmic plot of relative survival as function of incubation time. Shoulder points were avoided.

According to Duncan and Foster (1968 a,b) and Roevuori and Genigeorgis (1975), the nutrient broth containing various concentrations of NaCl, $NaNO_3$ and $NaNO_2$ was dispensed, in 100 ml aliquots, in 250 ml flasks and the pH was adjusted to 7.0 before sterilization. Final concentrations of NaCl were 2%, 4%, 6%, 8% and 10%. Those of $NaNO_3$ were 0.5%, 1%, 2%, 3% and 4%. $NaNO_3$ reached a final concentration of 0.05%, 0.1%, 0.2%, 0.4%, 0.6%, 0.8% and 1%. The flasks were inoculated with 1 ml spore suspension, incubated for 24 hours, at 30° C, with continuous shaking at 200 rpm. 1 ml of treated and control flasks was serially diluted, inoculated and incubated as previously mentioned before the viable count was recorded.

According to the previous same authors, the pH of 100 ml aliquots of the nutrient broth, in 250 ml flasks, were pH adjusted using 3N NaOH to give final value of 4.0, 5.0, 5.5, 6.0, 6.5, 7.5, 9.3 and 9.8 before sterilization, the pH were checked and readjusted after sterilization using sterile 3N HCl and 3N NaOH. The procedures then continued as mentioned above for the sodium salts.

Using the method described by Saleem and El-Delaimy (1982) and El-Delaimy and Ali (1988), the aqueous extract of garlic (*Allium sativum*) was prepared, in the ratio of 1:2 (w/v), using sterile distilled water. Garlic extract was added to sterile nutrient broth to give final concentration of 3%, 5% and 10%. The flasks were inoculated with 1 ml spore suspension, incubated at 30° C for 24 hours on a mechanical shaker (200 rpm). The viable count was determined as previously described.

RESULTS AND DISCUSSION

The data presented in (Fig. 1) reveal that the sensitivity of *B. cereus* spores increased by increasing the temperature from 90° C to 100° C. It is also clear that, within the first part of the curve, there was a sharp drop in the bacterial count at 90° and 95° C. This was followed by a decreased rate of spore inactivation. It was also found that, 100° C greatly affected *B. cereus* spores since D_{100} was 1.7 minutes and six log cycles reduction was obtained after 10 minutes. the same amount of reduction was reached after 60 and 40 minutes exposure to 90° C and 95° C respectively (D_{90} - 17.5 minutes; D_{95} = 10 minutes).

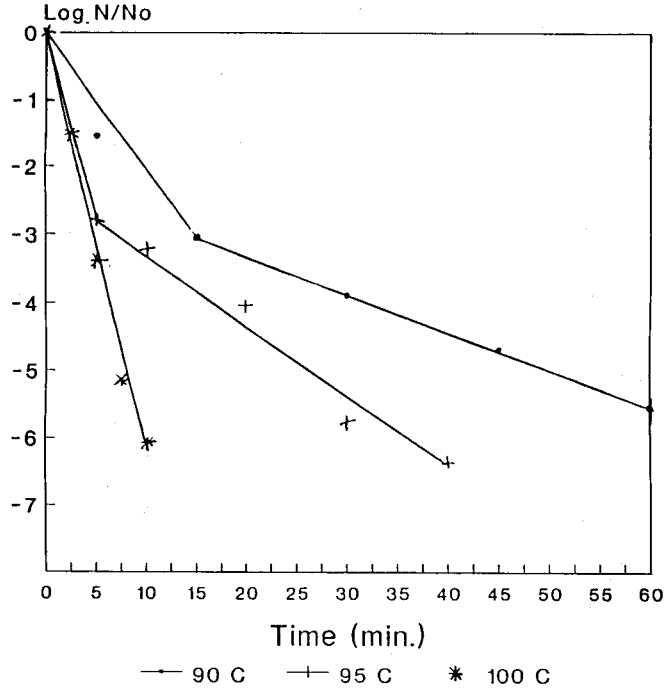

Fig. 1: Effect of different temperatures on the viability of *Bacillus cereus* spores.

The results in (Table 1) show that the growth of *B. cereus* spores first increased with increasing pH from 4.0 to 7.5, then decreased up to pH 9.8, the highest pH measured. The maximal count was at pH 6.5-7.5. The extreme pH values prevented the growth of the bacterium.

Table 1
Effect of pH value on the viable count of *Bacillus cereus* spores (Mean count in CFU/ml)

pH Value	Count	Log N
4.04	4.20×10^6	6.623
4.93	4.52×10^6	6.655
5.54	5.76×10^6	6.760
6.50	6.56×10^7	7.816
7.50	6.65×10^7	7.822
9.30	1.40×10^7	7.146
9.80	5.48×10^6	6.739

Table 2 shows that the highest population of *B. cereus* was obtained when the medium was enriched with 2% NaCl. Further increase in concentration lowered the bacterial count to the least density at 10% level (Fig. 2). All concentrations of $NaNO_3$ lowered the rate of multiplication of *B. cereus*, reaching almost 70% the original count at 4% concentration (Fig. 3). The rate of multiplication was severely attenuated by the gradual increase of $NaNO_2$ concentration from 0.1% to 1.0% (Fig. 4).

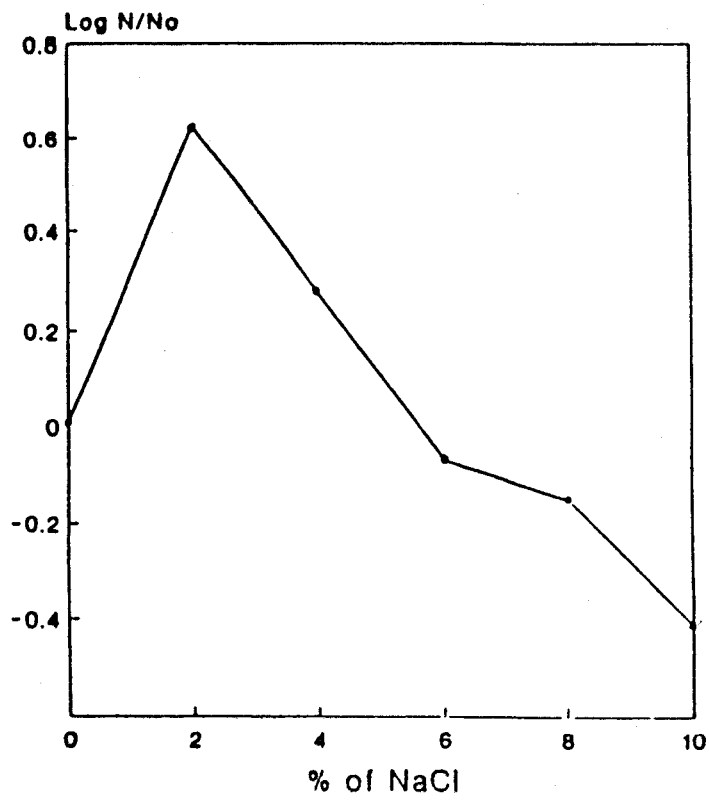

Fig. 2: Effect of sodium chloride on *Bacillus cereus* spore germination.

Table 2
Effect of curing agents on the growth of *Bacillus cereus* spores. (Mean count in C F U/ml)

Sodium Chloride				Sodium Nitrate				Sodium Nitrite			
Concentration	Count	Log N	Log N/No	Concentration	Count	Log N	Log N/No	Concentration	Count	Log N	Log N/No
0%	8.330×10^6	6.92	0	0%	9.610×10^7	7.98	0	0%	8.000×10^7	7.90	0
2%	3.510×10^7	7.54	+ 0.624	0.5%	8.900×10^7	7.94	-0.033	0.05%	7.430×10^6	6.87	-1.03
4%	1.600×10^7	7.20	+0.283	1%	7.550×10^7	7.87	-0.104	0.1%	1.705×10^6	6.23	-1.67
6%	7.060×10^6	6.84	-0.070	2%	5.280×10^7	7.72	-0.260	0.2%	1.566×10^6	6.19	-1.70
8%	5.900×10^6	6.77	-0.149	3%	3.700×10^7	7.56	-0.414	0.4%	1.490×10^6	6.17	-1.72
10%	3.166×10^6	6.50	-0.420	4%	2.880×10^7	7.45	-0.523	0.6%	1.125×10^6	6.05	-1.85
								0.8%	9.960×10^5	5.99	-1.90
								1%	9.680×10^5	5.98	-1.91

The results in (Table 3) revealed that the count of *B. cereus* spores decreased to almost 0.1g log cycle at 3% concentration of garlic extract. Raising the concentration to 10% lowered the bacterial count to almost one log cycle.

Long ago, the thermal inactivation of bacterial spores has been widely studied. Thermal processing is highly important in sterilization of many foods and medical products. The results of this investigation are in good agreement with those

Table 3
Effect of garlic extract on the viable count of *Bacillus cereus* spores. (Mean count in C F U/ml)

Concentration	Count	Log N	Log N/No
Control	1.45×10^7	7.16	0
3%	9.35×10^6	6.97	-0.192
5%	1.59×10^5	6.20	-0.961
10%	9.85×10^5	5.99	-1.169

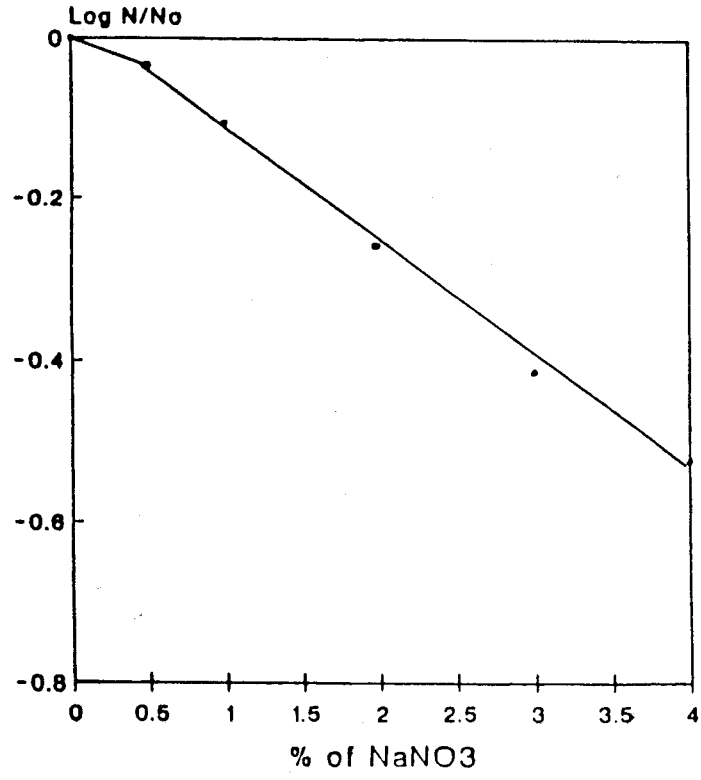

Fig. 3: Effect of sodium nitrate on *Bacillus cereus* spore germination.

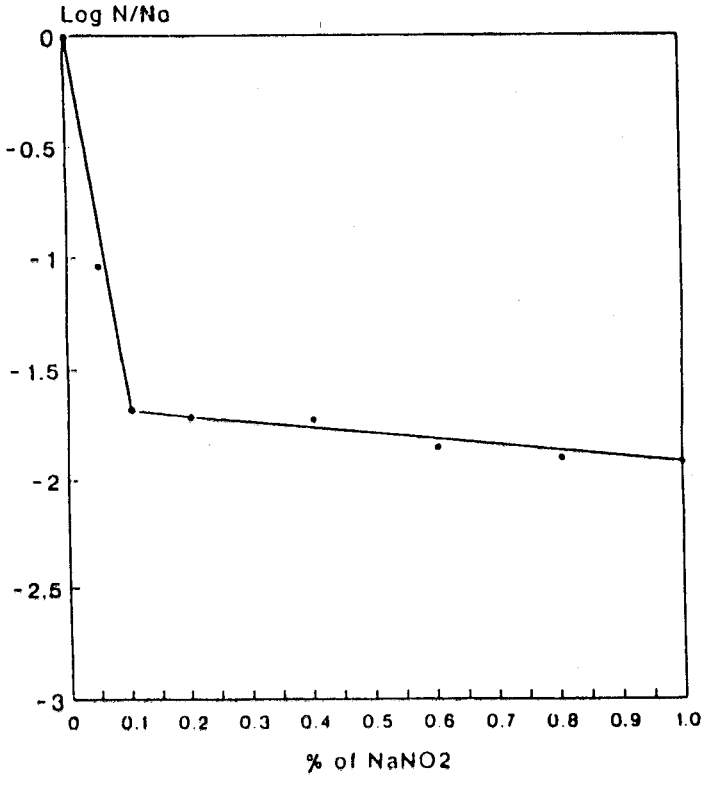

Fig. 4: Effect of sodium nitrite on *Bacillus cereus* spore germination.

reported by Roberts and Ingram (1966). Heat, in some way, injured or sensitized bacterial spores, making them less tolerant to meat-curing salts in the outgrowth medium. The D-values of thermosensitivity were 17.5, 10.0 and 1.7 minutes at 90 and 100° C respectively. Almost similar results were recorded by Vas and Proszt (1957). Wong *et al.*, (1988) found that D_{95} for the heat resistant *B. cereus* ranged between 1.2 and 36.0 minutes whereas D_{100} values ranged from 2.0 to 5.4 minutes. The initial high rate of destruction, followed by a slow exponential death rate might be explained by the presence of a system containing organisms of two different resistances, e.g. a mixture of vegetative cells and spores (Briggs, 1966).

There are conflicting reports on the effect pH on bacterial spores. Every microorganism has a range of pH for its growth. Not only growth is affected by pH but also the rate of survival during storage, heating, drying and other forms of processing (Frazier and Westhoff, 1979). In this study, the ranges of pH allowing growth of *B. cereus* ranged between 4.04 and 9.8. This is in agreement with Goepfert *et al.*, (1972) and Raevuori and Genigeorgis (1975) who found that *B. cereus* was able to grow in the range of pH 4.9 to 9.3. Mikolajcik *et al.*, (1973) reported that *B. cereus* vegetative cells failed to survive with increased acidity of milk but its spore count remained unchanged. Skim milk, at pH 5.0, arrested vegetative cell multiplication and spore germination ceased completely.

According to existing data (Kim and Geopfert, 1971; Troller, 1973) *B. cereus* is able to grow in 7% not in 10% NaCl concentration. The range between 4% and 7% inhibited outgrowth whereas above 10%, growth progressively declined and finally arrested at 15% NaCl. The survivor/concentration data of $NaNO_3$ indicated that the salt had minor effects on germination and outgrowth of *B. cereus* spores. Up to 4%, the viable count was only 0.5 log cycle. Similarly, Duncan and Foster (1968) reported that $NaNO_3$ had no apparent effect on germination and outgrowth of putrifactive 3679h spores at concentrations reaching 2%. This may be explained by assuming that bacteria are capable of utilizing nitrate as an electron acceptor and the possible reduction of nitrate to nitrite which is the most efficient of the two slats for meat preservation.

The survivor/concentration data of $NaNO_2$ indicated a sharp decline in the viable count reaching about 1.67 log cycle at 0.1% $NaNO_2$, followed by a slow rate of declension. The steep fall might be attributed to the toxic effect on the vegetative cells accompanying the spores in their suspension. Several explanations have been proposed for the mechanism of nitrite bacteriostasis. The bacteriostatic properties of nitrite were attributed to interference with the amino groups of the dehydrogenase systems of *E. coli* (Quastel and Woolridge, 1927). Also, hydroxylamine may be produced from nitrite by a number of organisms (Lindsey and Rhines, 1932) and this substance, in turn, is responsible to some extent for nitrite bacteriostasis. Ingram (1939) reported that at pH 6 oxygen uptake by *B. cereus* was strongly inhibited by nitrite, thus implying an interference with the cytochrome systems, or it might reactivate certain bacterial enzyme systems that possess an active sulphydryl group. Gould (1969) reported that spores of several *Bacillus* species germinated, though at reduced rates, in less than 0.03% $NaNO_2$ at pH 6.0.

Development was arrested immediately after germination, i.e. before lysis or rupture of spore coats. Higher concentrations of $NaNO_2$ (0.075 to 0.25%) prevented germination altogether at pH 6.0. Toxicity was three to five times greater at pH 6.0 than at pH 7.0.

In this study, 10% garlic lowered *B. cereus* spore count by one log cycle. This indicated that the bacterial count was reduced by 93.79%. Saleem and El-Delaimy (1982) found that addition of 10% garlic extract (v/v) completely inhibited growth of *B. cereus* cells on nutrient agar plates. The inhibition of *B. cereus* increased, almost in direct proportion, with increased extract concentration.

In conclusion, this study reveals the significance of nitrite and pH 6.0 in preserving food products. Sodium chloride is contributing, but sodium nitrate probably has no significant effect at the concentrations normally used in practice.

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