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Activity of Acetic Acid on *Bacillus stearothermophilus* and *Bacillus subtilis* Spores after Sublethal Heat Pretreatments

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ABSTRACT: This work has been led in view to find the influence of sublethal heat (45°C, 50°C, 60°C) on acid resistance of *B. subtilis* NCTC 3610 and *B. stearothermophilus* CNCH 5781 spores. Firstly, we have submitted *Bacillus* spores to 0.4% acetic acid pH 4.5 during the times of 1, 2 and 3 hours. Then another spores group were preheated at various sublethal temperatures, before be treated with acetic acid. The effect of acetic acid before and after preheat was evaluated by the culture of treated spores on agar medium and the number of colony obtained was compared with that of control culture (neither treated with heat nor acid) and control A culture (only treated with acid). We found that acetic acid was effective on the twice *Bacillus* spores with more effect on *B. stearothermophilus* CNCH 5781 spores. Furthermore we have noticed a significant increasing in percentages of recovery of colonies obtained from preheated and acid treated spores compared to those of control and control A cultures. This increase of recovery percentages could be demonstrated the manifestation of a "heat-induced acid resistance" phenomenon. Yet, this phenomenon was more accentuated for preheatings at 50 and 60°C during 3 and 2 hours, respectively for *B. subtilis* and *B. stearothermophilus* spores. This study suggest that sublethal heats could be play major role in protection of microorganisms to chemicals

KEYWORDS: sublethal heats, acid resistance, spores, *Bacillus*.

1 INTRODUCTION

The fight against microbial contamination is one major preoccupation in food industries, as far as microbial deterioration of foodstuffs can not only reduces the organoleptic and trading qualities of products, but can be also a cause of diverse food poisoning (**[1]**). Many cases of food poisoning issued from products made up of milk in Senegal, and street food in Cameroon were reported (**[2]**).

In industries of canned food, heat sterilization is the mostly used process in inactivation of sporulated forms in view to extend the preservative duration of product. Mean while, the intensity of heat treatment required for inactivation of

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sporulated forms could affect nutritive and organoleptic values of food. This is how, we observe for example more and more substitution of sterilization processes at high temperatures (T > 110ºC) by those sterilized by chemicals agents (**[3]**) such as short-chain fatty acids (acetic acid, propionic acid), sorbic and benzoic acids (**[4]**). Acetic acid is a weak organic acid and preservative agent found in vinegar. Due to its activity and its non toxic character, acetic acid has an expanded use in food industries, but also in home works cooking. On the other hand, the use of acids pH generally with the values lower or equal to 4.5 is largely employed into growth control of microorganisms in foods (**[5]**).

Nevertheless, further studies have proved that the stay of spores at sublethal temperatures had as disadvantage to develop in these spores a heat-induced dormancy phenomenon (**[6]**) and/or a heat-induced thermoresistance phenomenon (**[7]**). Regarding the implications of such observations in the process of sterilization in general, but also in food and pharmaceutical products conservation in particular, the question here is to know if the conservation and treatment of foods at sublethal temperatures (T< 100ºC) for spores could also induce an increase of chemical resistance of spores contained in these foods. From this interrogation, we proposed ourself to evaluate the effect of sublethal heat on behavior of *B. stearothermophilus* and *B. subtilis* spores in presence of acetic acid.

2 MATERIAL AND METHODS

2.1 ACID REAGENT

The reagent used for evaluation of acid resistance and heat induced acid resistance phenomenon was made up of acetic acid solution 0.4% at pH 4.5.

2.2 BACTERIAL STRAINS

Microorganisms used for evaluate the two phenomenon above were made up of two *Bacillus* species. This material was supplied to us in form of *B. stearothermophilus* CNCH 5781 spores obtained from Institute Appert of Paris, France and *B. subtilis* NCTC 3610 spores obtained from the culture collection of the Microbiology Laboratory, Institute of Food Research, Reading, UK. They were preserved at a temperature of 4°C before use.

2.3 SPORES PRODUCTION AND PURIFICATION

The spores used in this work were preliminarily produced from spore stocks in two steps. Firstly spore stocks were heat-activated at 80°C for 10 min (**[8]**) and spread on plate nutrient agar. The plates were incubated for 24 h at 35°C for *B. subtilus* and 63°C for *B. stearothermophilus*, and vegetative cells were obtained. Secondly, the spores were obtained from vegetative cells. Spores of *B. subtilus* were obtained according to the protocol described by **[9]** and those of *B. stearothermophilus* were obtained as described by **[10]**. Spores of both species were purified according to the standard method described by **[11]**. Cleaned spores were suspended in distilled sterile water and stored at 4°C for three months at least to ensure their stability.

2.4 DETERMINATION OF ACID RESISTANCE OF *BACILLUS* SPORES WITH ACETIC ACID

Fifteen microliters of non preheated spores (at $4{,}8x10^8$ UFC/mL) of both species were treated with an acetic acid solution 0.4% at pH 4.5 during 1, 2 and 3 hours. After treatment, in order to evaluate acid resistance by determination of percentage of recovery, the reactionnal mixture have been neutralized with a sodium hydroxide solution and one hundred microliters of appropriated decimal dilution of acid treated and heat-activated (**[8]**) culture were spread on GPB (*gélose glucosé au pourpre de bromocrésol*) agar medium. The preparations were incubated for 24 h at 35°C for *B. subtilus* and 63°C for *B. stearothermophilus.* After incubation, the number of colonies obtained from spores treated with acid (experimental culture or witness A spores) was enumerated and expressed in percentage of recovery as compared to that of non treated spore control (witness spore) subjected to same conditions, according to the formula below (**[12])** :

Percent of recovery (%) = (Number of colonies of experimental culture / number of colonies of control culture) x 100

2.5 EVALUATION OF HEAT INDUCED ACID RESISTANCE PHENOMENON OF *BACILLUS* SPORES

Firstly, Fifteen microliters of spores suspensions (at $4{,}8x10^8$ UFC/mL) of both species were pre-heated at fixed and sublethal temperatures of 45, 50, and 60°C during 1, 2 and 3 hours each according to the method described by **[13]**.

Secondly, each preheated spore suspension was treated with an acetic acid solution 0.4% at pH 4.5 during 3 hours**.** Control heat-activated spores neither preheated nor acid treated were submitted at the same conditions. After treatment, the pH of the medium was neutralized with NaOH solution. One hundred microliters of appropriated decimals dilution was spread on GPB agar medium and culture was done as described above. After incubation, the number of colonies obtained from heat activated spores preheated and treated with acetic acid was enumerated and expressed in percentage of recovery as compared to that of non treated spores control (witnesses spores), according to the same formula given above (**[12]**).

2.6 STATISTICAL ANALYSIS

The experiments were conducted in triplicate and the results expressed in terms of means. The difference between the control and treatments was made using a one-factor ANOVA and the Student Newman-Keuls test with IBM SPSS 20.0 for window at a 95% confidence level. Office/Excel software permitted us to draw the different diagram.

3 RESULTS AND DISCUSSION

The results compiled in figure 1 present effect of acetic acid treatments on recovery of colonies issued from *B. subtilus* and *B. stearothermophilus* spores. These results show that all percentage of recovery obtained from cultured treated spores (control A culture) was statistically lower than those of control spore (not treated) with an acetic acid solution 0.4% at pH 4.5. Indeed, we find that the decrease of the percentage of recovery is more pronounced with the duration of acid treatment with values of 54.23% and 43% respectively for *B. subtilis* and *B. stearothermophilus* spores species treated with acid during three hours. This increase in sensitivity of spores at the acetic acid was converted by a significant reduction ($p<0,05$) of recovery percentages, which could be justified by an effective sporistatic activity of acetic acid (**[14]**). Mean while, this antigerminative activity of acetic acid could be explained either by a covalent-bond of acetic acid on spores components (or terguments) (**[15]**), or by an acidification of sporal core (**[4]**) contributing to reinforce the dormancy of spore. We can also notice a sensibility to acetic acid mostly marked on *B. stearothermophilus* spores compared to those of *B. subtilis,* which confirms the observation established formaly on the variability of microorganisms resistance (**[7]**). This difference of sensibility could be linked to growing conditions of each species (**[16]**) but also could be explained by possible difference in number and accessibility on sites of fixation of acid substances on spore's components of different species (**[17]**).

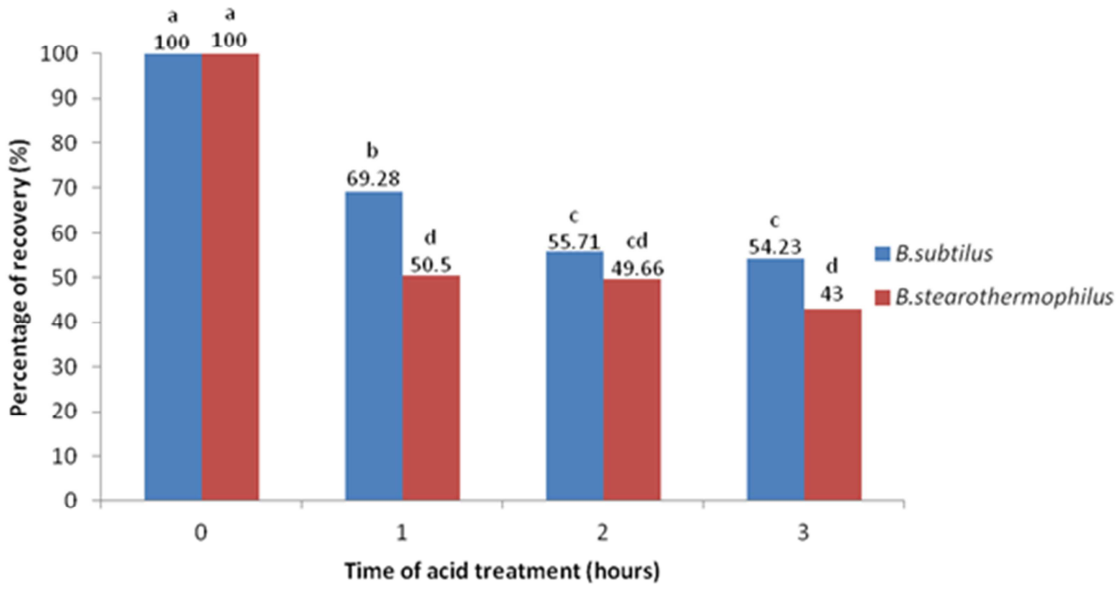

Figure 1: percentage of recovery of the colonies issued from B. subtilis and B. stearothermophilus spores treated with an 0.4% acetic acid solution at pH 4.5 during 1, 2 and 3 hours (control A culture). Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test (p<0.05).

The figure 2 and 3 below present respectively the results of effect of sublethal temperatures (45˚C, 50˚C, 60˚C) on the recovery of colonies from *B. stearothermophilus* and *B. subtilus* spores treated with acetic acid during 3 hours.

The results given by figure 2 below reveals the recovery percentages which are found between 66.67% and 97.5% respectively for *B. stearothermophilus* spores preheated at 50 and 60°C during 2 hours before their treatment with acetic acid 0.4%, pH 4.5. Concerning *B. stearothermophilus* spores preheated and treated with acetic acid 0.4% at pH 4.5 during 1 and 2 hours, we noticed the same observations of as those produced by figure 2 below. The analysis of figure values reveals that all recovery percentages of the preheated *B. stearothermophilus* spores and treated with acetic acid pH 4.5 during 3 hours are statistically higher than those of spores non preheated and treated with acetic acid (control A spores).

The figure 3 shows a variation of recovery percentages between 89,28 and 121,42%, respectively for *B. subtilis* spores preheated at 45 and 50°C during 2 and 3 hours before the acid treatment. The analysis of values on this figure brings out similar conclusion as that of figure 2. Similar observations as those carried on figure 3 were made with results obtained from spores of each species preheated and treated with acetic acid at pH 4.5 during 1 and 2 hours.

The increase of percentages of recovery as observed in figure 2 and 3 could illustrate a greater resistance of two *Bacillus* spores to acetic acid 0.4%, pH 4.5. These results clearify those of **[18]** which revealed a similar effect on *B. subtilis globigi* spores treated with peracetic acid. In fact these authors showed that the spores of *B. subtilis globigii* subjected to a temperature of 4 º C during 134 weeks were significantly more resistant to peracetic acid 0.04% compared with control untreated spores. Reference **[7]** explained this heat-induced resistance phenomenon by structural and conformationnal modifications of subsurface molecules (mainly those of tunic coat) in presence of sublethal temperatures, which will reduce the spore's permeability for chemicals. In fact these chemicals may simply react with the spore coats, thus reducing the amount of toxic agent which can attack more-central spore molecules such as enzymes or DNA in the spore core (**[19]**) and consequently could decreased spore's sensibility to effect of chemical agents. This heat-induced resistance phenomenon could be called heat-induced acid resistance. Thus, it appears that acid resistance can also be an inductible phenomenon at same title as thermoresistance (**[7]**).

In addition we notice on the basis of different recovery percentages shown in the Figure 2 and 3 that this phenomenon is more pronounced in *B. subtilis* spores compared to those of *B. stearothermophilus*. These results are in contradiction with the observations of **[20]** on the heat resistance of *Bacillus* spores and reinforce the hypothesis that heat-induced resistance would also vary from one species to another in within the same genus or a different strain within the same species.

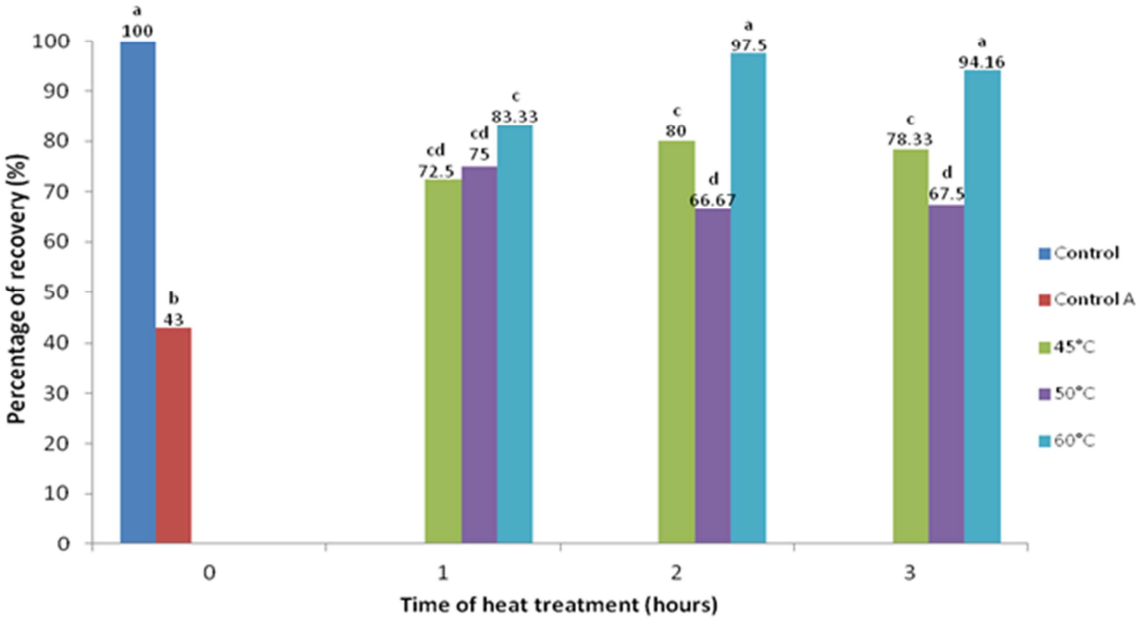

Figure 2: percentage of recovery of the colonies issued from B. stearothermophilus spores preheated at 45˚C, 50˚C and 60˚C then treated with acetic acid 0.4% at pH 4.5 during 3 hours. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test (p<0.05).

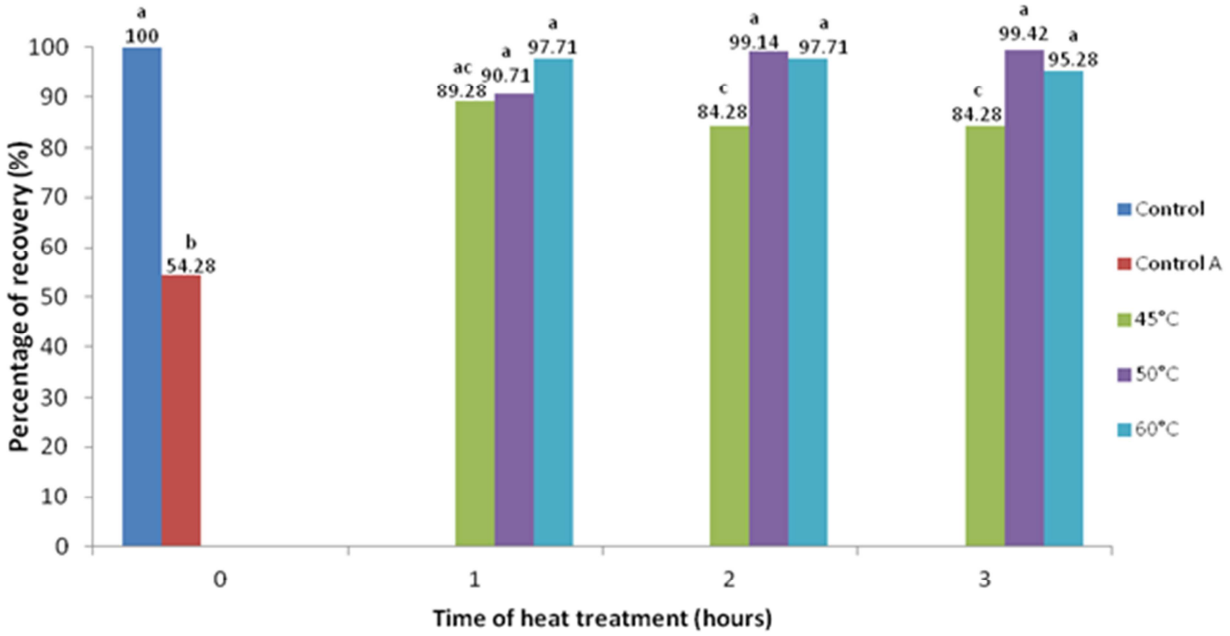

Figure 3: percentage of recovery of the colonies issued from B. subtilus spores preheated at 45˚C, 50˚C and 60˚C then treated with acetic acid 0.4% at pH 4.5 during 3 hours. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test (p<0.05).

4 CONCLUSION

At the end of our study, it can be noticed that preheatings at the temperatures of 45, 50 and 60°C induce on the two species of spores a certain increase of resistance to acetic acid. This phenomenon could be called in the example of heat-induced thermoresistance, a heat-induced acid resistance. Mean while, this phenomenon is less pronounced for preheatings at 45 and 50°C during 2 hours, on the other hand, it is more accentuated for preheatings at 50 and 60°C during 3 and 2 hours, respectively for each case on *B. subtilis* and *B. stearothermophilus*. This heat-induced acid resistance phenomenon can explain the damage of some acidic foodstuffs kept at low temperatures but also the resurgence of certain diarrhoeic diseases raise by some *Bacillus* species.

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REFERENCES

[1] **F-X. Etoa, B. Bougnom, and K. P. Bogne,** *Méthode alternative à l'isolement des spores bactériennes dans les aliments.* Maîtrise des procédés en vue d'améliorer la qualité et la sécurité des aliments, Ouagadougou, 2005.

[2] **M. Chauliac, and G. R. Pascal,** "L'enfant en milieu tropical : alimentation de rue," *Revue du Centre International de l'Enfance,* no. 213, p. 54, 1994.

[3] **J. Raso, G. Barbosa-Canovas and B. G. Swanson,** "Sporulation temperature affects initiation of germination and inactivation by high hydrostatic pressure of *Bacillus cereus,*" *Journal of Applied Microbiology*, no.85, pp. 17-24, 1998.

[4] **R. J. Lambert and M. Stratford, "**Weak-acid preservatives: modelling microbial inhibition and response," *Journal of Applied Microbiology,* no. 86, pp. 157-164, 1999**.**

[5] **P. J. McClure, M. B. Cole and J. P. P. M. Smelt,** "Effects of water activity and pH on growth of *Clostridium botulinum," Journal of Applied Bacteriology Symposium Supplement,* no. 76, pp. 105S-114S, 1994.

[6] **F-X. Etoa and L. Michiels,** "Heat induced resistance of *B. stearothermophilus* spores," *Letter of Applied. Microbiology,* no. 6, pp. 43-45, 1988.

[7] **F-X. Etoa and G.O. Adegoke, "** Changes in thermoresistance of some *Bacillus* spores after sublethal heat pre-treatment," *Revista. Española de Ciencia y Tecnologia de Alimento*s, vol. 3, no. 35, pp. 285-295, 1995 b.

[8] **Anonymous,** *Methods of microbiological examination for dairy purposes.* British Standards Institution Document. B.S. 4285 London, 1968.

[9] **K. M. Johnson, C. L. Nelson and F. F. Busta, "**Germination and heat resistance of *Bacillus cereus* spores from strains associated with diarrheal and emetic food-borne illnesses," *Journal of Food Science,* no. 47, pp. 101-121 1982.

[10] **Kim and B. Naylor, "**Spore production by *Bacillus stearothermophilus,"*. *Applied Microbiology,* no. 14, pp. 690-691 1966.

[11] **S. K. Long and O. B. Williams, "**Method for removal of vegetative cell from bacterial spore sporulation," *Journal of Bacteriol*ogy, no.76, pp. 332-332, 1958.

[12] **J. H. Hanlin and A. R. Slepecky, "**Mechanism of the heat sensitization of *Bacillus subtilis* spores by ethidium bromide," *Journal of Applied and Environmental Microbiology,* vol. 6, no. 49, pp. 1396-1400, 1985.

[13] **F-X. Etoa and G.O. Adegoke, "**Influence de la thermorésistance thermo-induite (TTI) sur l'isolement des spores bactérienne dans le lait," *Biosciences Proceedings*, no. 2, pp. 268-271, 1991.

[14] **F. Sally, Bloomfied and M. Arthur, "**Mechanism of inactivation and resistance of spores to chemical biocide," *Journal of Applied Bacteriology Symposium Supplement,* no. 78, pp. 91S-104S, 1994.

[15] **A. M. Wright, E. V. Hoxey, C. F. Soper and D. F. G. Davies, "**Biological indicators for low temperature steam and formaldehyde sterilization: effect of variations in recovery conditions on the response of spores of *Bacillus stearothermophilus," Journal of Applied Microbiology*, no. 82, pp. 552-556, 1997.

[16] **F. J. Sala, Ibarz, P. Palop, P. J. Raso and S. Condon, "**Sporulation temperature and heat resistance of *Bacillus subtilis* at different pH's," *Journal of Food Protection,* no. 58, pp. 239-243, 1995.

[17] **G. D. Wolgamott and N. N. Durham, "**Initiation of spore germination in *B. cereus,* a proposed allosteric mechanism," *Canadian Journal of Microbiology,* no. 17, pp. 1043-1048, 1971.

[18] **S. Leaper and K. Bloor, "**A note on the effect storage on the chemical resistance of spores of *Bacillus subtilis* SA22 and *Bacillus subtilis globigii* B 17," *Journal of Applied Bacteriology,* vol. 2, no. 64, pp. 183-186, 1988.

[19] **W. L. Nicholson, N. Munakata, G. Horneck , H. J. Melosh and P. Setlow, "**Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments," *Microbiology and Molecular Biology Reviews,* vol. 3, no. 64, pp. 548, 2000.

[20] **F-X. Etoa,** *Heat-induced resistance of Bacillus stearothermophilus and Bacillus cereus spores in processed foods.* Ph. D. thesis, University of Ibadan, 1993.