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Activity of conessine at various temperatures and pH on inhibition of germination of *Bacillus cereus* and *Bacillus stearothermophilus* spores

Steve Voundi Olugu[1], Maximilienne Nyegue[1,2]*, Patrice Bogne Kamga[3], James Ronald Bayoï[4] and François-Xavier Etoa[1]

[1]Laboratory of Microbiology, Department of Biochemistry, University of Yaounde I, BP 812 Yaounde - Cameroon.
[2]Laboratory of Phytobiochemistry and Medicinal Plantes, Department of Biochimistry, University of Yaoundé I, BP 812 Yaounde - Cameroon.
[3]Laboratory of Microbiology, Department of Food Science and Nutrition, University of Ngaoundere , BP 455 Ngaoundere - Cameroon.
[4]Laboratory of Bioscience, Department of Environemental Science, University of Maroua, BP 46 Maroua - Cameroon.

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This work reports the activity (at various conditions) of conessine isolated from methanolic extract of *Holarrhena floribunda,* on the inhibition of the germination of two *Bacillus* spores species. This activity was studied by treating spores of *Bacillus cereus* T and *Bacillus stearothermophilus* CNCH 5781 with effective concentrations of conessine at various temperature, pH and treatment times. The inhibition of germination was evaluated by the culture of treated spores on agar medium and the number of colony obtained was compared with that of control culture (not treated with conessine). We found that conessine used at 50 and 100 µg/ml for 20 min each decreased considerably the germination of spore of *B. cereus* T and *B. stearothermophilus* CNCH 5781. The maximum temperature of conessine activity for *B. cereus* T was at 30 and 60°C for *B. stearothermophilus* CNCH 5781 spores. Furthermore, the activity of conessine was sensitive to pH change and was more effective at pH 6 on both bacterial spore strains. The treatment of spores with conessine at various lengths of time demonstrated that, the activity of the compound on both bacterial spores was strongly related to the bacterial species. This study suggested that the activity of conessine on the inhibition of germination of *Bacillus* spore depends on physico-chemical factors and the bacterial species.

Key words: Conessine, germination, spores, *Bacillus*.

INTRODUCTION

In many food industries, bacterial spores are forms of microbial contaminants that are most harmful. First, they are difficult to be removed because of their high resistance to physical and chemical agents used in food sterilization. Furthermore, the inactivation of bacterial spores requires high temperatures often combined with pressure (Kramer and Gilbert, 1984; Gerhardt and Marquis, 1989). This causes significant losses of protein and vitamin in foods. At last, several foodstuffs have a short shelf life because, in industry, they are pasteurized to avoid a change in their organoleptic characteristics, leaving within them, a high content of spores.

*Corresponding author. E-mail: maxy_nyegue@yahoo.fr. Tel: 00237 99 95 60 68.

The existence of bacterial spores is not a problem exclusive to only industries. The microbiological quality of many local foods, to craft production in Cameroon comes into play with the high concentrations of bacterial spores.

Such is the case with many sold soups for consumption by children which many studies have revealed spore concentrations exceeding the standard (Bougnom, 2005; Feudjio, 2005). Alarming results were found in several honey samples across the country, as well as the suspected offending food of infant botulism (Etoa and Adegoke, 1996)

However, it should be noted that, although is a major contaminant, bacterial spore itself is of no danger because it can not cause any harm due to its very low metabolism as compared to a vegetative cell. In addition, bacterial spores cannot divide to give new ones. But, spores can respond to specific compound called germinant to give a vegetative cell by the process called germination (Moir, 1990). For instance, spores of *Bacillus cereus* can produce after germination, Gram-positive bacteria widely spread in several foods and drugs, are able to grow in aerobic and anaerobic conditions as well. Furthermore, during germination, some bacterial spore's species can produce food-spoiling toxins. This is the case of *B. cereus* vegetative cell that causes two different types of food poisoning: the emetic syndrome cause by production of non-protein heat stable toxin and the diarrheal syndrome due by an entero-toxin (Granum, 1994). Spores of *Bacillus stearothermophilus*, on its own, can give germination heat resistant vegetative cells that are non pathogenic, but cause outbreaks of several foods.

Control of pathogenic and toxigenic spore strains could result from the ability, either to stop completely spore germination, so that subsequent growth and multiplication could not occur. This kind of process is already applied in some canned food and drug using nysin or tylosin, two antibiotics produce by microorganisms (Meyer et al., 1988). However, few studies concerning the effect of non-germination of bacterial spores by plant compounds have been reported.

In order to promote the use of plant extract to decrease germination of bacterial spore, the investigation done by Bogne (2008) showed that methanolic extract of *Holarrhena floribunda* can decrease germination. At some concentrations of the extract, number of colonies obtained from treated spores was statistically lower than those of non spore control treated with the extract. This activity was later ascribed as conessine (Bogne, 2008), an alkaloid often present in various Apocynaceae that many studies has revealed its important anti-amoebic, antibacterial and antifungal activities (Burn, 1915).

The aim of this work was to evaluate the effect of temperature, pH and exposure time on conessine activity against the germination of spores of *B. cereus* and *B. stearothermophilus*.

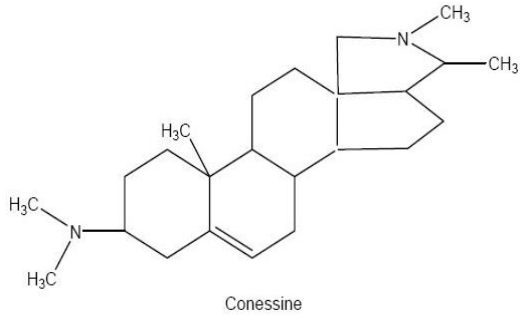

Figure 1. Structure of conessine revealed by spectroscopy (Bogne, 2008).

EXPERIMENTAL

Conessine

Conessine was obtained from the Microbiology Laboratory of the University of Yaoundé I. The methanolic crude extract of *H. floribunda* was used to obtain the molecule using a 72-h maceration of stem bark in methanol. The crude extract was acidified with 5% HCl, the aqueous solution obtained was brought to alkaline pH with ammonia and extracted with ethyl acetate (EA). The methanolic, EA and the remaining fraction resulting from the precedent operations were screened for antigermination activity. The bioactive fraction (EA) was flash-chromatographied on aluminia column under eight solvent systems composed of hexane (Hex) and ethyl acetate (EA) as solvents. The Hex-EA fraction (25-75%) from our active extract was purified by flash chromatography on aluminia column gradiently eluted with Hex-EA to obtain ten fractions (FA1 to FA10). The active fraction FA1 was crystallized from acetone to yield shiny pink crystals identified as conessine (Figure 1) on the basis of spectroscopic data and comparison with reported data (Bogne, 2008).

Bacterial strains

Activity of conessine against germination was evaluated on two *Bacillus* spores: *B. cereus* T spores, obtained from the culture collection of the Microbiology Laboratory of the Institute of food research of Reading, UK and *B. stearothermophilus* CNCH 5781 spores, obtained from the Institut Appert of Paris. These materials were maintained at 4°C before use.

Spore 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 (Neyman and Buchanan, 1985) and spread on plate nutrient agar. The plates were incubated for 24 h at 35°C for *B. cereus* and 63°C for *B. stearothermophilus*, and vegetative cells were obtained. Secondly, the spores were obtained from vegetative cells. Spores of *B. cereus* were obtained according to the protocol described by Johnson et al. (1982) and those of *B. stearothermophilus* were obtained as described by Kim and Naylor (1966). Spores of both species were purified according to the standard method of Long and Williams (1958). Cleaned spores were suspended in distilled sterile water and stored at 4°C for three months.

Determination of effective concentration of conessine for inhibition of germination

The determination of effective concentration of conessine inhibiting germination was done according to the method described by Bogne (2008). Fifteen microliters of heat-activated spores (at 1.8×10^7 spores/ml) of both species were treated at various concentrations of conessine of 100, 50, 25 and 0 µg/ml at 30°C for 20 min. After treatment, in order to evaluate germination, 100 µl of appropriated decimal dilution of treated culture were spread on GPB (*gélose glucosé au pourpre de bromocrésol*). The plates were incubated for 24 h at 35°C for *B. cereus* and 63°C for *B. stearothermophilus*. The number of colonies was enumerated and expressed in percentage germination as compared to that of non treated spore control, subjected to same conditions. The lesser the percentage of germination, the higher the activity of conessine (Hanlin and Slepecky, 1985).

Percent germination (%) = (Number of colony of experimental culture / number of colony of control culture) x 100

Determination of conessine activity at various temperatures

Fifteen microliters of heat-activated spores (at $1.8x10^7$spores/ml) of each bacterial was treated at effective concentration of conessine (50 µg/ml for *B. cereus* and 100 µg/ml for *B. stearothermophilus*) at temperatures of 30, 40, 50 and 60°C for 20 min. Control heat-activated spores none exposed to conessine were treated in the same conditions. Appropriate decimal dilution was spread on agar medium and culture was done as described above. Colonies were enumerated and percentage of germination calculated.

Determination of conessine activity at varying pHs

Fifteen microliters of heat-activated spores (at $1.8x10^7$ spores/ml) of each species were treated at effective concentration and optimum temperature activity of conessine (50 µg/ml at 30°C for *B. cereus* and 100 µg/ml at 60°C for *B. stearothermophilus*) at various pH of 5; 6; 7 and 8 for 20 min. pH were obtained by adding little amounts of HCl 0.2% and NaOH 0.4%. Control heat-activated spores none exposed to the conessine were treated at the same condition. After treatment, the pH of the medium was neutralized and final volume completed at 500 µl. One hundred microliters of appropriated decimals dilution was spread on agar medium and culture was done as described above. Colonies were enumerated and percentage of germination calculated.

Influence of treatment time on activity of conessine

Fifteen microliters of heat-activated spores (at $1.8x10^7$ spores/ml) of each species were treated at effectiveness concentration, optimum temperature and pH of activity of conessine as described above, but at different times of <1; 10; 20; 30; 40 min. Control heat-activated spore not exposed to the conessine was treated at the same condition. After each treatment time, neutralization of the pH of the medium and culture of 100 µl of appropriated decimal dilution were done as described above. Colonies were enumerated and percentage of germination calculated as already described.

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.

RESULTS

Figure 2 presents the results of effect of concentration of conessine, on decrease of percent germination of spores of *B. cereus* and *B. stearothermophilus*. These results show that, some amount of percent germination obtained from cultured treated spores were statistically lower than those of control spore (not treated) at some concentration of conessine, this depend on the bacterial species (at all concentrations of conessine for *B. cereus* spores and only at 100 µg/mL for spores of *B. stearothermophilus*). This suggests that conessine can decrease germination with a strong differential sensitivity depending both on bacterial species and conessine concentration. *B. cereus* appears more sensitive, with maximum decrease of germination observed at 50 versus 100 µg/ml. of conessine for spores of *B. stearothermophilus*. Over 50 µg/ml the effect of conessine on spores of *B. cereus* remained constant.

The comparisons of percent germination of control spore, with those of experimental spores treated at 20 min with effective concentration of conessine (50 µg/ml for *B. cereus* and 100 µg/ml for *B. stearothermophilus*) at various temperatures are shown in Figure 3. The results obtained showed that, inhibition of germination of spores of *B. cereus* is effective only at 30°C. Temperatures equal and more than 40°C did not allow an inhibitory activity of conessine on germination of this strain.

For spores of *B. stearothermophilus,* temperatures of 30, 50 and 60°C showed the percent germination obtained from culture of spores treat with conessine statistically lower than those of control spore (non treated with conessine). These temperatures allowed effective activity of conessine. The maximum activity of compound against germination of spore of *B. stearothermophilus* CNCH 5781 is observed at 60°C.

The percent germination obtained after culture of control spores of *B. stearothermophilus* (non treat with conessine) was greater with increased temperature more than 50°C. For the study of the effect of pH on conessine activity, bacterial spores were treated with 50 µg/ml of conessine at 30°C for *B. cereus* and 100 µg/ml at 60°C for *B. stearothermophilus* at various pHs. The results illustrated in Figure 4 show that, although all the pH used allow an antigerminative effective activity of conessine on all spore species, conessine maximum pH of activity was 6 on both species.

The results of Figure 5 show that, conessine activity depends on treatment time of spores. So, the activity of conessine was effective after 20 min of treatment on spores of *B. cereus* and 10 min on spores of *B. stearothermophilus*.

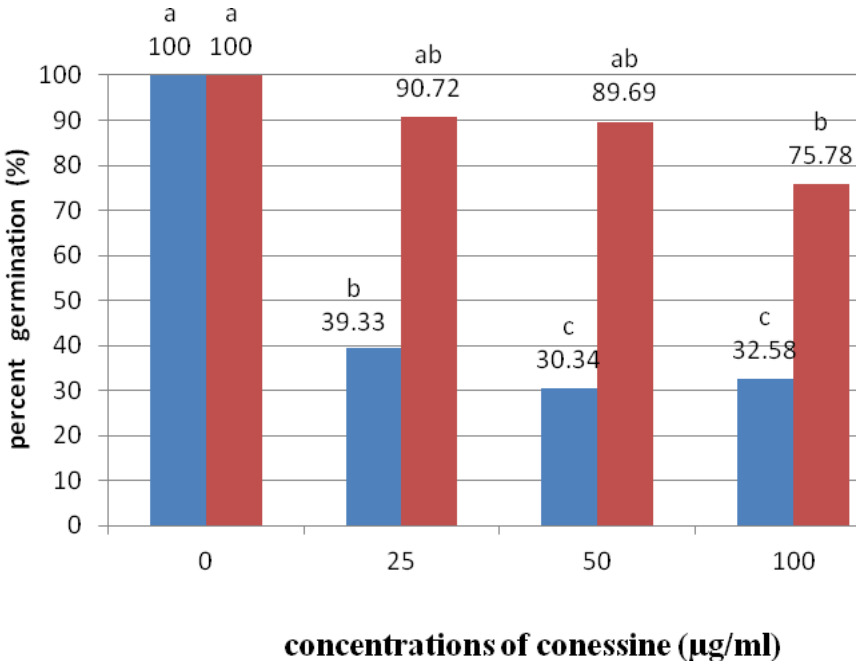

Figure 2. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various concentrations of conessine on germination. Different letters square of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

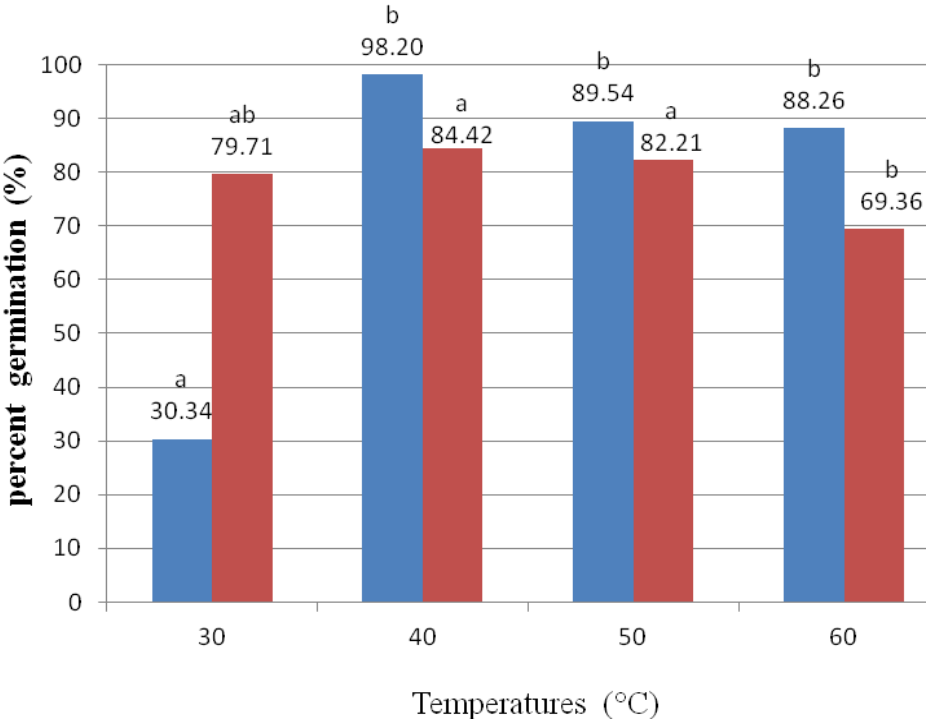

Figure 3. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various temperatures of treatment with conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

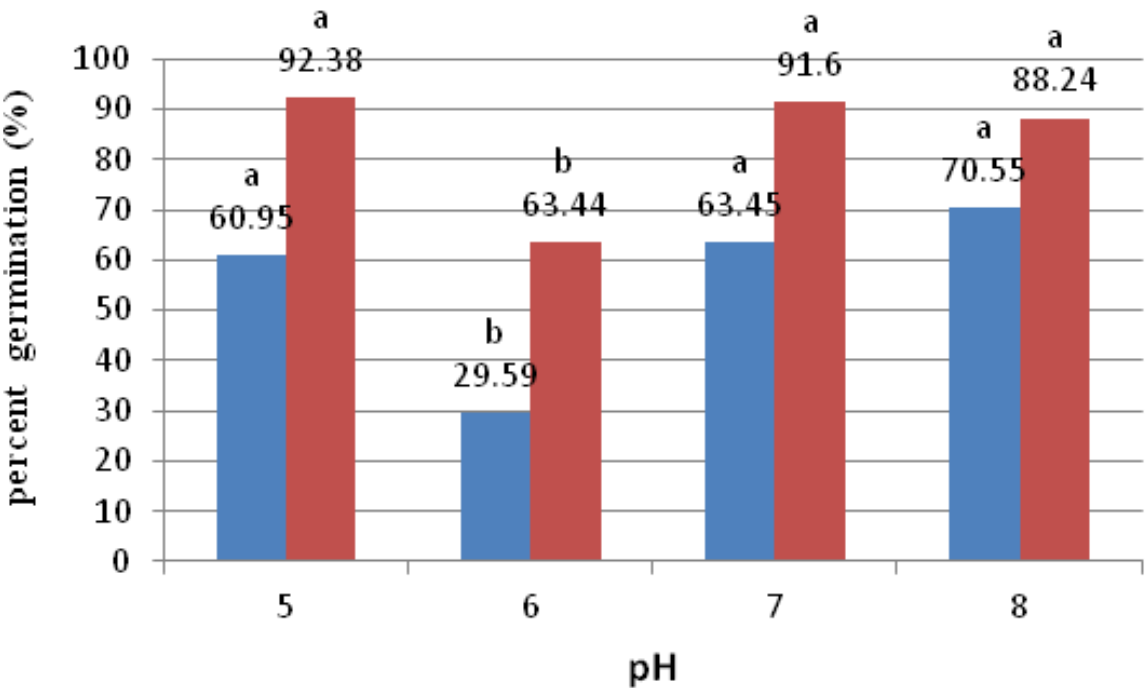

Figure 4. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various pH treatment of conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

At 40 min of treatment, the activities began to slow down.

It was also seen that antigerminative activity of conessine was not immediate because no activity was observed after incubation time which is less than 1 min. Activity of conessine therefore needed treatment time which depended on bacterial species.

DISCUSSION

The results obtained in this study confirm the previous work of Bogne (2008), where it was shown that conessine inhibits germination of spores of *B. cereus* and *B. stearothermophilus*. We think that, conessine could bind to spore surface layer (coat and exosporium), thus contributing to reinforce the dormancy of spore and their resistance to respond to germination. This activity was already observed by Edima et al. (2010) who treated spores with some Cameroonian beers. Another hypothesis could be that, conessine specifically reacts with germination sites of spore and therefore acting as specific inhibitor of germination agents.

Although conessine inhibits germination of two bacterial spores, spores of *B. cereus* were more sensitive than those of *B. stearothermophilus*. Difference of sensitivity of conessine could be explained by possible difference in number and accessibility on sites of fixation of antigerminative substances on spores of different species (Wolgamott and Durham, 1971). Constance activity of conessine on spores of *B. cereus* treated at the concentrations equal or more than 50 µg/ml may be explained by saturation of those active sites on spores.

This work also shows that treatment of spores of both species with conessine is more effective at temperature ranges of 28-35°C for *B. cereus* and 55-65°C for *B. stearothermophilus*. This may be due to the fact that, germinant receptors are generally proteins (Gould, 1970). So, at temperature of optimal growth, those receptors would have specific conformation to react with germinant or inhibitor of germination like conessine.

In addition, lack of conessine activity observed at temperatures equal or more than 40°C on spores of *B. cereus* and at 40°C on spores of *B. stearothermophilus* may be due to the fact that, conessine did not reach or did not attach to its fixation sites at those temperatures. On the other hand, the increase of numbers of colonies of control spores of *B. stearothermophilus* exposed at temperature more than 50°C may be explained by the continuation of activation step already observed on those spores by Etoa (1985).

Activity of conessine depends on pH medium. This factor can influence both compound state (solubility and ionization state) and site of fixation on spore. In this report, we can say that, effect of conessine depending on

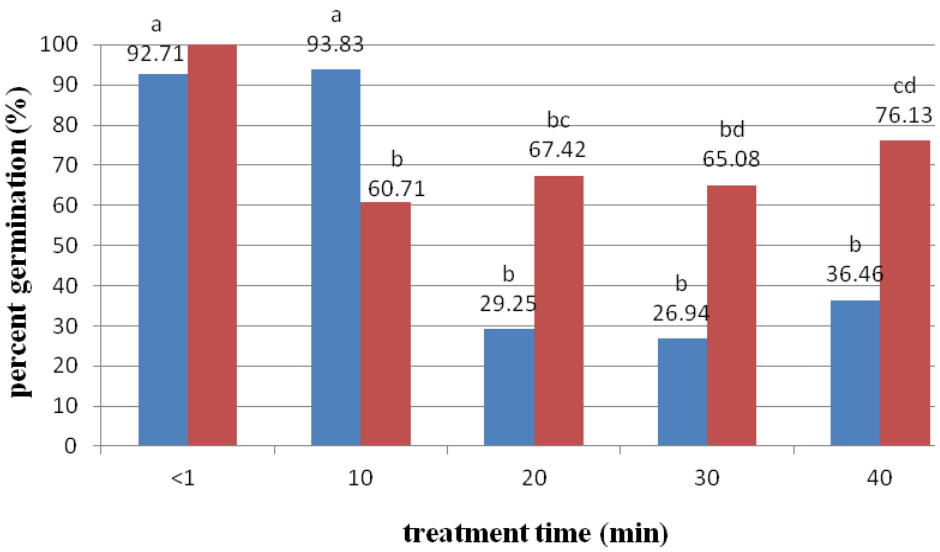

Figure 5. Percent germination of spore of *B. cereus* ( ) and *B. stearothermophilus* ( ) at various treatment time of conessine. Different letter squares of recovery percentage indicate significant difference using Student Newman-Keuls test ($p<0.05$).

pH is due to difference of solubility in solvent used at different pH. Indeed, it was shown that, alkaloid are more soluble in polar solvents (Bruneton, 1999). So, conessine would be more soluble in polar solvents used at acid pH (5 and 6) as compared to the neutral and basic pH used (7 and 8). However, at pH 5, medium would be more acidic to alter spore coats because acid activates spores germination by damage in an irreversible manner spores coats. Then a maximum activity was observed at pH 6 as compared to pH 5.

We have also seen that, conessine activity depends on treatment time which also depends on *Bacillus* species. Antigerminative activity of conessine is not immediate, because no activity was observed after incubation time less than 1 min. The activity needed treatment time which depends on bacterial species. Spores of *B. cereus* which appeared above to be more sensitive needed more time as compared to those of *B. stearothermophilus*. This could be explained by different accessibility of conessine at the site of fixation of spores of both species. Further studies must been done on another *Bacillus* species and *Clostridium.*

Conclusion

In this work, results obtained suggest that, conessine considerably decreased germination of spores of both *B. cereus* T and *B. stearothermophilus* CNCH 5781. This activity depended on physico-chemical factors and the bacterial species. This compound could be used as food additive to extend food shelf-life by inhibiting bacterial spores growth, however, further studies must been done on another *Bacillus* species and *Clostridium*.

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