CHEMICAL SANITIZER DAMAGE OF *BACILLUS CEREUS* T SPORES*)

Suhadi Darmosuwito **)
and
Francis F. Busta ***)

R I N G K A S A N

Telah dipelajari daya kematian dan kerusakan mikrobia dari sodium hipokhlorit (NaOCl) terhadap perkecambahan dan kehidupan spora *Bacillus cereus* T (Tipe Liar). Perhitungan sel hidup dilakukan dengan medium Trypticase Soy Agar (TSA) yang ditambah NaCl pada kadar-kadar tertentu. Perkecambahan spora diamati dengan spektrofotometer dan mikroskop fase kontras.

Daya kematian hipokhlorit ternyata meningkat selaras dengan (a) kenaikan kadar khlor bebas, (b) penurunan pH, (c) lamanya perlakuan, dan (d) pemanasan pendahuluan pada spora. Makin tinggi kadar NaCl dalam TSA ternyata makin sedikit jumlah spora yang tahan hidup.

Pengaruh hipokhlorit pada kadar rendah terhadap perkecambahan bervariasi tergantung pada (a) pemanasan pendahuluan, (b) adanya NaCl dalam medium perkecambahan, dan (c) stimulan yang digunakan.

Khlorinasi sedikit mengurangi response perkecambahan spora terhadap adenosine atau l-alanine. Kombinasi adenine dan l-alanine atau l-alanine dan inosine mempunyai pengaruh sinergitik pada perkecambahan spora yang telah dikhlorinasikan, sedang kombinasi adenosine dan inosine tidak berpengaruh. NaCl dalam medium perkecambahan mendorong terjadinya response bifase pada spora yang telah dikhlorinasikan.

Khlorinasi pada spora yang telah dipanaskan sedikit mengurangi perkecambahan, tetapi tidak mengurangi pertumbuhan keluar dari spora. Khlorinasi menyebabkan "luka" pada spora. Spora-spora ini mempunyai response yang tinggi dalam medium pertumbuhan yang mengandung NaCl (3%).

S U M M A R Y

The germicidal and damaging effect of sodium hypochlorite on the germination and viability of spores of *Bacillus cereus* T (Wild Type) have been studied. Viable counts were evaluated in Trypticase Soy Agar (TSA) containing different concentration of NaCl. Germination was monitored spectrophotometrically and microscopically.

The sporocidal effect of chlorine was found to increase with : (a) the increase of free available chlorine (FAC) concentration; (b) the decrease of pH; (c) the lengthening of exposure time; and (d) the preheat treatment of the spores. Addition of different concentrations of NaCl into TSA medium propotionally reduced the apparent number of the surviving spores.

*) Taken from a plan B project paper submitted to the Faculty of the Graduate School of the University of Minnesota by Suhadi Darmosuwito in partial fulfilment of the requirements for the degree of Master of Science.

**) Department of Microbiology in the Faculty of Agriculture of the Gadjah Mada University. Yogyakarta Indonesia.

***) Professor in Food Microbiology, Director of Graduate Study in the Department of Food Science and Nutrition of the University of Minnesota, St. Paul, Minnesota, U.S.A. 55101.

The effect of the chlorine treatment (25 ppm, pH 7,30 sc) on the germination varied with : (a) preheat treatment; (b) the presence of NaCl in the germination media; and (c) the specific stimulant used. Chlorine treatment slightly reduced the germinal response of unheated spores to adenosine or l-alanine. A combination of adenosine and l-alanine or l-alanine and inosine showed a synergitic effect on the germination of chlorinated and unchlorinated spores, while a combination of adenosine and inosine did not. NaCl in some germination media induced a biphasic response of unheated or heated chlorinated spores. Chlorine treatment on heated spores slightly reduced germination but did not reduce outgrowth of germinating spores. Chlorine treatment enhanced injury of heated spores. These spores responded highly in the presence of NaCl in the recovery medium.

INTRODUCTION

Chlorine as a chemical sanitizer is widely used. It is used in food processing plants, municiple water treatment, swimming pools, sewage treatment, etc. The application of chlorine, included sodium hypochlorite, has a long history.

The bacterial activity of hypochlorite is a function of concentration and the time of exposure. Other factors are pH, temperature, the presence of organic matter, the presence of water hardness compounds, etc. (12). Hays *et al.* (15) showed that bacterial efficiency of hypochlorite is greatly increased when the solution is acidified below pH 6. He noted that at various level of pH 4.0 – 9.0 there is still little loss of available chlorine ($<$ 1 ppm) within 24 hrs.

Odlough and Pfug (18) recalculated the data from several reports in term of time required for a 90% reduction in spores as function of hypochlorous acid concentration. From these data a single graph was prepared. Results in the analysis indicate that *Bacillus* spores are more resistant to chlorine than *Clostridium* spores. The sporocidal effect of chlorine solution increases with (a) an increase in free available chlorine (FAC), (b) a decrease in pH, and (c) an increase in temperature.

The bacilli are considerably more tolerant than the chlostridia to the inclution of NaCl in the recovery medium, but after heating their tolerance are similar (22). It has been shown that lightly heated spores of several species of *Bacillus* need salt concentration of about 15% to prevent germination, while 4 – 7% suffice to prevent out growth (22). NaCl seems possible that its influence is in some way osmotic, and perhaps depends fundamentally on the activity of water in the system.

The transformation of a bacterial endospore into a vegetative cell involves at least three sequential processes : activation, germination, and outgrowth (5). It has been suggested that activation changes the tertiary structure of a protein responsible for a dormant state of spores, and that activation could be considered as a reversible denaturation of the protein (16). Most items which bring about activation are known to cause structural changes in macromolecules (5). Activation by heat, reducing agent, and low pH may involve a reversible reduction of disulfide bond resulting either in an "unblocking" of an enzym system, or in a change in the permeability of a structure controling the dormant state of the spores (16).

During germination of *B. cereus* spores, the following sequence of events are observed : loss of heat resistant, release of Ca^{++} and DPA, release of peptidoglycan, and fall of extinction (8). Phase contrast microscopy observations show that the germination of *B. cereus* and *B. subtilis* spores can be studied a few minutes after the addition of adenosine, and adenosine can be identified in minute concentration.

Adenosine appears to act as an indicator of suitable condition for vegetative existance instaneously inducing physical changes in spores (20). It has been found that a mixture of l-alanin and inosine and various ions is the best germinative solution for most strains of *Bacillus* spores (9).

MATERIALS AND METHODS

Organism

The spores of *Bacillus cereus* T (Wild Type) were used throughout this study.

Media

The growth medium used for building up the inoculum and for production of spore crops was a modification of G-medium as described previously (16, 22). The medium contained : $FeSO_4 \cdot 7H_2O$, 0.0001%; $CuSO_4 \cdot H_2O$, 0.001%; $ZnSO_4 \cdot 7H_2O$, 0.001%; $MnSO_4 \cdot H_2O$, 0.01%; $MgSO_4 \cdot H_2O$, 0.04% $(NH_4)_2SO_4$, 0.1%; glucose 0.1%; K_2HPO_4, 0.05%; $CaCl_2 \cdot 2H_2O$, 0.008%; and yeast extract 0.2%. Concentrated stock solutions of glucose, K_2HPO_4 and $CaCl_2 \cdot 2H_2O$ were prepared in 100 x of concentrations and yeast extract in 50 x of concentration, sterilized at 121°C for 15 min, and stored at 3°C before using. A solution of amonium sulfate was separately prepared in 100 x of concentration (solution A), and the four other minerals were mixed in one solution of 100 x of concentration (solution B). The broth was freshly prepared from solution A and B, adjusted to pH 7.8, dispensed into Erlenmeyer flasks and sterilized at 121°C for 15 min. The final pH is about 7.0 – 7.2. To avoid precipitation during sterilization and storage, the sterile stock solutions were added aseptically to each flask at the time of inoculation.

Trypticase Soy Agar (TSA) was used for viable counts of spores. The medium contained pepton (BBL), 1.5%; Phyton pepton (BBL) 0.5%; NaCl, 0.5%; and Bacto-agar (Difco), 1.5%. Additional NaCl of various concentrations between 0 and 10% were added to the medium.

Preparation of Spore Crops

The active cultures for building up the inoculum were successively prepared in G-medium. The first culture was prepared by inoculating 100 ml G-medium with a spore suspension to obtain 0.02 – 0.03 OD (about 10^6 spores/ml) agitated at 190 rpm in an incubating shaker at 30° C for approximately 3 hr until the OD reached 0.2 – 0.3 (about 10^7 spores/ml). The second culture was prepared as the first with 10% inoculum from the first culture and shake-incubated for 1½ hr until the OD reached 0.2 – 0.3. The third culture was prepared from the second with the same procedure.

The sporulating culture was prepared in 1L G-medium with 10% inoculum of the third culture. Complete sporulation was obtained in about 15 hr in an incubating shaker. The spores were harvested and washed as described by Gould (13). The yield of spore crops from three cultures was about 60 ml, with the population mean of 5.6×10^{10} spores/ml, as determined with a Petroff–Hausser bacteria counter. The spore crops were stored at 3° C in water.

Heat Activation

The spores were adjusted to desired concentrations in 50 mM pre-equilibrated phosphate buffer pH 7 and heated at 70° C for 10 min. After heat treatment the spores were cooled in ice for 5 min.

Chlorine Treatment

The spores were adjusted to desired concentrations in 50 mM phosphate buffer pH 7, 6, or 5 and treated with sodium hypochlorite solution (pH 7, 6 or 5) at desired final concentration of free

available chlorine (FAC) for certain exposure times as described by Weber and Black (29) with some modification. The chlorine solutions were freshly prepared from bleach containing about 5% sodium hypochlorite. The FAC expressed as ppm (part per million) was determined by Iodometric titration (2, 3, 15). The neutralizer was 0.05 N sodium thiosulfate solution.

Viable Count

The spores were adjusted to desired concentrations in 50 mM phosphate buffer pH 7 or in sterile deionized water as required. After dilutions were made, 0.1 ml of appropriate suspension was surface-plated in duplicate on TSA containing different concentrations of additional NaCl. The plates were incubated at 30° C for 24 hr. The plates containing 3% NaCl or more needed 42 – 48 hr of incubation to develop countable colonies. The colonies were counted with a Darkfield Quebec colony counter, and were expressed in Colony Forming Units (CFU) per ml.

Germination

The spores were adjusted to a final concentration of about 5×10^7 spores/ml (OD 0.3 – 0.4) in temperature-controled cuvettes at 30° C containing germination medium. The OD changes of the spore suspensions were continuously monitored at 650 mm with a Bosch and Lomb Spectronic 20 spectrophotometer. The germinants used were adenosine, l-alanine, glucose, l-glycine, and inosine; each with a final concentration of 10 mM in 50 mM phosphate buffer pH 7. The germinants were tested separately and in combination with eachother. For controls, deionized water, phosphate buffer (50 mM, pH 7) and Trypticase Soy (TS) medium (TSA without agar) were used as germination media; each without and with addition of 3% NaCl. The data were converted to the percentage of initial OD by using the following equation : percent initial $OD = OD_t/OD_i \times 100$, where OD_i represents the OD at the time of administration of spores into germination media, and OD_t is the OD at time t (23).

Phase-Contrast Microscopy

A Unitron phase-contrast microscope equipped with a 100 x objective lens was used for dark-field phase-contrast microscopy. A drop of spores (about 10^7/ml) were put on a slide layered with TSA medium (without or with additional NaCl), covered with a cover slip and examined under the microscope periodically. Bacterial spores appear bright under phase-contrast illumination and become dark during normal germination. Outgrowth was seen as elongation of germinated spore. The percentages of germinating and outgrowing spores were recorded periodically. The slides were incubated at 30° C.

RESULTS

Effect of Sodium Chloride in TSA

Figure 1 shows the effect of NaCl added to TSA medium on the viability of unheated and heat-activated (70° C, 10 min) spores. The data show that the higher NaCl content, the less CFU indicating less viable spores. When the medium did not contain additional NaCl, the CFU of heat-

activated spores were higher than unheated spores. When the media contained 1 – 5% additional NaCl, heat-activated spores gave CFU numbers less than unheated spores. The greatest difference in CFU numbers was between 3 and 4% NaCl added to media. There was little difference in the number of CFU of heated and unheated spores when NaCl concentration was more than 5%. When spores were heated at 90° C for 10 min (data not shown), their viable counts in the presence of NaCl were much lower. The data indicate that heat-activated spores were more sensitive to inhibitory effect of NaCl.

Effect of Free Available Chlorine (FAC) Concentration

Figure 2 indicates the effect of FAC concentration (pH 7) on the reduction rates of spores grown in TSA containing 0, 3, and 5% additional NaCl. The reduction rate was expressed in time for 90% reduction in the number of viable spores, as done previously (21). These numbers were obtained from the survivor curves of different FAC concentrations. Each graph in Figure 2 represents the chlorine-concentration time curve of the spores in TSA containing different concentrations of NaCl. Z value represents the slope of the curve, especially at the first 10 to 25 ppm FAC concentration range. It is also the number of ppm of FAC change necessary to change the chlorine-reduction time by one log cycle. Z value in TSA containing 3% additional NaCl was the smallest (Z = 8 ppm). This medium had the optimum effect in reducing the viable counts with increasing of FAC concentrations. Each graph shows that above 25 ppm FAC, the reduction rate of chlorine decrease. This indicates that after a marked decrease in number of survivor, the remaining spores were the most resistant; or that concentration became less influential as it increased from 20 to 50 ppm.

Effect of Heat Activation, pH and Time of Chlorine Exposure

Figure 3 shows graphically the effect of heat activation of spores, pH, and time of exposure of chlorine treatment on the viable counts of spores grown in TSA media containing 0, 3, and 5% additional NaCl. These data indicate that there was a reduction in number of CFU; (a) with the increasing of additional NaCl in media from 0 to 5%; (b) with lowering the pH of chlorine treatment from pH 7 to 6; (c) with heating the spores at 70° C for 10 min before chlorine treatment; and (d) with lengthening the time of chlorine exposure. Compared with unheated spores, heat-activated spores (unchlorinated and chlorinated) produced lower CFU numbers when grown in TSA without and with an additional 3% of NaCl. When they were grown in TSA with 5% additional NaCl, they produced higher CFU numbers. This shows that 3% additional NaCl in TSA was the most effective in helping to reduce the viable spores after heat treatment and/or chlorine treatment. The data confirm that efficiency of chlorine increased with lowering the pH and lengthening the time of exposure.

Germination of Unheated Spores

The nature of the response to the specific germinant and the effect of chlorine treatment and heat-activation are shown in Figures 4 to 8. For each case, the response of unheated spores (portion A), is compared with heated spores (portion B). In each portion, the response of unchlorinated spores is compared with chlorinated spores and the response in the absence of NaCl is compared with that in the presence of NaCl.

Without heat activation, the responses of spores to germination were very small, especially the response to l-alanine (Figure 7A) and in buffer (Figure 4A). Chlorinated spores were slightly less

responsive to the germinant than unchlorinated spores. Addition of NaCl decreased their responses, but chlorinated spores showed slightly higher responses than unchlorinated.

In TS medium (Figure 5A), chlorinated and unchlorinated spores had coincident responses. At first, the response of chlorinated spores was slightly lower, but after 60 min it was slightly higher than unchlorinated spores. This indicates that chlorine treatment on unheated spores slightly retarded the germination in TS medium.

When adenosine was the stimulant (Figure 6A), the slow responses of unchlorinated and chlorinated spores were still increasing at 90 min; when NaCl was added these responses were practically constant at a low level. When inosine was the germinant (Figure 8A) the responses were the highest, although the germination rates were still slow. Chlorine treatment apparently did not affect the response. Chlorine treated spores in the presence of NaCl showed a biphasic response to inosine, while unchlorinated spores exhibited their slow response through 90 min. This indicates that chlorine treatment slightly enhanced the response of unheated spores to inosine stimulation in the presence of NaCl.

When adenosine plus l-alanine (data not shown) were the germinants, they showed a synergetic effect on unheated spores, even when the spores were treated with chlorine. The other synergetic effects were also obtained when l-alanine plus inosine (Figure 9) were the germinants, but not when adenosine plus inosine were used (data not shown). When NaCl was added their synergetic effects disappeared. Their effects were somewhat additive.

Germination of Heat-Activated Spores

Heat-activated spores showed more active responses to germinant stimulation. Their responses were much higher than unheated spores (Figures 4 to 8). The germinations in buffer and in TS media were about one-half and two-thirds respectively, compared with those in the presence of adenosine, alanine or inosine.

In buffer solution (Figure 4B), chlorine treatment on heat-activated spores reduced the germination by approximately one-half. The same result was obtained from heated-unchlorinated spores when NaCl was added. A greater effect was obtained when heat-activated spores were treated with chlorine, and NaCl was added to the buffer medium.

TS medium (Figure 5B) showed a good stimulation effect of outgrowth. Chlorine treatment of heat-activated spores reduced the germination in TS, but didn't inhibit the outgrowth. NaCl in TS medium retarded the germination of heat-activated spores and inhibited the outgrowth, but did not reduce the germination.

When adenosine (Figure 6B) was the germinant, the response of heat-activated spores was very high. Chlorine treatment of these spores reduced the germination but did not inhibit outgrowth. Addition of NaCl did not reduce the germination, but inhibited the outgrowth. The very similar results were observed when inosine was germinant (Figure 8B). NaCl seems to enhance injury to heated-chlorinated spores stimulated by adenosine or inosine. Their germinal responses appeared biphasic.

The effect of alanine (Figure 7B) as a stimulant to heat-activated spores was medium fast. Chlorine treatment of these spores reduced germination to about one-third. About the same result was obtained when NaCl was added, but the response was slower. When NaCl was added, the response of heated-chlorinated spores to alanine stimulated germination markedly decreased.

The response of heat-activated spores to various combinations of two or more germinants were maximum high (about 60% germination). Chlorine treatment of these spores reduced germination, and NaCl in the germination media only retarded germination. The biphasic responses in the presence of NaCl in these media was not readily apparent. Data for the alanine-inosine combination are shown in Figure 9.

Germination With Other Stimulants

The stimulation response to glucose (data not shown) was very low (OD drop < 5%), even when the spores were heat-activated (OD drops < 20%). The heated-chlorinated spores showed the highest response to glucose. Chlorine treatment of heated spores seems to enhance the stimulation effect of glucose. Addition of NaCl in the germination medium slightly reduced the germination.

L-glycine (data not shown) gave almost the same results with glucose. The stimulation effect of l-glycine was not actively apparent. The highest response was obtained when unheated spores were chlorinated (OD drops about 20%). Chlorine seems to enhance the stimulation effect of glycine.

When l-alanine plus l-glycine were the stimulants (data not shown) the responses of unheated spores (chlorinated and unchlorinated, with and without NaCl addition) were almost at the same low level with the responses to l-alanine alone. When the spores were heated, their responses to alanine plus glycine were higher than those to glycine but lower than those to alanine. These indicate that l-glycine reduced the stimulation effect of l-alanine on heated spores, as shown previously (11).

Outgrowth

Microscopic examination of germinating spores (Figure 10) indicate that chlorine treatment of unheated spores did not clearly reduce germination, however, it reduced outgrowth. The data supported the previous data in Figure 5. Although there was a reduction in outgrowth, the final results of CFU numbers of unchlorinated and chlorinated spores were the same (Table 1). These indicated that chlorine treatment of unheated spores did not apparently reduce germination nor outgrowth nor, vegetative growth. When NaCl was added (Figures 10A, B) chlorine treatment seemed to reduce the germination only, the same as indicated in Figure 5, and resulted in about 35% reduction of CFU number (table 1).

When spores were heated, chlorine treatment very slightly reduced germination and retarded outgrowth (Figure 10C, D), and resulted in about 60% reduction in CFU number (Table 1). When NaCl was added, chlorine treatment slightly increased germination and outgrowth (Figure 10C, D), and resulted in about a 30% increase in CFU number (Table 1). These indicated that heated spores were more sensitive to chlorine, and that chlorine causes injury to the spores. When NaCl was added to the medium, the injured spores recovered better.

Data in Table 1 show that less than 50% of the total spores were capable of forming colonies in TSA. When the spores were heated, about three-fourths of them could germinate, but only some of the amount resulted in outgrowth and vegetative growth (Figure 10, Table 1). This fact may explain that although the germination and outgrowth levels of unheated spores were very low, they could produce a fairly high number of CFU compared with heated spores.

DISCUSSIONS

The results of the present study agree with the previous data (21) that *Bacillus cereus* T was able to grow in 7% NaCl, but was not in 10% NaCl. There is a reduction of survivor spores with increased NaCl concentrations from 0 to 7.5%. When the spores were heat-activated (70^{o} C, 10 min), they were more sensitive to NaCl toxicity. Addition of NaCl between 1 and 5% was found to be more active

toward heat-activated spores than toward unheated spores. These results supported the previous finding (22) that aerobic and anaerobic spores given increasing heat treatments became progressively more sensitive to the effect of curing agents in growth media. The same results were reported for heated spores of putrefactive anaerobes (6). The effect of NaCl partly depends on the media used (21).

The data confirm that the sporocidal effect of chlorine solution increases with; (a) an increase of FAC concentration (4, 17, 18, 26); (b) a decrease in pH of treatment (12, 15, 17, 18); (c) a lengthening of exposure times (27), and also with preheat treatment of the spores. The last recognition was also supported by data of germination experiments. After various treatments of heat and chlorine the very resistant spores still survived and were able to germinate and grow. This may indicate that population of bacterial spores is heterogenous as regards germinability and viability.

The data of germination indicate that the influence of chlorine treatment on the germicidal responses varies with heat-activation of spores, the presence of NaCl in germination media, and the specific stimulant used. Without heat activation, chlorine only slightly affected the germinal response to adenosine, l-alanine, inosine, TS medium, or buffer. Glycine, an inhibitor of l-alanine for *B. megaterium* (11) highly induced the germination of chlorine treated spores of *B. cereus* T. The presence of NaCl in the germinative media slightly affected the response of chlorinated and unchlorinated spores to various stimulants. When inosine was the germinant, chlorine treatment slightly enhanced the response of unheated spores in the presence of NaCl. Few strains are capable of substantial germination in NaCl solution alone (9). NaCl does not seem to have a stimulation effect of *B. cereus* T spores.

When the spores were heat-activated, chlorine treatment reduced germination but did not inhibit the outgrowth of germinating spores. When chlorine is put in the germination media (28), its effect is stimulation to germination followed by inactivation of germinated spores. Our data indicate that heat-activated spores were more sensitive to chlorine treatment. Heat activation has been observed to induce at least four changes (5). It increases the germination rate, activates enzymes which are dormant in the resting spores, changes germination requirements and induces changes in morphology, permeability and spore composition.

The mode of action of chlorine on spores has been shown to cause to release of DPA (1, 8) and ninhidrine-positive material (8). It has been shown that heating at 80° C for 10 min was too drastic for optimal recovery of chlorine-treated spores of *Cl. welchii* (8).

It is suggested that heat-activated spores, because of permeability and spore composition changes, permit the penetration of active germicidal principles into the spore. The chemical combination of this ingredient with protoplasm is responsible for the damage or death of the spores. The spores which survived at 25 ppm chlorine treatment were able to germinate and outgrow.

The data indicate that the presence of NaCl in germination media induced a biphasic response from unheated-chlorinated spores in inosine and from heated-chlorinated spores in adenosine and inosine. The nature of the biphasic response is not caused by two subpopulations of spores, one with minimal microlag time, the other with much longer microlag time (23, 24). The biphasic response may be due to the loss of DPA and heat resistance of spores during the initial phase of germination (14, 23). Treatment with chlorine solution alone reduced the germination but didn't induce a biphasic response.

The occurrence of heat injury seems to result in more susceptibility to certain chemicals. Shacter and Hasimoto (23) gave some explanation; (a) such injury makes the chemical more easily and selectively accessible to the critical sites of reactions vital for the initiation or the progress of the second phase germination; (b) sublethal dose of heat may partially inactivate some of the enzymes involved in the reactions mentioned above; or, (c) extended heat treatment may cause the alteration of the structural components of spores in such a manner that the rapid degradation of these structures normally occurring during germination become difficult.

Outgrowth is characterized by a significant rise of respiration activity and by the ordered synthesis of new macromolecules i.e., nucleic acids and cell wall peptido-glycans (5, 25). Therefore, their step may be inhibited by many common inhibitors. It is suggested that inhibition of protein synthesis results in a rapid blockage of further development of outgrowing bacterial spores (25). The data of outgrowth indicate that heated spores were more sensitive to chlorine action, and resulted in chlorine injury of the spores and so a lower number of CFU.

The present data indicate that only some of the total spores were able to grow and produce CFU. The previous finding (20) explains that the media in which vegetative forms grow readily for many subcultures do not necessarily induce spore germination. Another explanation is the existance of a superdormancy (10). Although all spores are dormant, some spores in any population are more dormant than the others. The spores are reluctant to germinate, even with various activations. Such spores would remain essentially viable and recoverable if they could be germinated by some technique.

The results of this study concluded that the sporocidal effect of sodium hypochlorite increased with; (a) the increase of free available chlorine concentration; (b) the decrease of pH; (c) the lengthening of exposure time; and, (d) the heat treatment of spores. Addition of different concentrations of NaCl into Trypticase Soy Agar medium reduced proportionally the apparent number of the surviving spores.

The effect of the chlorine treatment on germination varied with; (a) pre-heat treatment; (b) the presence of NaCl in the germination media; and, (c) the specific stimulants used. Unheated spores showed less active responses than heated spores, and chlorine treatment only slightly affected their germinal responses. Combination of two stimulants might result in a synergetic, additive, or inhibitive response of germination, and chlorine treatment did not affect this response. NaCl in some germination media induced the biphasic response of unheated and heated-chlorinated spores.

Chlorine treatment on heated spores slightly reduced germination but did not reduce the outgrowth of germinating spores. Chlorine treatment enhanced injury of heated spores. When NaCl was added to the recovery medium, the injured spores recovered better.

ACKNOWLEDGEMENTS

This investigation was supported by MUCIA – AID – Indonesian Higher Agricultural Education Project and was done in the Department of Food Science and Nutrition of the University of Minnesota. They are sincerely appreciated.

REFERENCES

1. Alderton, G. and Halbrook, W.U. 1971. Action of chlorine on bacterial spores. Bacterial Proc., 12.
2. Anon. 1960. Standard methods for the examination of water and wastewater. American Public Health Assoc., Inc., New York.
3. Anon. 1972. Standard methods for the examination of diary products. 13th ed. by W.J. Hausler Jr. American Public Health Assoc., Inc., Washington, D.C.

4. Benarde, M.A., Snow, W.B., and Olivieri, V.P. 1967. Chlorine dioxide disinfection temperature effects. J. Appl. Bacteriol. 30(1), 159 – 167.

5. Berg, R.W. and Sandine, W.E. 1970. Activation of bacterial spores. A review. J. Milk Food Technol. 33. 435 – 441.

6. Duncan, C.L. 1970. Arrest of growth from spores in semi-preserved foods. J. Appl. Bacteriol. 33, 60 – 73.

7. Dring, G.J. and Gould, G.W. 1970. Sequence of events during rapid germination of spores of *Bacillus cereus*. J. Gen. Microbiol. 65, 101 – 104.

8. Dye, M. and Mead, G.C. 1972. The effect of chlorine on the viability of clostridial spores. J. Food Technol. 7, 173 – 181.

9. Foerster, H.F. and Foster, J.W. 1966. Response of *Bacillus* spores to combination of germinative compounds. J. Bacteriol. 91(3), 1168 – 1177.

10. Gould, G.W. 1970. Germination and the problem of dormancy. J. Appl. Bacteriol. 33, 34 – 49.

11. Gould, G.W. 1971. Methods for studying bacterial spores. *Methods in Microbiology*. Vol. 6A. Academic Press, London and New York. 327 – 381.

12. Hadfield, W.A. 1957. Chlorine and chlorine compounds. Introduction and historical review. In Anticeptic, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. by G.F. Reddish. Lea and Febige, Philadelphia. 558 – 580.

13. Halverson, H.O. 1957. Rapid and simultaneous sporulation. J. Appl. Bacteriol. 20(3), 305 – 314.

14. Hasimoto, T., Frieben, W.R. and Conti, S.F. 1972. Kinetics of germination of heat-injured *Bacillus cereus* spores. Spores V. 409 – 415. Ed. by Halvorson, R. Hanson, and L.L. Cambel. American Society of Microbiology, Washington, D.C.

15. Hays, H., Elliker, P.R., and Sandine, W.E. 1963. Effect of acidification on stability and bactericidal activity of added chlorine in water supplies. J. Milk Food Technol. 26(5), 147 – 150.

16. Keynan, A., Evenchik, Z., Halvorson, H.O., and Hastings, J.W. 1964. Activation of bacterial endospores, J. Bacteriol. 88(2), 313 – 318.

17. LaBree, T.R., Field, M.L., and Desrosier, N.W. 1960. Effect of chlorine on spores of *Bacillus coagulans*. Food Technol. 14(12), 632 – 634.

18. Odlaug, T.E. and Pflug, I.J. 1976. Sporocidal properties of chlorine compounds : Applicability to cooling water for canned foods. J. Milk Technol. 39(7), 493 – 498.

19. Pelcher, E.A., Fleming, H.P., and Ordal, Z.J. 1963. Some characteristics of spores of *Bacillus cereus* produced by a replacement technique. Canadaian J. Microbiol. 9, 251 – 258.

20. Pulvertaft, R.J.V., and Haynes, J.A. 1951. Adenosine and spore germination; Phase contrast studies. J. Gen. Microbiol. 5, 657 – 663.

21. Raevuori, M. and Genigeorgis, C. 1975. Effect of pH and sodium chloride on growth of *Bacillus cereus* in laboratory media and certain foods. Appl. Microbiol. 29(1), 68 – 73.

22. Roberts, T.A. and Ingram, M. 1966. The effect of sodium chloride, potassium nitrate and sodium nitrite on the recovery of heated bacterial spores. J. Food Technol. 1, 147 – 163.

23. Shachter, S.M. and Hashimoto, T. 1975. Bimodal kinetics of germination of *Bacillus cereus* T spores. Spores V, 531 – 538. Ed. by P. Gerhadt, R.N. Costilow, and H.L. Sadoff. American Society for Microbiology, Washington, D.C.

24. Sogin, M.L., McCall, W.A., and Ordal, Z.J. 1972. Effect of heat activation conditions on the germinal response of *Bacillus cereus* T spores. Spores V, 363 – 367. Ed. by H.O. Halvorson, R. Hanson, and L.L. Campbell. American Society for Microbiology, Washington, D.C.

25. Vinter, V. 1970. Germination and outgrowth: Effect of inhibitors. J. Appl. Bacteriol. 33, 50–59.

26. Waites, W.M., Wyatt, L.R. and Arthur, B. 1972. Effect of alkali treatment on the germination and morphology of spores of *Clostridium bifermentans.* Spore V, 430 – 436. Ed. by H.O. Halvorson, R. Hanson, and L.L. Campbell. American Society for Microbiology, Washington, D.C.

27. Weber, G.R. and Black, L.A. 1948. Laboratory procedure for evaluating practical performance of quarternary amonium and other germicides proposed for sanitizing food utensils. American J. Public Health. 2, 282 – 287.

28. Wyatt, L.R. and Waites, W.M. 1973. The effect of hypochlorite on the germination of spores of *Clostridium bifermentants.* J. Gen. Microbiol. 78, 383 – 385.

Table 1

Effect of Sodium Hypochlorite Treatment on the Viability of *B. cereus* T spores

Treatment	Spores/ml	Number of Determination
Direct Microscopic Count (DMC)		
– untreated spores (control)	5.6×10^6	3
Viable Count (CFU/ml in TSA)		
– unheated, unchlorinated, no NaCl	2.6×10^6	14
– unheated, unchlorinated, NaCl added	2.0×10^6	13
– unheated, chlorinated, no NaCl	2.6×10^6	6
– unheated, chlorinated, NaCl added	1.3×10^6	6
– heated, unchlorinated, no NaCl	2.1×10^6	6
– heated, unchlorinated, NaCl added	8.1×10^5	5
– heated, chlorinated, no NaCl	6.9×10^5	3
– heated, chlorinated, NaCl added	1.1×10^6	3

DMC – as determined with Petroff-Hausser bacteria counter
Heated – 70° C, 10 min, pH 7
Chlorinated – 25 ppm FAC, pH 7, 30 sec
No NaCl – TSA contain 0.5% NaCl
NaCl added – 3% NaCl added to TSA

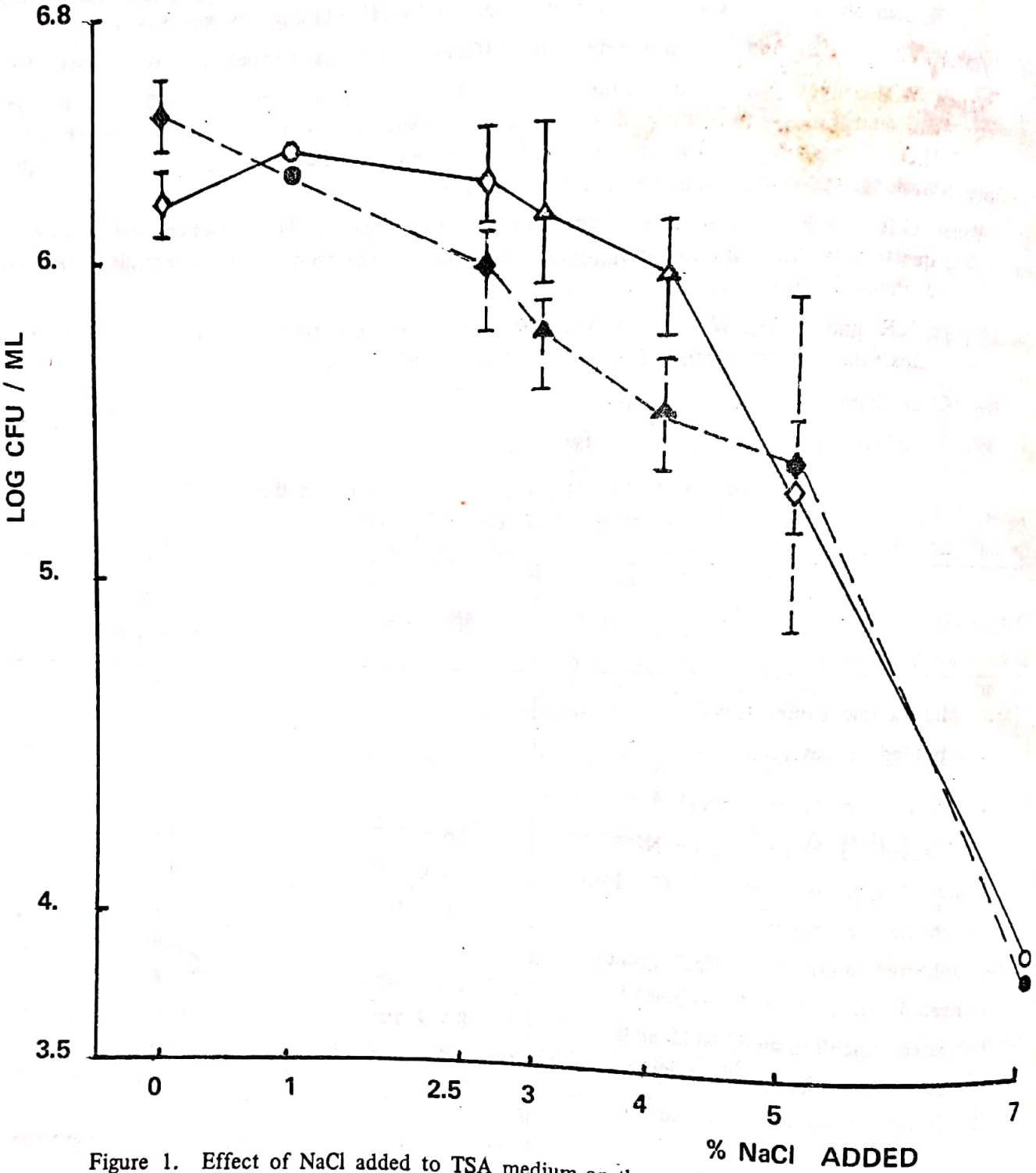

Figure 1. Effect of NaCl added to TSA medium on the viability of unheated and heated (70°C. 10 min, pH 7) spores of *B. cereus* T. *Symbols* : open, unheated; close heated; O. Δ, and ◇ represent results of 1, 2, and 3 experiments respectively; bars are one standard deviation either side of the mean, solid line for unheated and dashed line for heated spores.

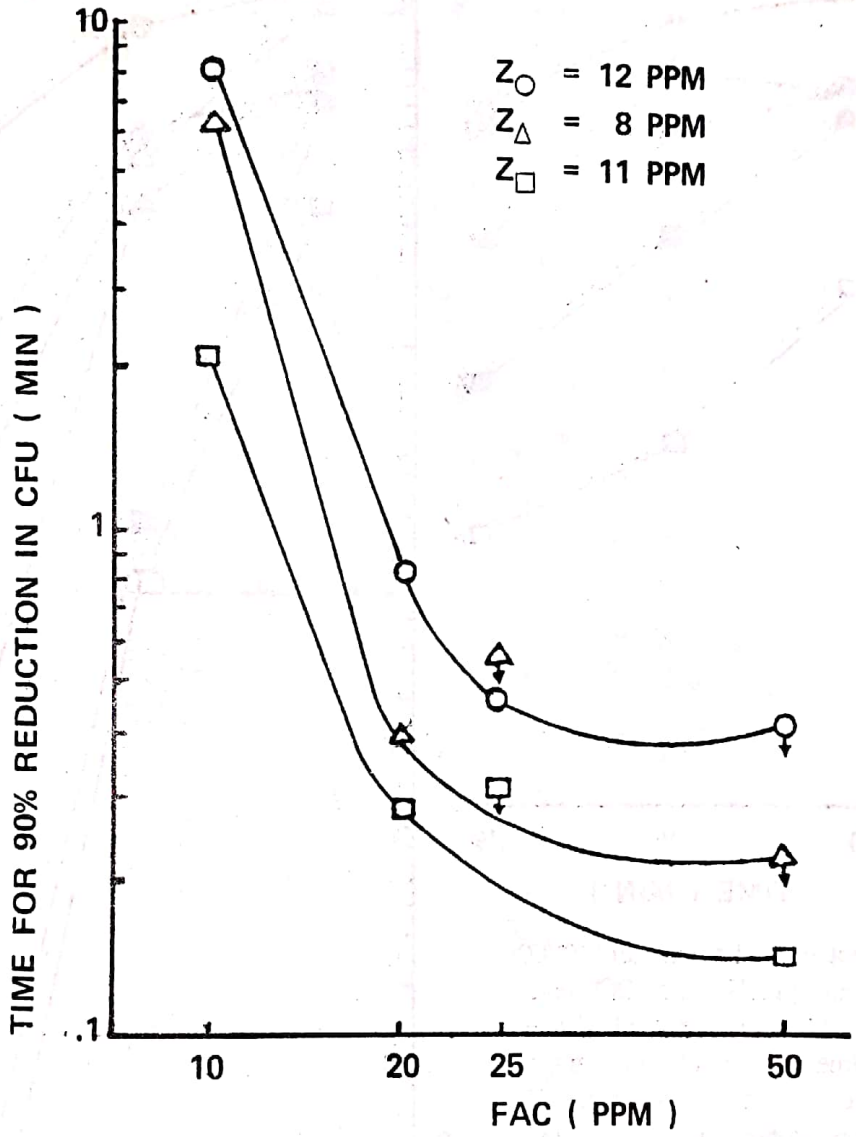

Figure 2. Effect of NaOCl (pH 7) concentration treatment of unheated spores on the reduction rate of *B. cereus* T spores grown on TSA containing 0% (○), 3% (△), and 5% (□) additional NaCl.

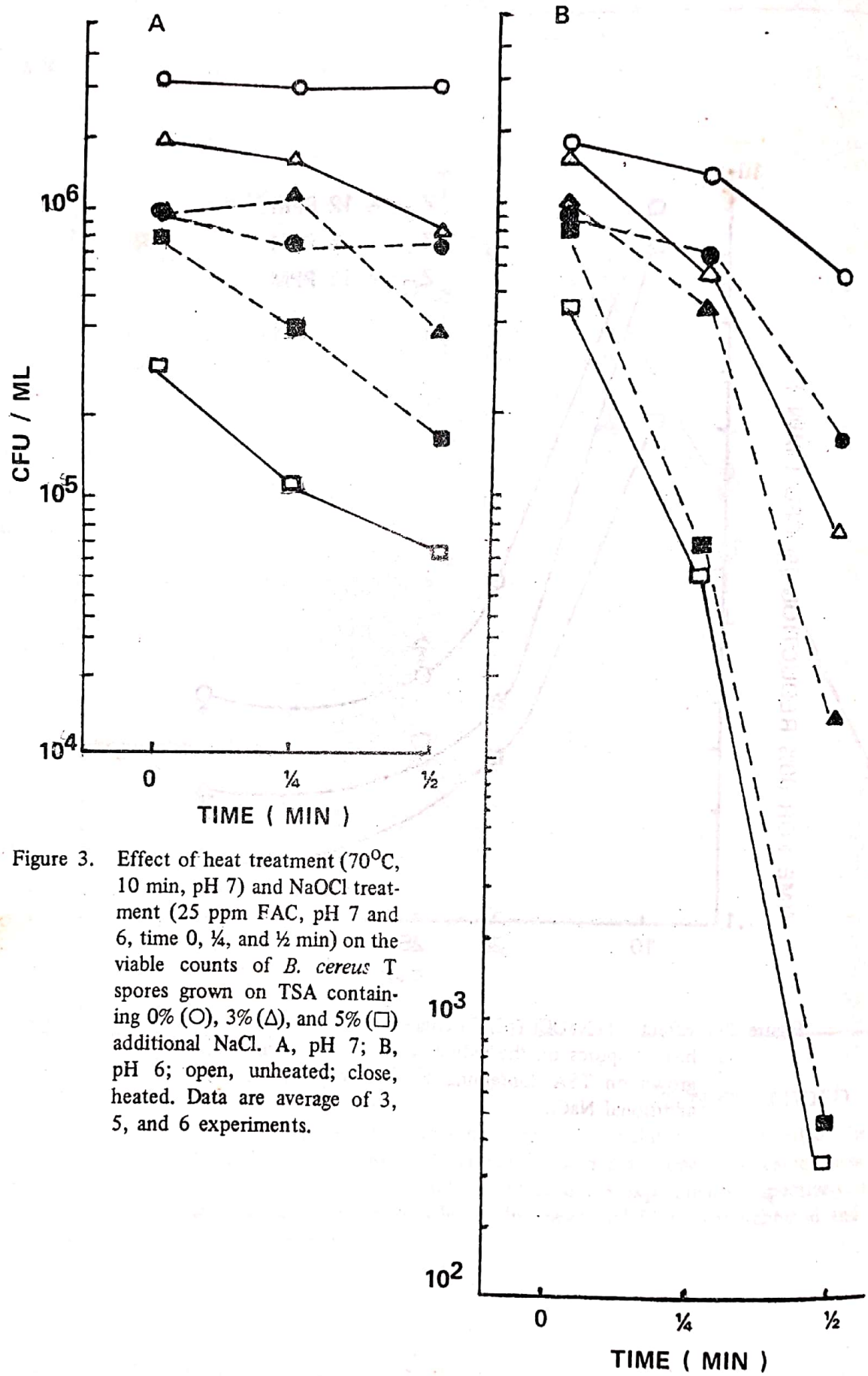

Figure 3. Effect of heat treatment (70°C, 10 min, pH 7) and NaOCl treatment (25 ppm FAC, pH 7 and 6, time 0, ¼, and ½ min) on the viable counts of *B. cereus* T spores grown on TSA containing 0% (○), 3% (△), and 5% (□) additional NaCl. A, pH 7; B, pH 6; open, unheated; close, heated. Data are average of 3, 5, and 6 experiments.

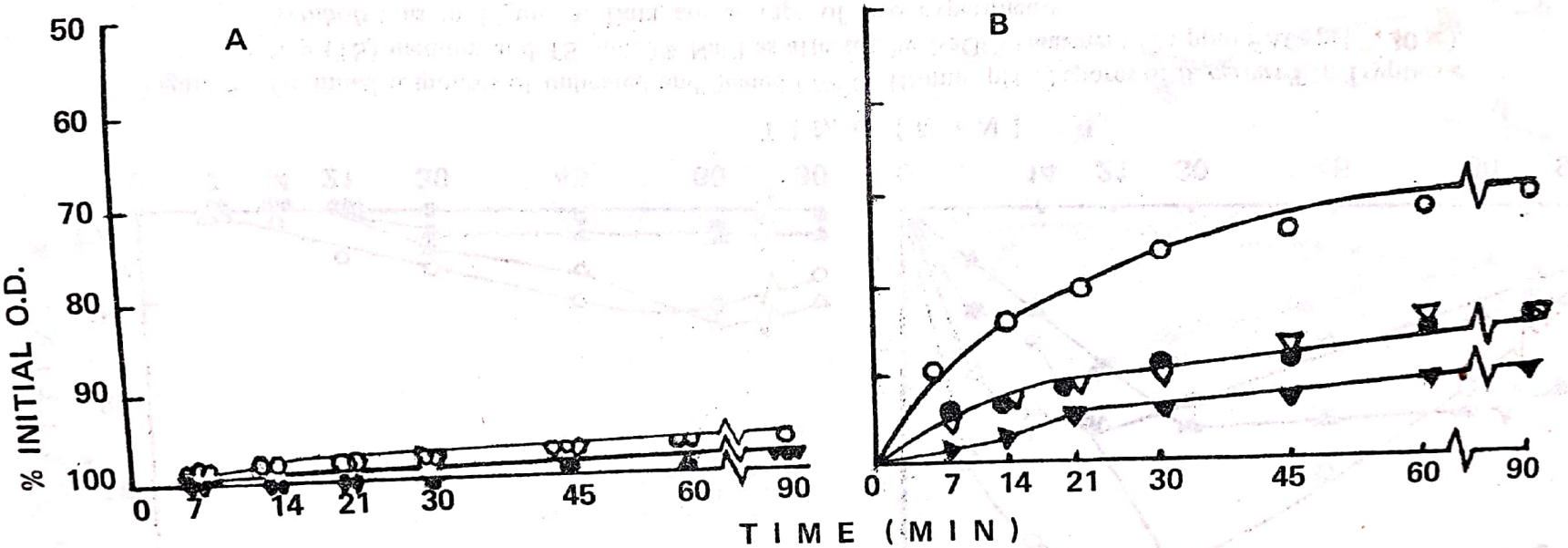

Figure 4. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T in phosphate buffer (50 mM, pH 7) and in buffer plus 3% NaCl as affected by NaOCl treatment (25 ppm FAC, pH 7, 30 sc). *Symbols* : A, unheated; B, heated; ○, unchlorinated; ▽, chlorinated; open, no NaCl added; close, 3% NaCl added. Data are average of two experiments.

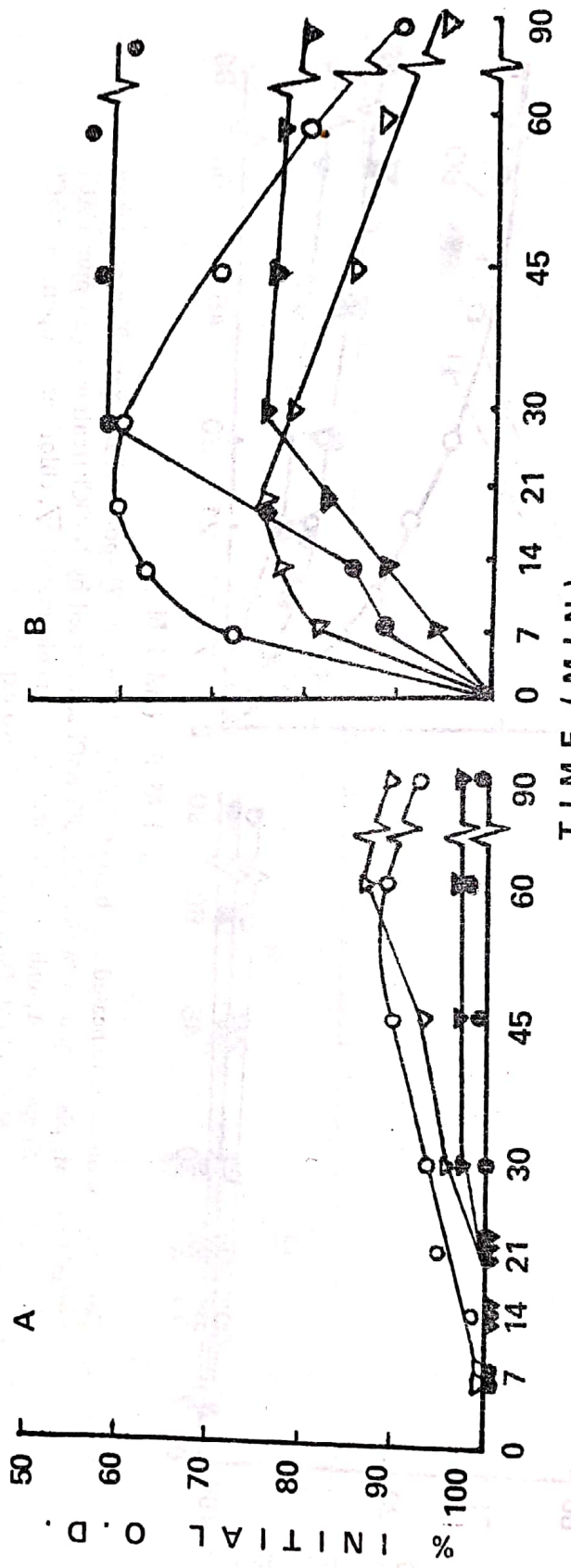

Figure 5. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T in Trypticase Soy (TS) medium and TS plus 3% NaCl as affected by NaOCl treatment (25 ppm FAC, pH 7, 30 sc). *Symbols* : as in Figure 4. Data are average of two experiments.

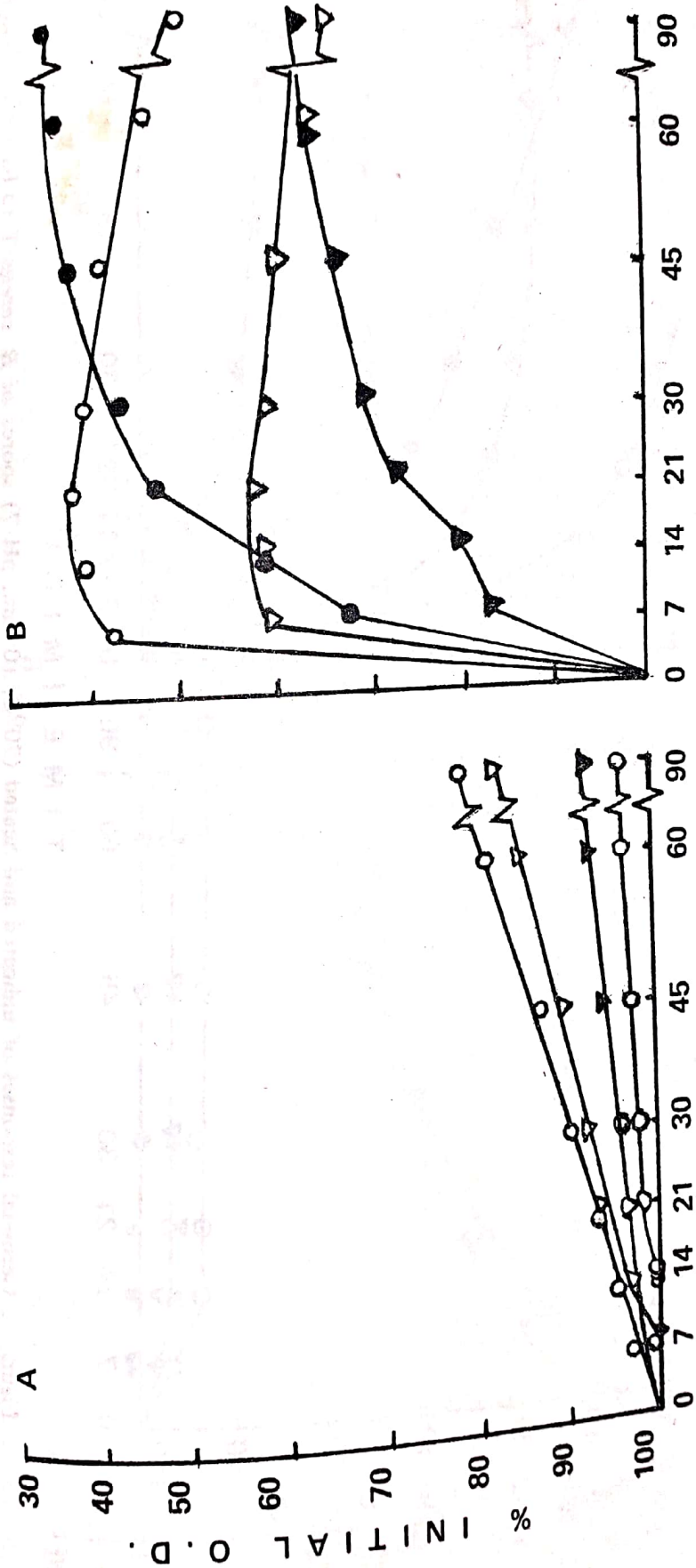

Figure 6. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T to adenosine (10 mM) and adenosine plus 3% NaCl as affected by NaOCl treatment (25 ppm, pH 7, 30 sc).
Symbols : as in Figure 4. Data are average of two experiments.

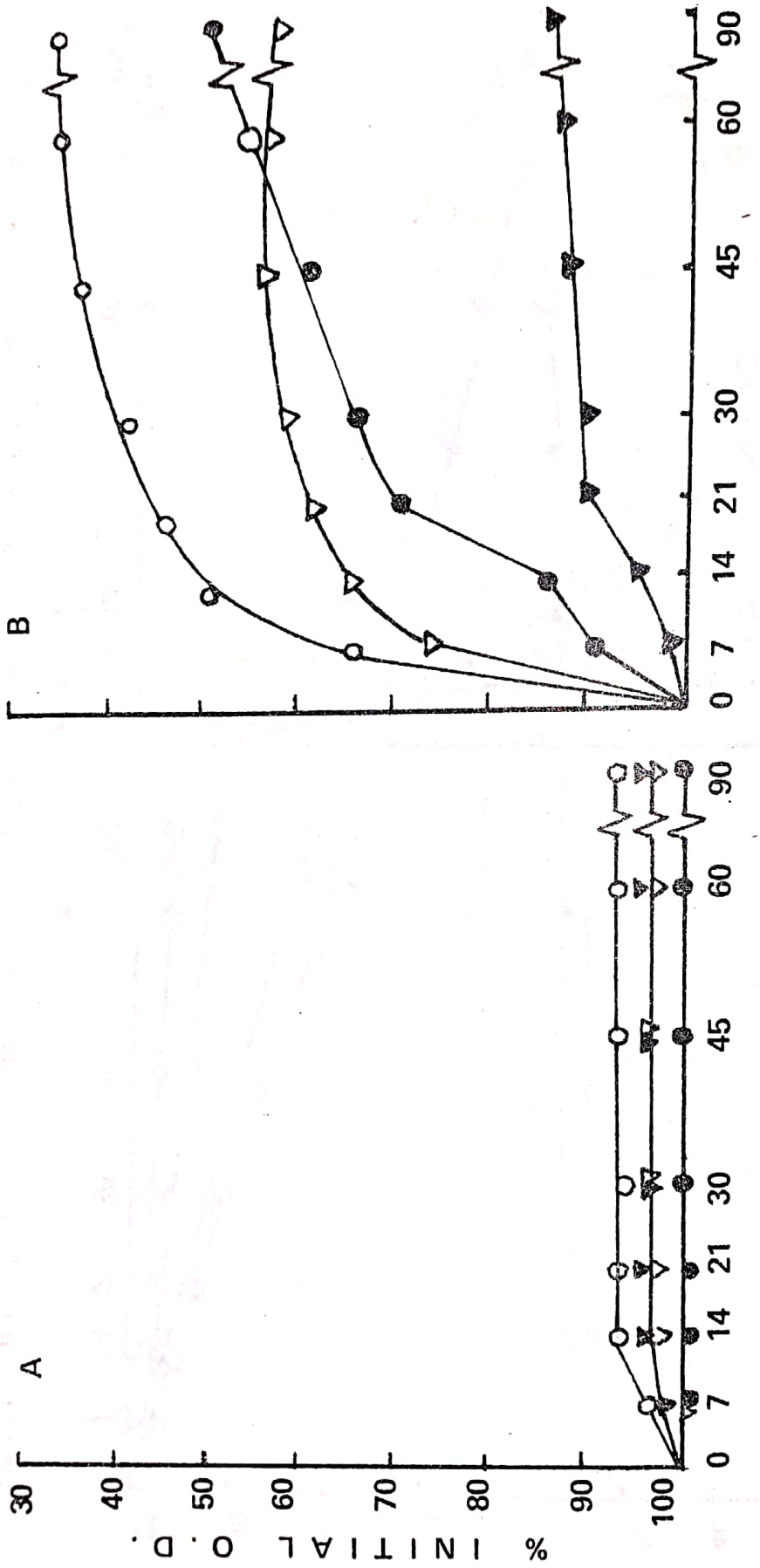

Figure 7. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T to l-alanine (10 mM) and l-alanine plus 3% NaCl as affected by NaOCl treatment (25 ppm, pH 7, 30 sc). *Symbols* : as in Figure 4. Data are average of two experiments.

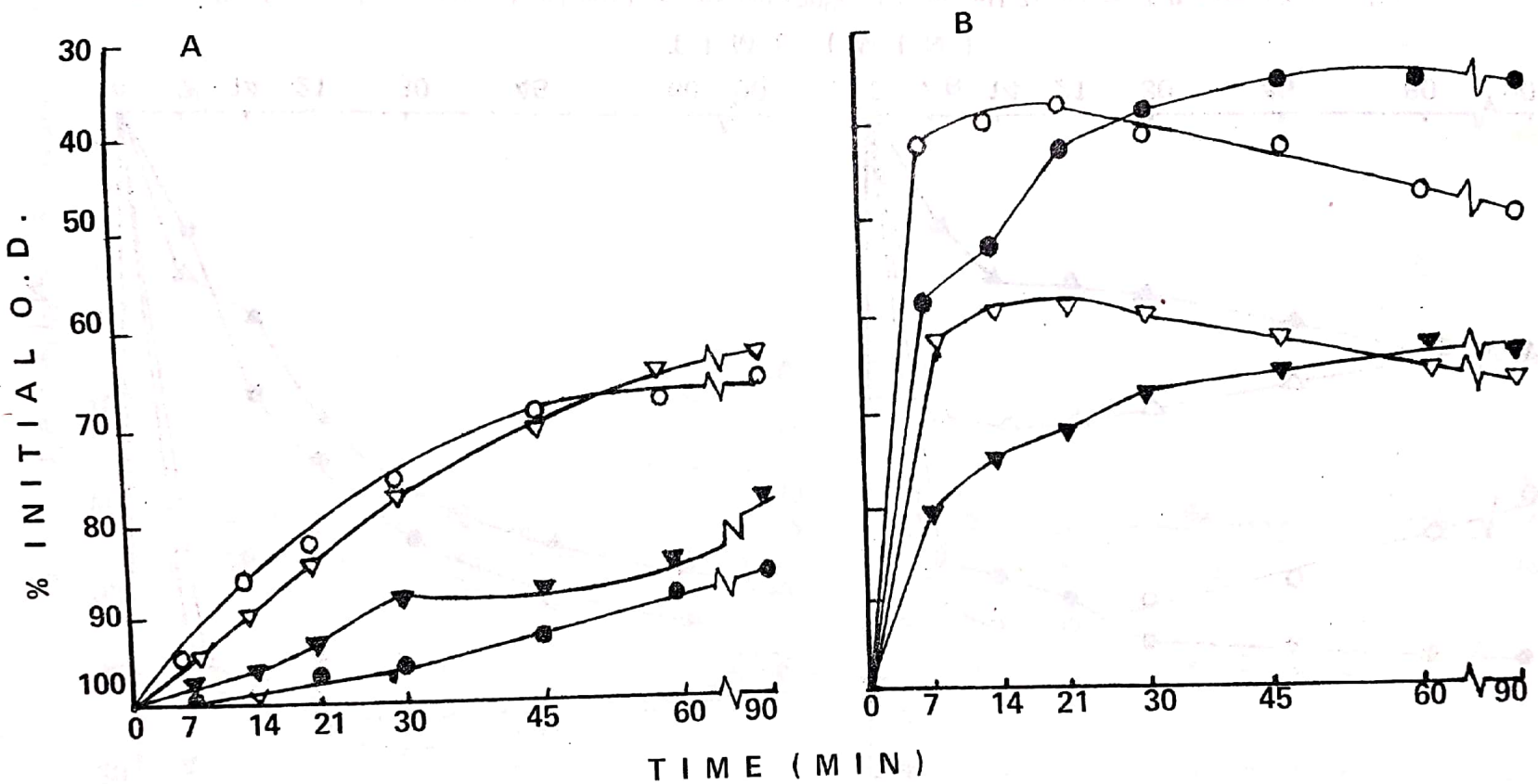

Figure 8. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T to inosine (10 mM) and inosine plus 3% NaCl as affected by NaOCl treatment (25 ppm, pH 7, 30 sc).
Symbols : as in Figure 4. Data are average of two experiments.

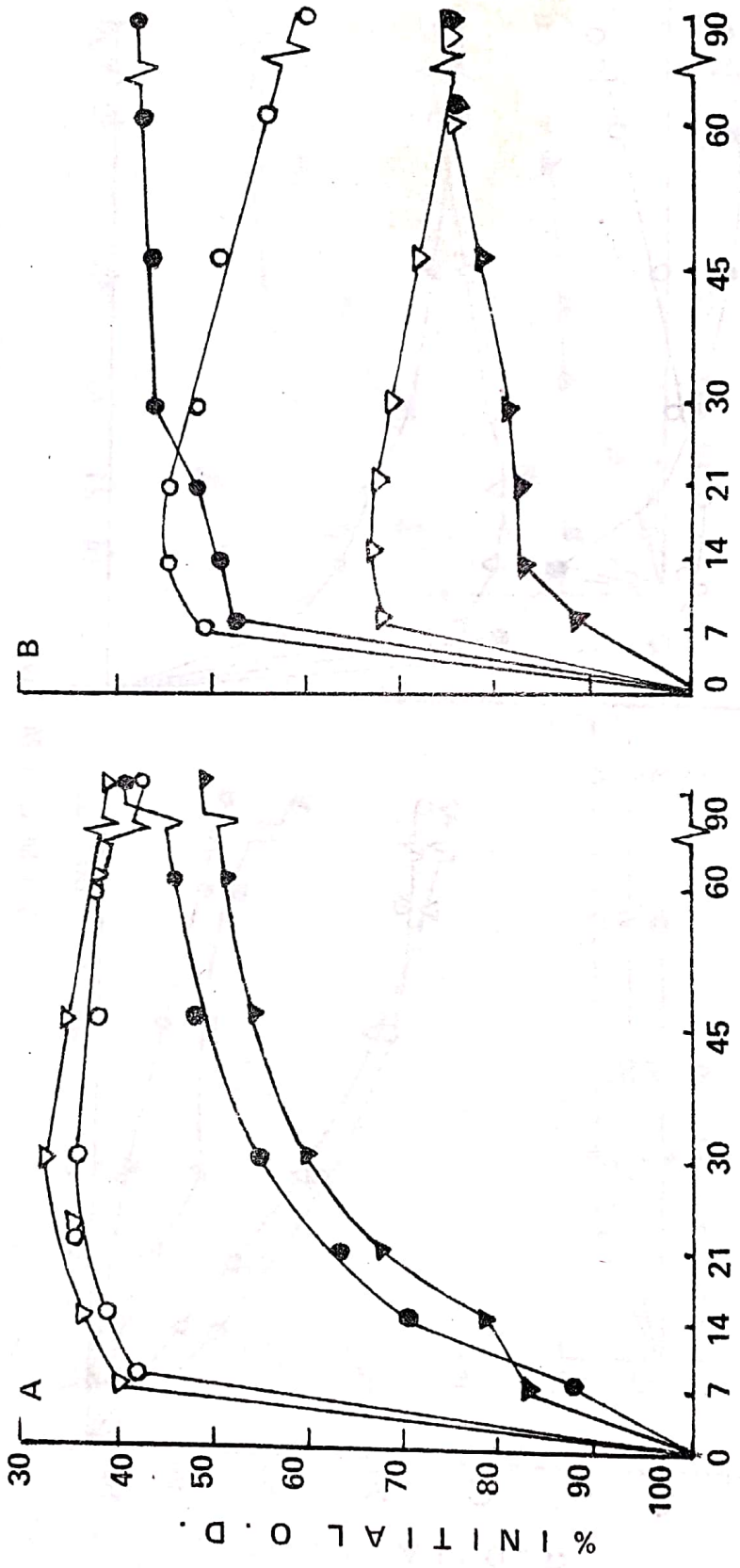

Figure 9. Germinal responses of unheated and heated (70°C, 10 min, pH 7) spores of *B. cereus* T to l-alanine inosine combination (10 mM each), without and with 3% NaCl addition, as affected by NaOCl treatment (25 ppm, pH 7, 30 sc).
Symbols : as in Figure 4. Data are average of two experiments.

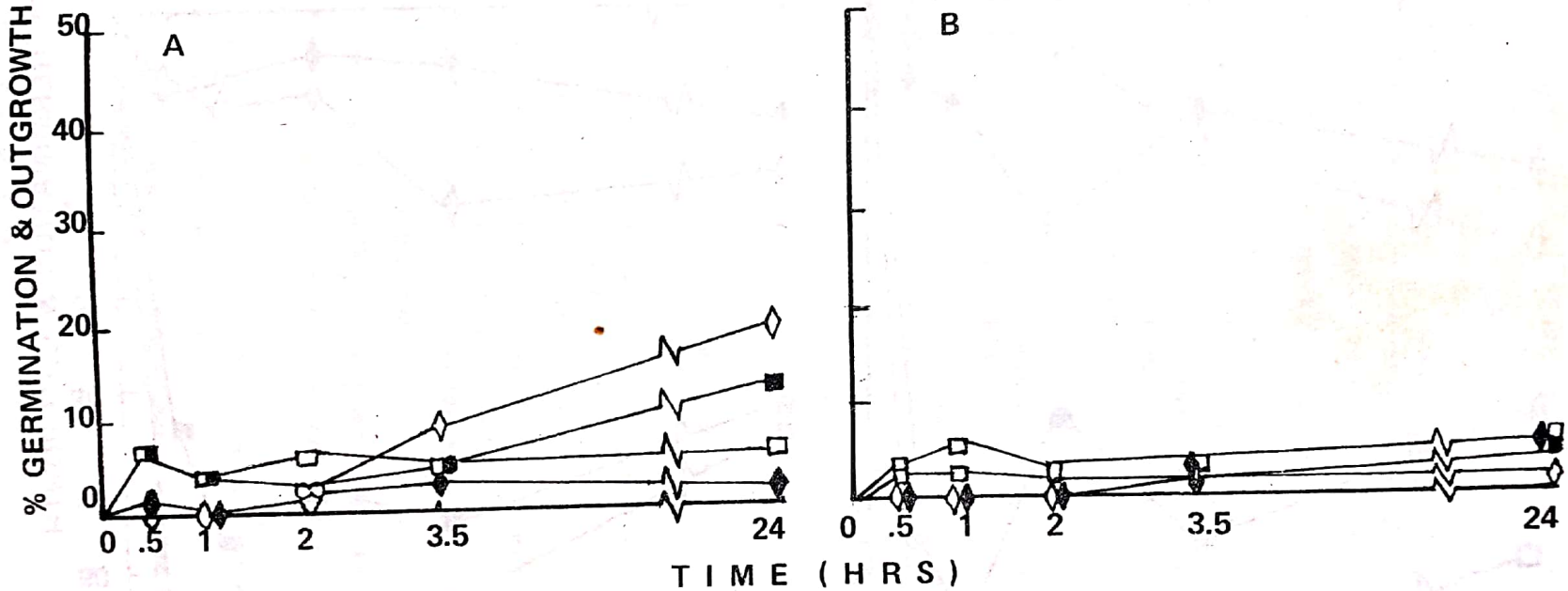

Figure 10. Effect of NaOCl treatment (25 ppm, pH 7, 30 sc) on the germination and outgrowth of *B. cereus* T spores on TSA without and with 3% NaCl additional.
Symbols : A, unheated - unchlorinated; B, unheated - chlorinated; C, heated - unchlorinated; D, heated - chlorinated (C and D next page); □, germination; ◊, outgrowth; open, no NaCl added ; close, 3% NaCl added. Data are average of three experiments.

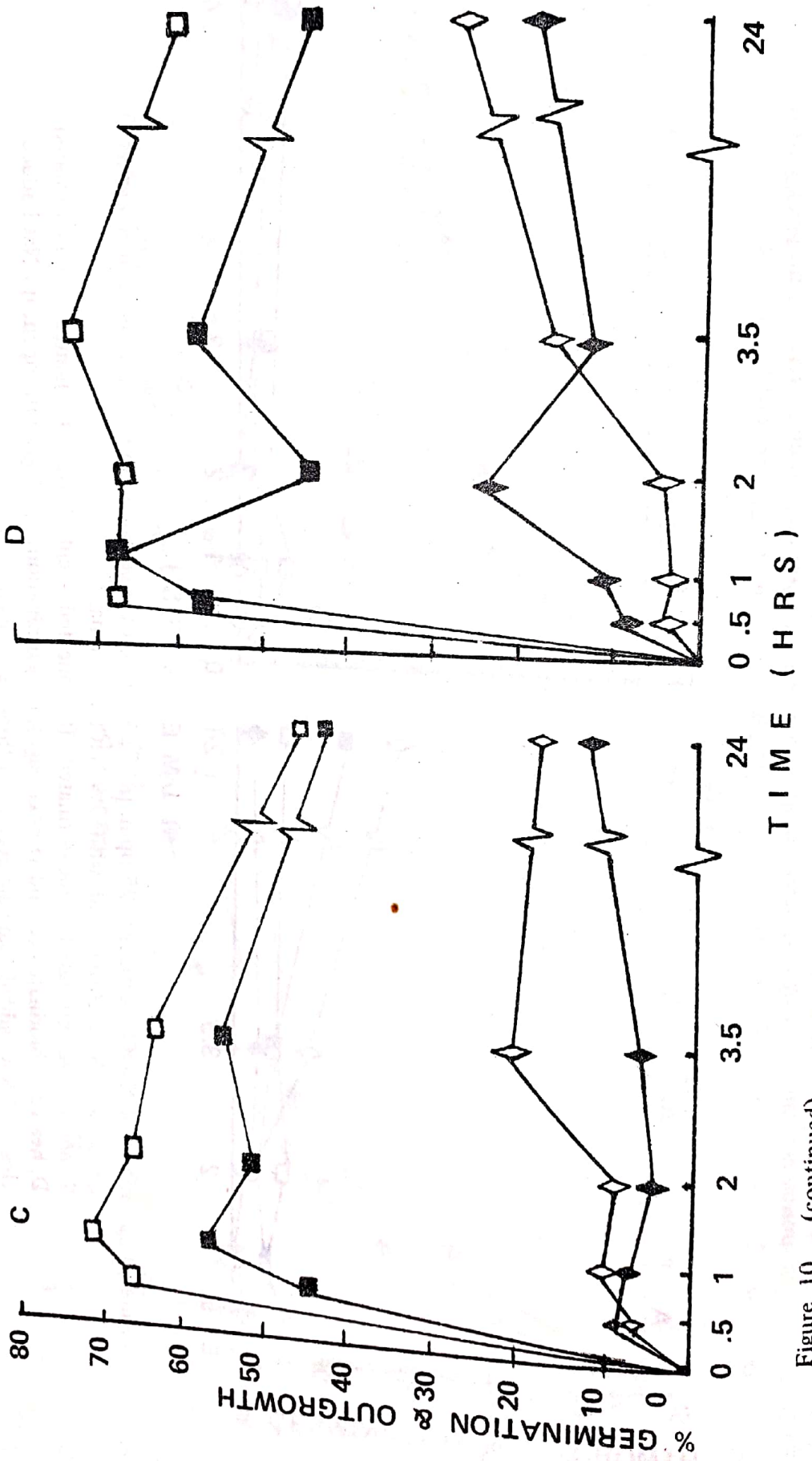

Figure 10. (continued)