EFFECT OF TEMPERATURE ON THE GERMINATION OF SPORES OF *BACILLUS CEREUS* AND *BACILLUS THURINGIENSIS*

by

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CHAPTER I

INTRODUCTION

Bacillus thuringiensis and *Bacillus cereus* are aerobic, gram-positive, rod-shaped, spore-forming soil microorganisms. They are considered to be in the same group of bacteria, and their relationship has been shown by various classification methods (2).

In 1952, Smith, Gordon, and Clark (11) suggested *B. thuringiensis* should replace the name *cereus* var. *thuringiensis* because of the chemical composition and morphology of the spores and the insecticide properties of the bacteria to larvae. The major difference between these two species is the presence of a parasporal crystal in *B. thuringiensis*, but not in *B. cereus*. The crystal is formed along side the exosporium--spore membrane and is toxic to *Lepidopteran* larvae (5, 12). This crystal is a proteinaceous bipypramidal inclusion (protoxin) and is produced during the process of sporulation.

The conversion of a spore to an actively metabolizing vegetative cell occurs in three stages: 1) activation, 2) germination, and 3) outgrowth (8, 9). Activation is a reversible process and is defined as a treatment which conditions the dormant state to allow rapid germination. To date, activation of the spore is known to be initiated with heat, low pH, or the presence of reducing agents (6). Germination is the irreversible degradation of the rigid spore wall and leads to the complete hydration of the spore (9). Outgrowth is the stage from germination to subsequent vegetative growth.

The bacterial endospore is characterized by its high refractility, resistance to heat, and impermeability (3, 4, 8). In the period of germination, refractility, and heat resistance are lost, while stainability and permeability are increased. Part of the dipicolinic acid content is lost, and the spore is dark when examined by phase contrast microscopes (15). During germination, no macromolecules (RNA, DNA, protein, etc.) are synthesized (10).

Germination requires specific chemical agents, germinant, to occur. Chemical agents such as L-alanine, adenosine, or glucose can serve as germinants; however, the mixture of L-alanine and adenosine provided optimal germination for B. cereus spores (13). Vary and Halvorson (13) divided the germination stage into two events: microlag time, which lasts from the addition of germinant to the heat-activated spores until the beginning of loss in refractility, and micro-germination time, which is the time from the end of microlag to the complete loss in refractility.

Enzymes play an important role in spore germination and are widely found on the surface of the spore coat exosporium and in the spore cortex. It was suggested that spore enzymes may differ from those found in vegetative cells (1).

Aronson and Pandey found that the spores of B. cereus germinate more rapidly than do those of B. thuringiensis. A possible reason for this is the production of parasporal crystal by B. thuringiensis, which causes a decrease in spore coat protein. This deficiency in spore coat protein probably affects the germination system (1).

Since B. thuringiensis is an important microbial insecticide, it is of interest to test the factors (chemical and physical) that influence their survival in nature. Temperature is one of the important environmental factors that influences the growth and development of the microorganism. Very few studies have shown the effect of temperature on spore germination. In 1964, Knaysi (7) reported that in the genus Bacillus, the optimum for the spore germination lies between 30 and 37°C, but in some members of the genus, it may be 41°C or even 50°C (7). In 1957, Wolf and Mohmound (16) supplied evidence that spores of B. cereus germinate slowly at 8°C although no vegetative growth occurs at that temperature. This study occurred before the activation and germination process were clearly defined.

The purpose of this study was to test the influence of low temperature on the spore germination of B. cereus and B. thuringiensis.

CHAPTER II

MATERIALS AND METHODS

Organisms and Cultures

The organisms used in this study were Bacillus thuringiensis ssp. Karstaki (HD-1) isolated from a commercial insecticidal formulation, Dipel, (Abbott Laboratories, North Chicago, IL) and Bacillus cereus 569 (Robert Shaw, Department of Biochemistry, Texas Tech University). Stock cultures were grown on nutrient agar slants at 30°C and maintained at 4°C.

Preparation of Spores

The procedures for spore growth and maintenance were described by A. Keynan et al. (6). Nutrient agar (Difco Laboratories) was used for isolation and growth of inoculum. The cells were checked for purity with a phase-contrast microscope (American Optical Phasestan). A single colony was transferred to a test tube containing 5 mls of sterile Brain Heart Infusion (BHI) broth (Difco Laboratories) and mixed well by vortex mixer. The cell suspension in BHI was inoculated on to nutrient agar plates by using sterile cotton swabs to streak over the medium surface. The plates were incubated at 30°C for 3 days. All the spores were harvested with a sterile metal spatula and suspended in sterile 50 ml polypropylene centrifuge tubes containing 30 mls of 0.1M NaCl with 0.1% Triton X-100 (United States Biochemicals). The spore suspension was allowed to stand for 30 minutes at room temperature and then mixed by vortex mixer. The spore

suspension was then centrifuged at 3,000 rpm for 30 minutes. Spores were washed three times in cold distilled water and stored at 4°C until used.

Effect of Low Temperature on Germination

Fresh spores were suspended in 4 mls of water in 13x100 mm test tubes at 4°C and 30°C respectively. For spore activation, spores at 4°C or 30°C water were heat-shocked at 75°C in a water bath for 10 minutes and then cooled to room temperature.

The concentration of spores was adjusted to 0.4 Optical Density (O.D.) at 66 nm (6) (Bausch and Lomb Spectronic 21). The germinant (an equimolar mixture of L-alanine and adenosine, Sigma Chemical Company) was added to the spore suspension to give a final concentration of 4mM. At time zero, 0.1 mls of germinant was added and O.D. readings were recorded every 5 minutes for 30 minutes. In the spore suspension with heat activation, germinant was added at 4°C. The O.D. reading was recorded for 20 minutes. The sample was separated into two parts: one part was transferred to a 30°C incubator and the O.D. was recorded every 5 minutes for 30 minutes. Another was incubated for 24 hours; then the O.D. reading was recorded.

The procedures for study of spore germination in 0.1M NaCl with 0.1% Triton X-100 were the same as the procedures in water. The amount of germination was expressed as the percent decrease in O.D. (6) after 30 minutes. With the phase contrast microscope, the final percent of germination was the percent of spores that were dark (6).

Germination of fresh spores was tested at different temperatures with heat shock or without heat shock, or with germinant or without germinant in water or in NaCl-Triton-X-100 solution.

CHAPTER III

RESULTS

Table 1 shows that nonheat-activated spores of B. thuringiensis ssp. Kurstaki suspended in distilled water at 30°C obtained 43 percentage germination, and about 20 percent of the B. cereus spores germinated. After 10 minutes heat shock, about 83 percent of B. thuringiensis germinated, and over 95 percent of B. cereus spores showed no germination. Heat activated spores of B. thuringiensis in the absence of germinant showed 4.8 percent of germination, and no germination was observed in B. cereus.

Table 2 indicates that nonheat-activated spores of B. thuringiensis ssp. Kurstaki suspended in distilled water at 4°C showed 17 percent germination, and about 12 percent of B cereus spores germinated. After heat activation, about 66 percent of B. thuringiensis ssp. Kurstaki germinated, and 75 percent germination was observed in B. cereus spores. After spore suspensions were transferred from 4°C to 30°C for 30 minutes, there was about 10 percent increase in germinated B. thuringiensis spores, but there was no increase in the germination of spores of B. cereus. When the incubation time was increased to 24 hours, germination of B. thuringiensis spores increased over 20 percent, and B. cereus spores showed less than 20 percent germination. Without adding germinant, nonheat-activated spores showed no germination. Heat activated spores of B. thuringiensis in the absence of germinant showed 5 percentage germination, and no germination occur in B. cereus spores.

Table 3 shows that 69 percent of nonheat-activated spores of B. thuringiensis ssp. Kurstaki in the presence of 0.1 M NaCL with 0.1% Triton X-100 at 30°C, and about 85 percent of B. cereus spores germinated. The percent germination was much higher than in distilled water. After heat shock, 88 percent of B thuringiensis spores germinated, and over 95 percent of B. cereus spores germinated. In the absence of germinant, nonheat-activated spores of B. thuringiensis ssp. Kurstaki and B. cereus showed the same result as those of spores in distilled water. Heat activated spores of B. thuringiensis ssp. Kurstaki without added germinant showed low germination and no germination occurred in B. cereus spores.

Table 4 shows that 70 percent of nonheat-activated B. thuringiensis ssp. Kurstaki spores germinated in the solution of NaCl with Trition X-100 at 4°C and about 90 percent of B. cereus spores germinated. This result is much higher than those of spores in distilled water at 4°C. After heat shock, 73 percent of B. thuringiensis ssp. Kurstaki spores germinated, and 88 percent of B. cereus spores germinated. This result shows that spores with heat shock show the similar percent germination tp spores without heat activation in NaCl with Triton X-100. After spore suspensions were transferred from 4°C to 30°C, only a slight increase in the spore germination of B. thuringiensis and B. cereus for both incubation times (30 minutes and 24 hours) was observed. In the absence of germinant, B. thuringiensis spores showed a very low germination without or with heat activation.

Table 5 indicates that nonheat-activated spores of B. thuringiensis ssp. Kurstaki show 13 percent germination in distilled water at 30°C and about 46 percent germination with heat shock. In the absence of germinant, nonheat-activated spores of B. thuringiensis ssp. Kurstaki show no germination, and heat-activated spores show low percent decrease in O.D. (Figure 1). At 4°C in distilled water, there was a 14 percent decrease in O.D. after 30 minutes. This result shows temperature does not influence the spore germination (measured with O.D. at 4°C and 30°C). Heat activated spores of B. thuringiensis ssp. Kurstaki at 4°C showed 34 percent decrease in O.D. after 30 minutes. This is only a slight decrease in the extent of germination compared with that of the spores at 30°C.

After the spore suspension was transferred from 4°C to 30°C (Figure 2), there was a slight increase in germination at 30 minutes and 24 hours. Non-heat activated spores of B. thuringiensis without adding germinant showed a low level of germination, and a similar result was obtained from spores with heat shock in the absence of germinant (Figure 2.).

Figure 3 shows that the nonheat-activated spores of B. thuringiensis in solution of 0.1M NaCL with 0.1% Triton X-100 at 30°C have a substantial increase in germination as also shown by heat-activated spores. Heat activated spores of B. thuringiensis showed a slight increase in germination as compared with spores at 4°C. In the absence of germinant, both nonheat-activated and heat-activated spores showed no significant germination. The effect

of temperature on the spores is not shown by nonheat-activated spores in a solution of 0.1M NaCl with 0.1 percent.

With heat activation, spores of B. thuringiensis had a slight decrease in germination at 4°C (Figure 4) as compared with those spores at 30°C (Figure 3). After the heat-activated spore suspension was transferred from 4°C to 30°C, a slight increase in the germination was observed after 24 hours incubation (Figure 4). In the absence of germinant, there was no significant germination for both nonheat-activated and heat-activated spores in NaCl with Triton X-100 solution (Figure 4.).

Table 6 shows B. cereus spores have a similar decrease in O.D. as spores of B. thuringiensis in Table 5, but B. thuringiensis spores show a slightly lower germination measured with O.D. (Figures 5-10).

Table 1. Effect of temperature on the germination of B. thuringiensis and B. cereus spores as measured with phase contrast microscope.

Spore Treatment	Percentage Germination (%) 30°C Spores Suspended in Distilled Water	
	B. thuringiensis	B. cereus
No Heat Shock and Germinant	43	21
Heat Shock and Germinant	83	98
No Heat Shock and No Germinant	2	0
Heat Shock and No Germinant	4	0

Table 2. Effect of low temperature on the germination of B. thuringiensis and B. cereus spores as measured with phase contrast microscope.

Spore Treatment	Percentage Germination (%) 4°C Spores Suspended in Distilled Water	
	B. thuringiensis	B. cereus
No Heat Shock and Germinant	17	12
Heat Shock and Germinant	66	75
Heat Shock and Germinant Transferred to 30°C for 30 Minutes	78	76
Heat Shock and Germinant Transferred tO 30°C for 24 Hours	94	94
No Heat Shock and No Germinant	2	0
Heat Shock and No Germinant	5	0

Table 3. Effect of temperature on the germination of B. thuringiensis and B. cereus spores measured with phase contrast microscope in NaCl with Triton.

Spore Treatment	Percentage Germination (%) 30°C Spores Suspended in NaCl w Triton X-100	
	B. thuringiensis	B. cereus
No Heat Shock and Germinant	69	85
Heat Shock and Germinant	88	99
No Heat Shock and No Germinant	2	0
Heat Shock and No Germinant	7	0

Table 4. Effect of low temperature on the germination of B. thuringiensis and B. cereus spores as measured with phase microscope in NaCl with Triton.

Spore Treatment	Percentage Germination (%) 4°C Spores Suspended in NaCl w Triton X-100	
	B. thuringiensis	B. cereus
No Heat Shock and Germinant	70	89
Heat Shock and Germinant	73	88
Heat Shock and Germinant Transferred to 30°C for 30 Minutes	86	89
Heat Shock and Germinant Transferred tO 30°C for 24 Hours	92	99
No Heat Shock and No Germinant	2	0
Heat Shock and No Germinant	5	0

Table 5. Effect of temperature on the germination of B. thuringiensis spores as measured with O.D.

Spore Treatment	Germination Decrease in O.D. (%)			
	4°C		30°C	
	Spores Suspended in		Spores Suspended in	
	Distilled Water	NaCl with Triton X-100	Distilled Water	NaCl with Triton X-100
No Heat Shock and Germinant	14	34	13	45
Heat Shock and Germinant	34	32	46	45
Heat Shock and Germinant Transferred to 30°C for 30 Minutes	45	35		
Heat Shock and Germinant Transferred to 30°C for 24 Hours	53	50		
No Heat Shock and No Germinant	2	4	0	2
Heat Shock and No Germinant	3	0.2	5	0

Table 6. Effect of temperature on the germination of B. cereus spores as measured with O.D.

Spore Treatment	Germination Decrease in O.D. (%)			
	4°C		30°C	
	Spores Suspended in		Spores Suspended in	
	Distilled Water	NaCl with Triton X-100	Distilled Water	NaCl with Triton X-100
No Heat Shock and Germinant	15	42	12	46
Heat Shock and Germinant	45	45	47	54
Heat Shock and Germinant Transferred to 30°C for 30 Minutes	49	48		
Heat Shock and Germinant Transferred to 30°C for 24 Hours	57	60		
No Heat Shock and No Germinant	2	0	5	1
Heat Shock and No Germinant	6	0	0	1

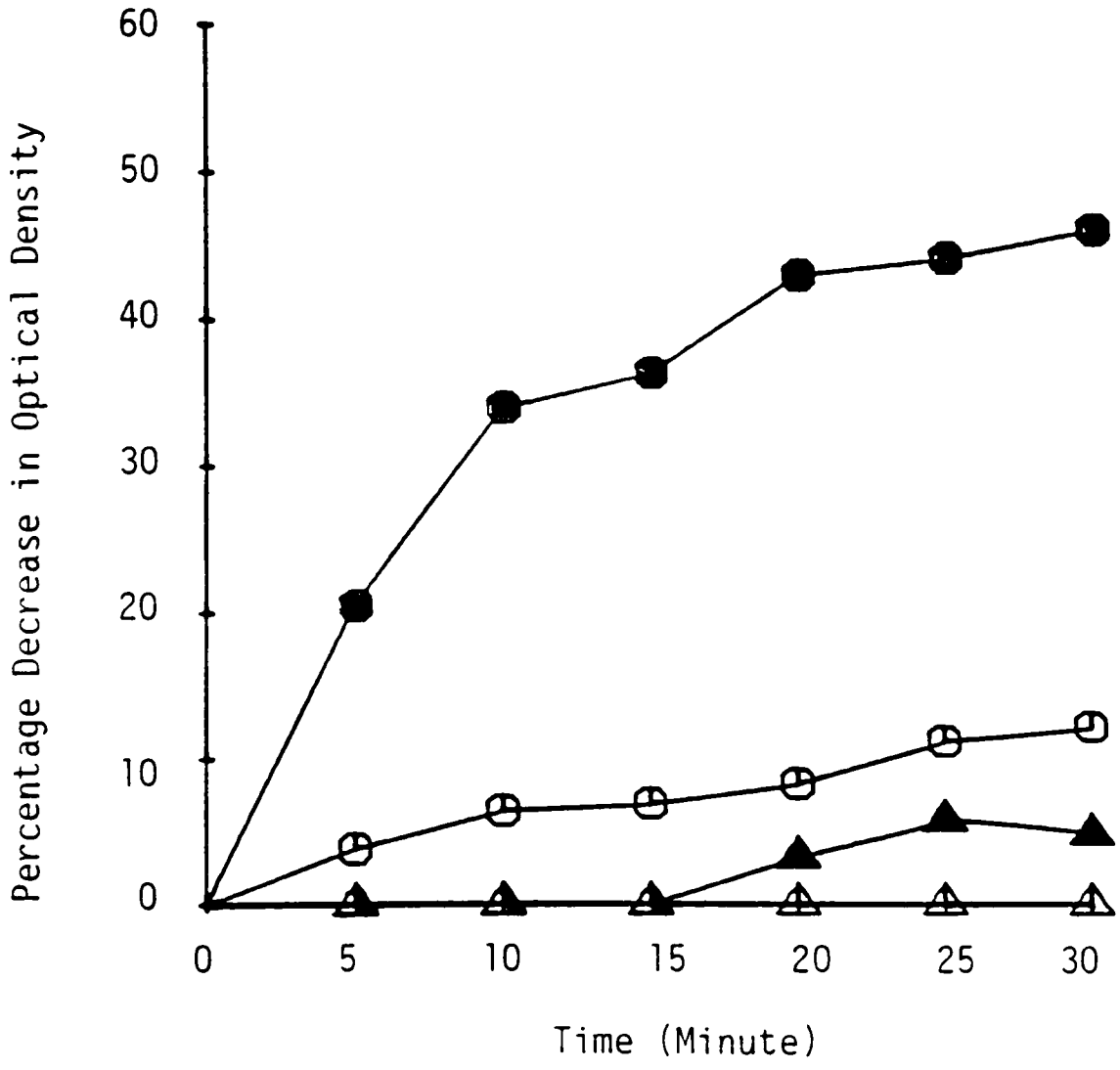

Figure 1. Effect of temperature on the germination of B. thuringiensis spores. Nonheat-activated spore suspensions in distilled water at 30°C (⊖), heat activated spore suspensions in distilled water at 30°C (●), nonheat-activated spore suspensions without adding germinant in distilled water at 30°C (△), heat activated spore suspensions without adding germinant in distillec water at 30°C (▲).

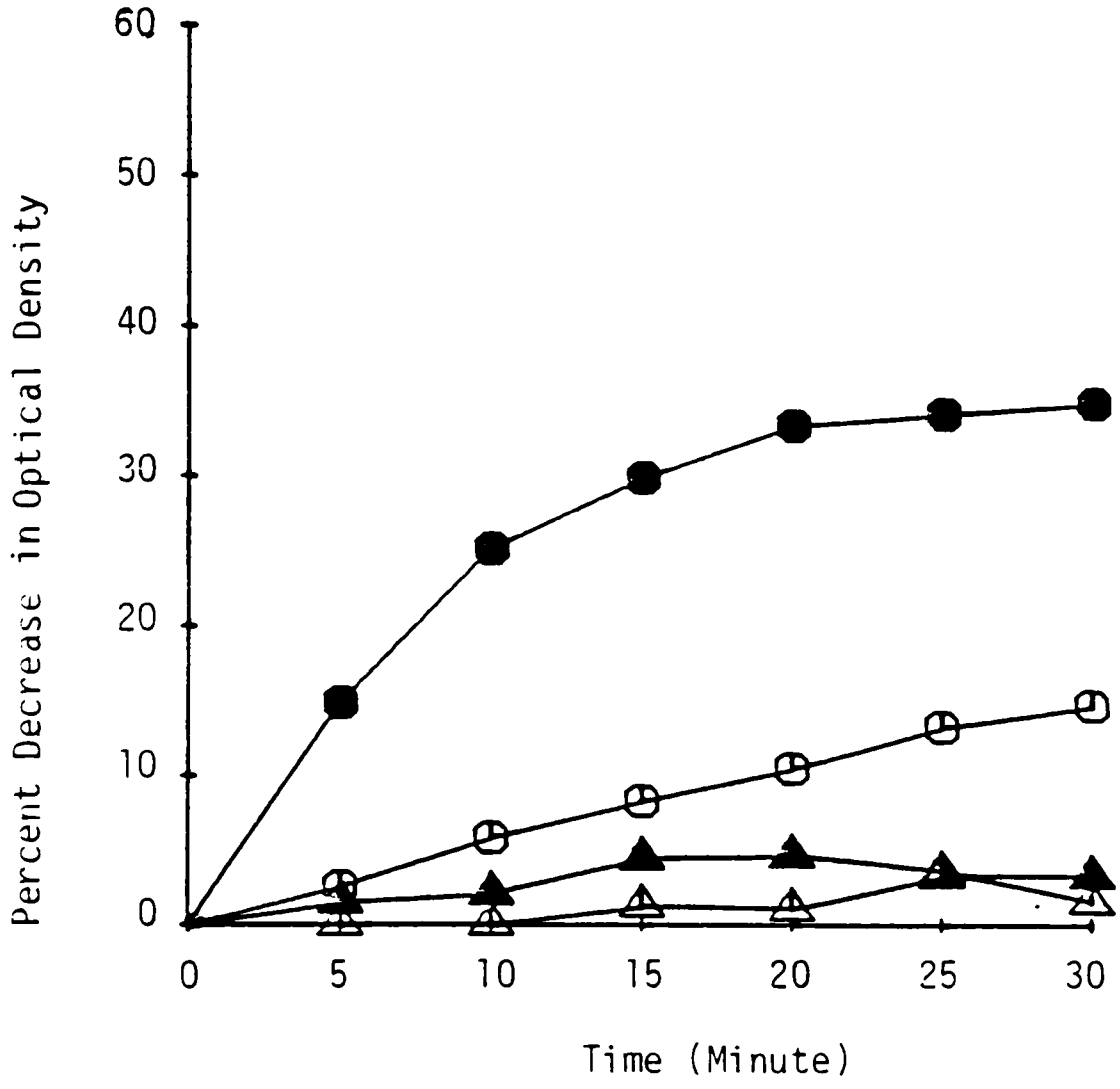

Figure 2. Effect of low temperature on germination of B. thuringiensis spores. Nonheat-activated spore suspension in water at 4°C (⊖), heat-activated spore suspension in water at 4°C (●), nonheat- activated spore suspensions without adding germinant in water at 4°C (△), heat-activated spore suspensions without adding germinant in water at 4°C (▲).

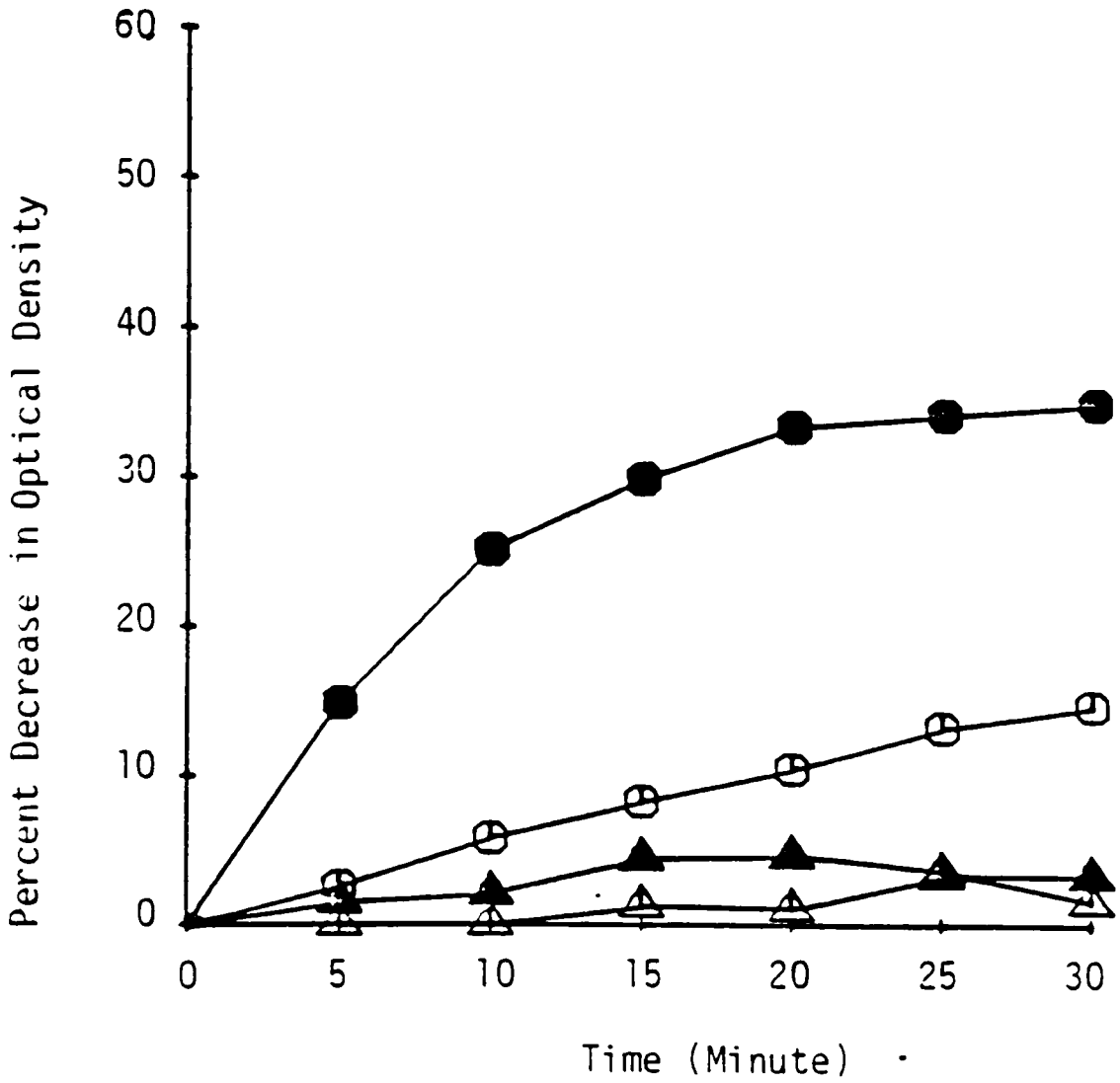

Figure 3. Effect of temperature on germination of B. thuringiensis spores in NaCl with Triton. Nonheat-activated spore suspension in NaCl with Triton X-100 at 30°C (⊖), heat-activated spore suspensions in NaCl with Triton X-100 at 30°C (●), nonheat-activated spore suspension without adding germinant in NaCl with Triton X-100 at 30°C (△), heat-activated spore suspension without adding germinant in NaCl with Triton X-100 (▲).

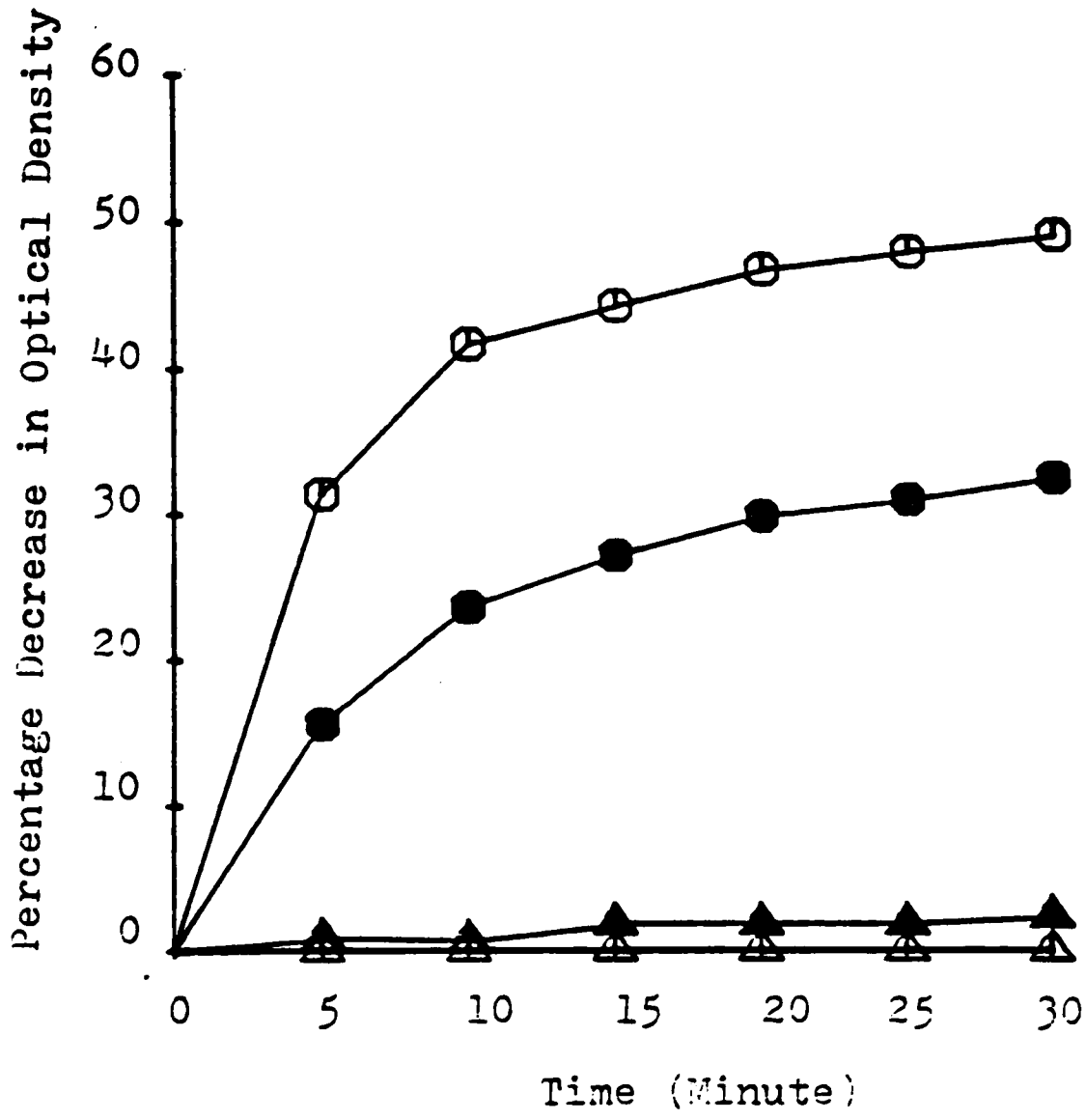

Figure 4. Effect of low temperature on germination of P. thuringiensis spores in NaCl with Triton. Nonheat-activated spore suspension in NaCl with Triton X-100 at 4°C (◵), heat-activated spores suspensions in NaCl with Triton X-100 (●), nonheat-activated spore suspension without adding germinant in NaCl with Triton X-100 (◬), heat activated spore suspension without adding germinant in NaCl with Triton X-100 (▲).

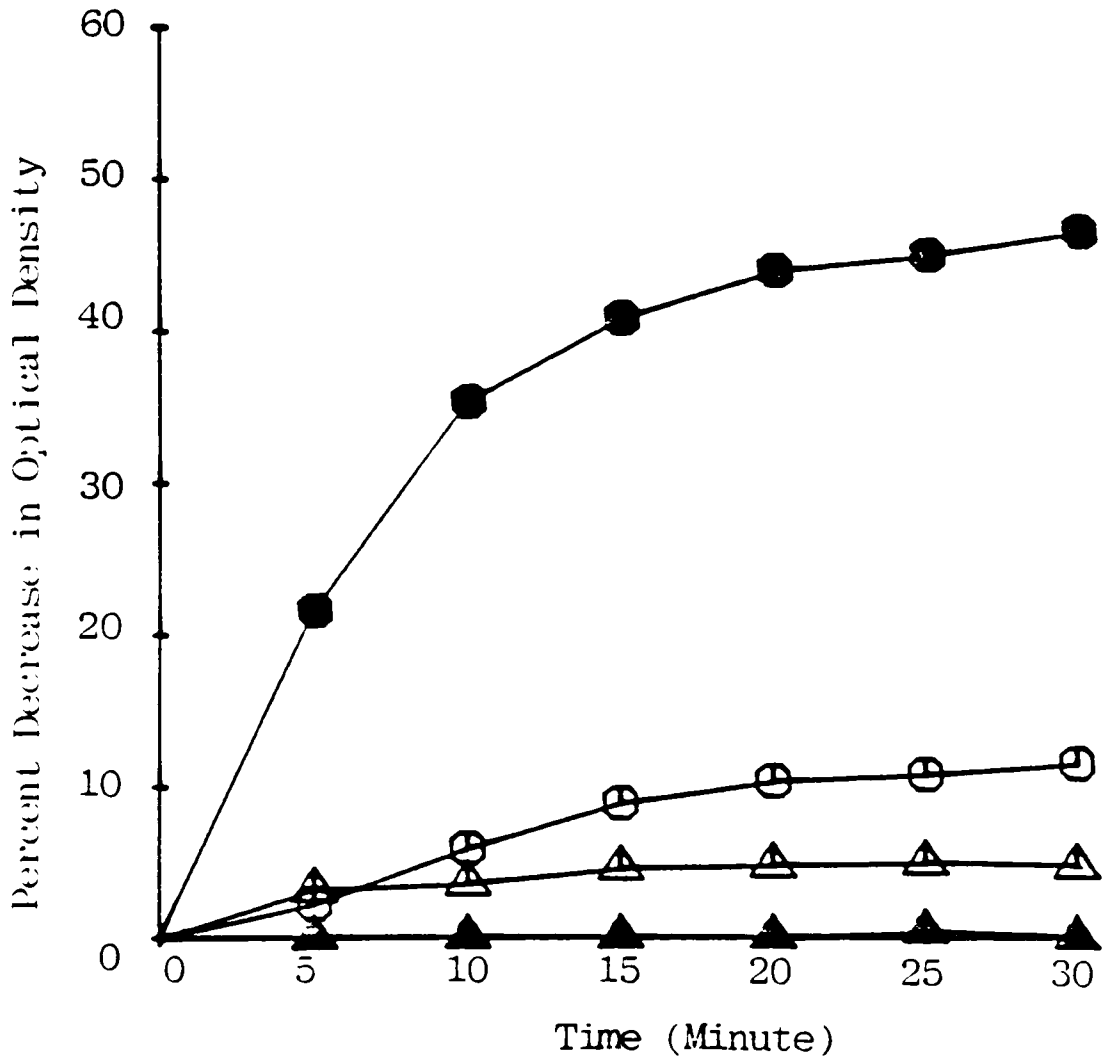

Figure 5. Effect of temperature on germination of B. cereus spores. Nonheat-activated spore suspension in distilled water at 30°C (⊖), heat activated spore suspension in distilled water at 30°C (●), nonheat-activated spore suspensions without adding germinant in distilled water at 30°C (△), heat activated spore suspensions without adding germinant in distilled water at 30°C (▲).

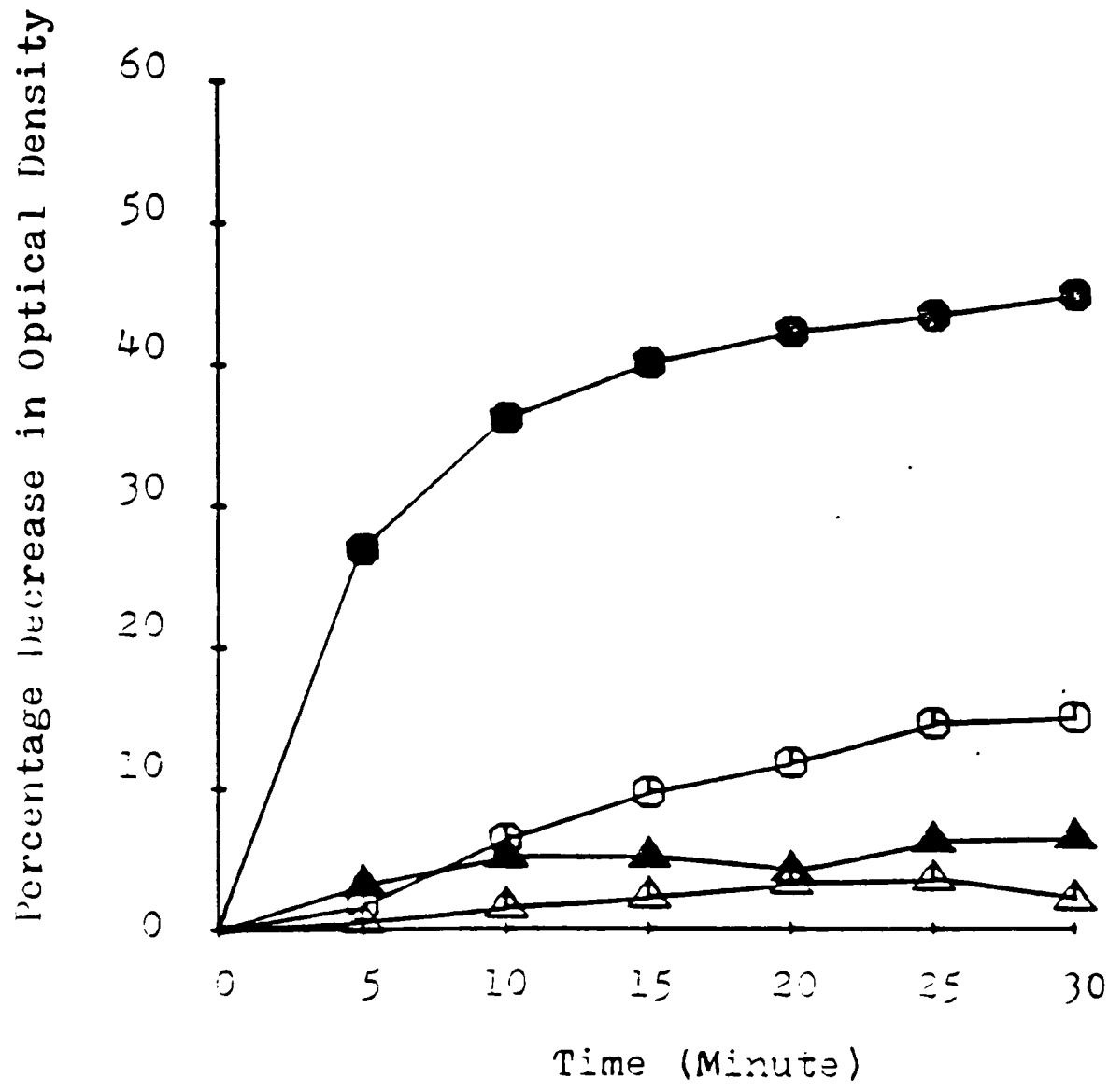

Figure 6. Effect of low temperature on germination of B. cereus spores. Nonheat-activated spore suspension in distilled water at 4°C (⊖), heat-activated spore suspensions in distilled water at 4°C (●), nonheat-activated spore suspensions in distilled water at 4°C (△), heat-activated spore suspension in distilled water at 4°C (▲).

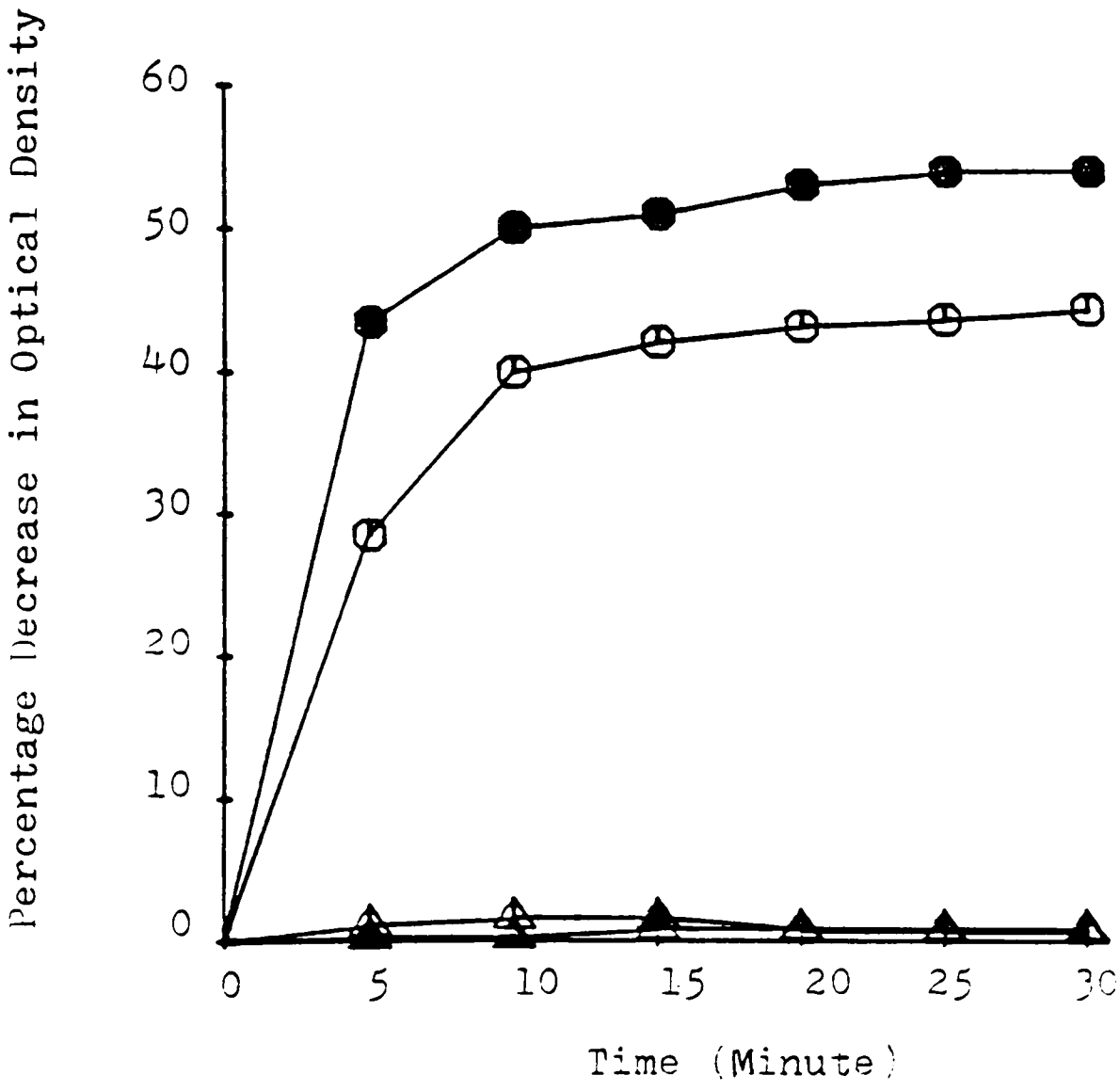

Figure 7. Effect of temperature on germination of B. cereus spores in NaCl with Triton. Nonheat-activated spore suspensions in NaCl with Triton X-100 at 30°C (⊖), heat-activated spore suspensions in NaCl with Triton X-100 at 30°C (●), nonheat-activated spore suspension without adding germinant in NaCl with Triton X-100 at 30°C (△), spore suspensions without adding germinant in NaCl with Triton X-100 at 30°C (▲).

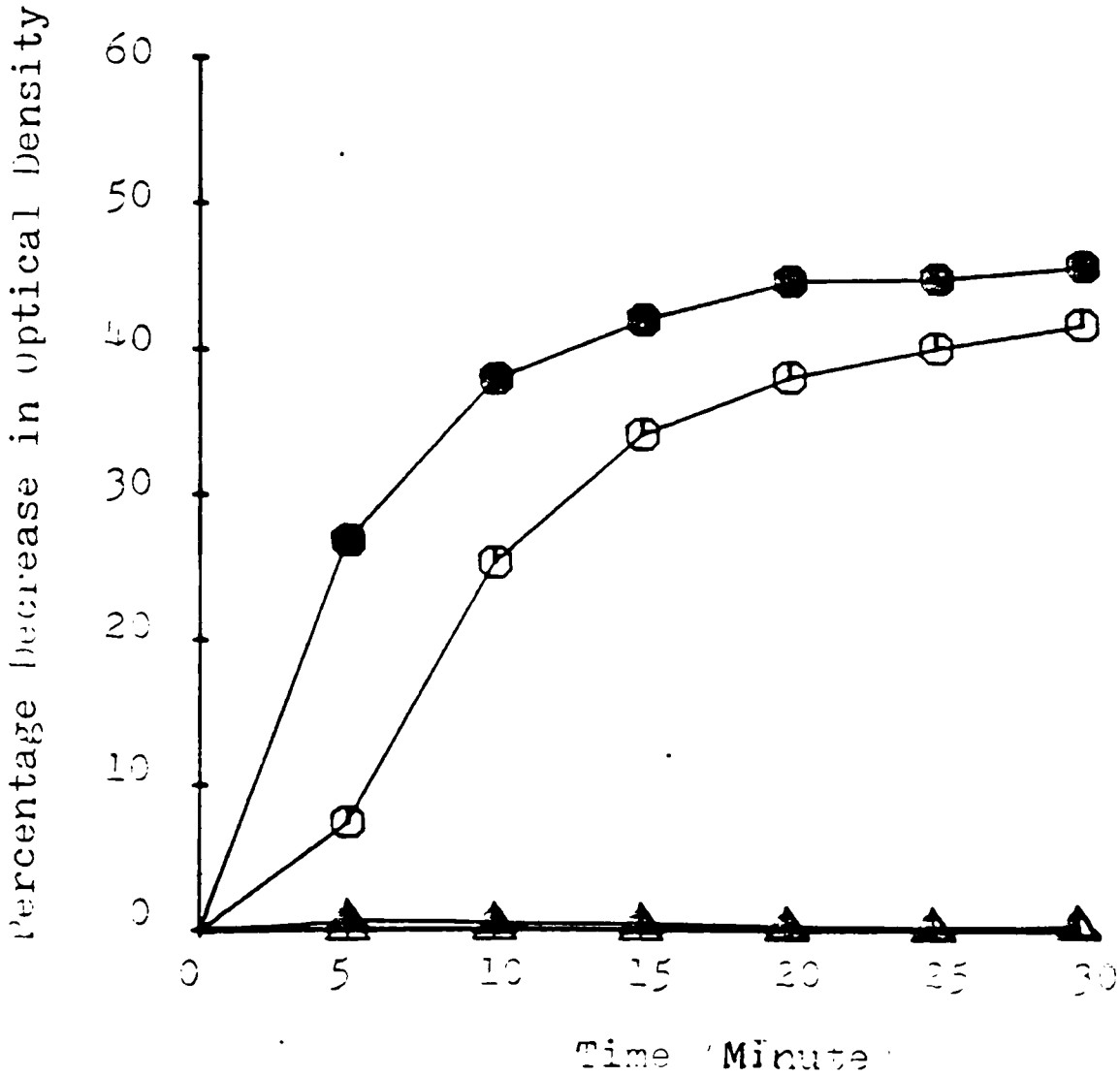

Figure 8. Effect of temperature or germination of B. cereus spores in NaCl with Triton. Nonheat-activated spore suspensions in NaCl with Triton X-100 at 4°C (⊖), heat-activated spore suspensions in NaCl with Triton X-100 at 4°C (●), nonheat-activated spore suspension without adding germinant in NaCl with Triton X-100 at 4°C (△), heat activation spore suspensions without adding germinant in NaCl with Triton X-100 at 4°C (▲).

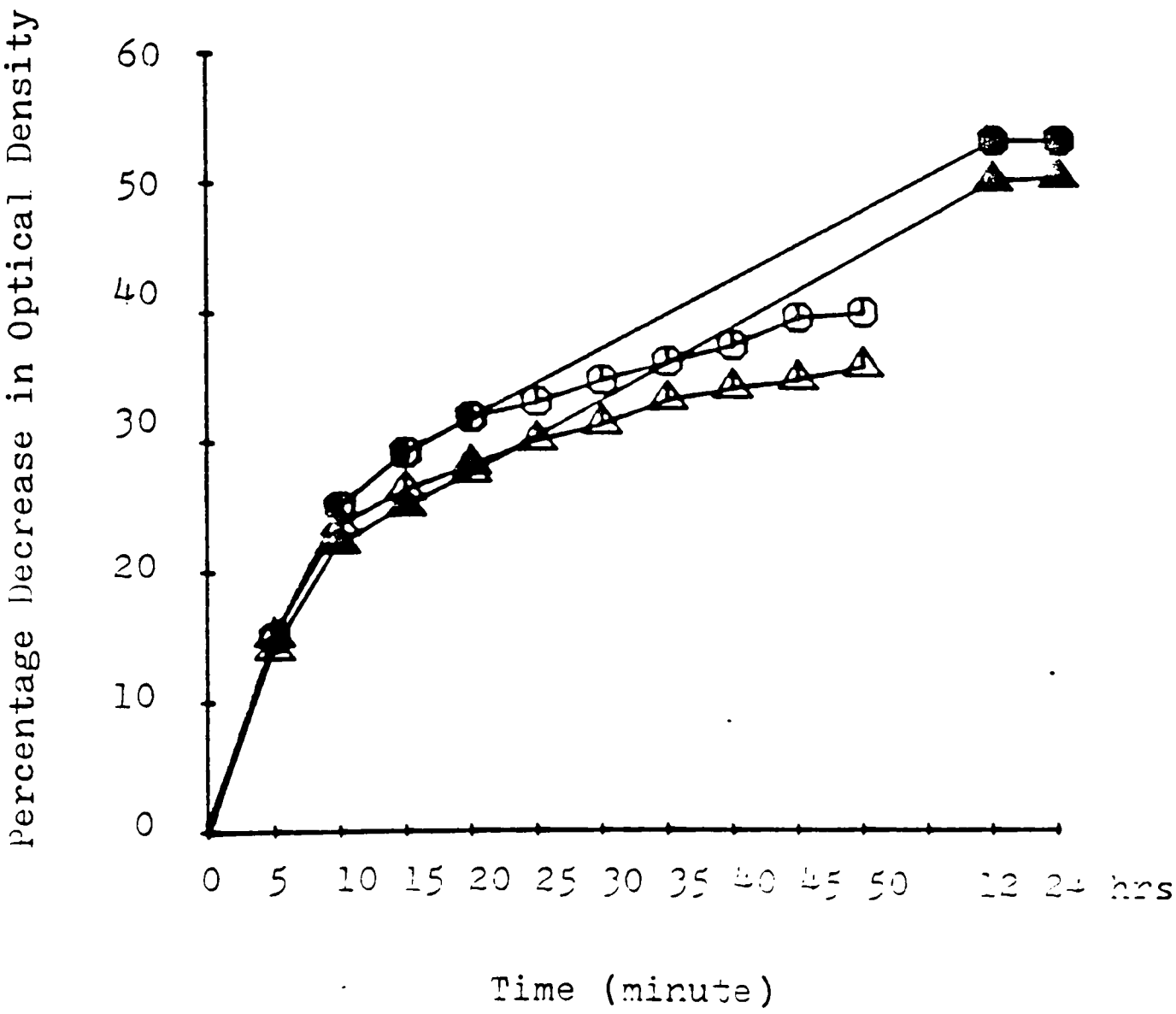

Figure 9. Effect on temperature on the germination of B. thuringiensis spores transferred from 4°C to 30°C with heat activation. Spore suspension in distilled water transfer to 30°C for 30 minutes (⊖), for 24 hours (●), spore suspensions in NaCl with Triton X-100 transfer to 30°C for 30 minutes (△), for 24 hours (▲).

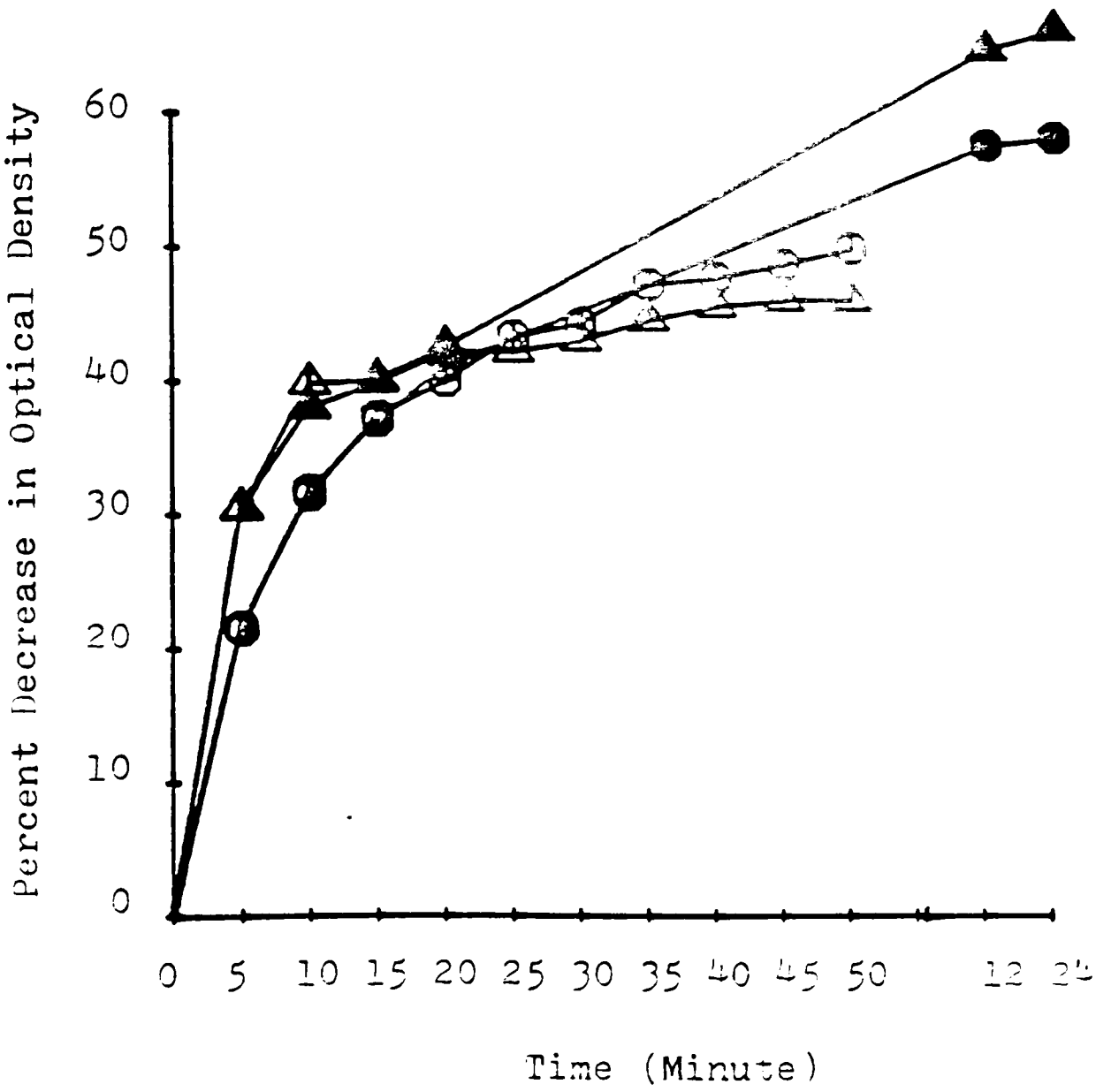

Figure 10. Effect of temperature on the germination of B. cereus spores transferred from 4°C to 30°C with heat-activation. Spore suspension in distilled water transferred to 30°C for 30 minutes (⊖) spores in distilled water incubate at 30°C for 24 hours (●), spores in NaCl with Triton X-100 transfer to 30°C for 30 minutes (△), for 24 hours (▲).

CHAPTER IV

DISCUSSION

Results of this study show that low temperature (4°C) causes a low germination for both spores of B. thuringiensis ssp. Kurstaki and B. cereus measured with the phase contrast microscope. However this effect did not show on the germination measured with percent decrease in O.D. which gave similar decreases in O.D. at 4°C and 30°C. This difference between phase contrast and O.D. is inconsistent with the result of Keynan et al. (6) who obtained the same results with both procedures. The explanation could be that phase contrast represents early internal changes in the spore while O.D. measures physical degradation of the spore coat. It may be indirectly related to the two events of microlag and microgermination described by Vary and Halvorson, even though their time scale is shorter. Hence phase contrast and O.D. probably could be used as tools to further study spore germination.

For nonheat-activated spores of both B. thuringiensis ssp. Kurstaki and B. cereus, Triton X-100 additon produced an effect similar to heat activation, and showed a substantial increase in germination. B. cereus spores show higher germination than those of B. thuringiensis. Triton X-100 could act as activator and is more effective for B. cereus spores. Trition X-100 is a non-ionic detergent with a specific solubilizing effect on the cell membrane of microorganism, but not on the cell wall. Triton X-100 solubilizes exosporium and releases some materials which may affect the spore coat

protein. Triton X-100 probably affects spore activation directly or indirectly. It would be of interest to study the effect of Triton X-100 on the activation of B. subtilis which does not contain exosporium.

Without heat shock, B. thuringiensis ssp. Kurstaki spores in distilled water at 30°C show a higher germination than B. cereus spores. This could have some relationship to differences in the spore coat protein. B thuringiensis ssp. Kurstaki does not contain protective protein which is a keratin-like structural protein (1). This could result in the loss of resistance to the unfavorable environment. B. thuringiensis spores probably do not require activation for their germination. Perhaps these spores develop the potential to gain access to a favorable protected environment in which spores do not need a protective layer of spore coat or a germination system (1). B. cereus spore coat has a series of protective layers which contain important enzymes for germination. These enzymes provide important characteristics for survival in various ecological niche.

B. cereus shows higher and more rapid germination rates in most conditions. This is probably because of the difference in spore coat protein between B. thuringiensis spp. Kurstaki and B. cereus. There is no effect of changing the temperature from 4°C to 30°C after 30 minutes. Probably the spore coat of B. cereus is affected by low temperature.

Non-heat activated spores in the absence of germinant at 4°C or 30°C show poor germination, so spores of both B. thuringiensis ssp.

Kurstaki and B. cereus require some factor to help them in germination.

Therefore, it is concluded that low temperatures effect the germination of spores measured with phase contrast, not with O.D., and Triton X-100 may be used as an activator. The ability for spores to germinate may be determined by the difference in the spore coat protein in B. thuringiensis ssp. Kurstaki, and B. cereus, and this could affect the survival of their spores in nature.

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