APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1976, p. 731-734
Vol. 32, No. 5

Printed in U.S.A.

Catalase: Its Effect on Microbial Enumeration

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Received for publication 12 July 1976

The addition of catalase to the surface of selective medium plates permitted increased enumeration of physically or chemically injured (stressed) microorganisms. Catalase acted by preventing the accumulation of hydrogen peroxide in, or around, injured cells. Heat-injured *Staphylococcus aureus* cells had decreased catalase activity, and heat-inactivated catalase had no effect on enumeration.

The debilitating effects of physical and chemical stress on the microbial cell have been well documented (4, 16). A variety of cellular lesions may be produced, some of which have been characterized by biochemical and molecular means. It has also been established that the injured but viable cells have been physiologically altered. One important consequence of this alteration is that such cells are commonly unable to produce colonies on media that are regularly used in standard or recommended procedures used in their enumeration. Public health concern has therefore been directed towards the possibility that viable food pathogens might go undetected in processed foods when conventional selective media are used. The failure of selective media to allow detection of injured cells is thought to result from hypersensitivity to secondary stress, such as the presence of selective agents.

Cultures of *Staphylococcus aureus* MF-31 were grown in Trypticase soy broth (TSB). The cells from a 12-h culture were collected by centrifugation, washed once in 100 mM potassium phosphate buffer at pH 7.2, and finally suspended in 0.1 volume of the original culture in phosphate buffer. The washed suspension was then added to 0.9 volumes of phosphate buffer preheated to 52°C. The suspension was heated for 20 min, and the cells were then cooled in an ice bath. *Salmonella typhimurium* 7136 was grown overnight in TSB, centrifuged, and suspended in 100 mM potassium phosphate buffer, pH 6.0. The cells were then suspended in buffer of reduced water activity (adjusted to 0.92 with glycerol). The suspension was stored at room temperature and tested after 7 days. *Pseudomonas fluorescens* was grown overnight in TSB and heated for 2 h at 43°C. A late-exponential-phase culture of *Escherichia coli* grown in TSB was centrifuged and resuspended in 0.1% peptone. The washed cells were added to 300 mM sodium acetate buffer, pH 4.2, and incubated for 1 h at 32°C. The media used for enumeration are described in the tables. Catalase (3,400 units/mg) was obtained from Sigma Chemical Co. Catalase solutions were prepared by dissolving the catalase in water and passing the solutions through a sterile 0.45-μm membrane filter.

When cells of *S. aureus* are subjected to a sublethal heat treatment, the treatment produces a variety of repairable lesions (9, 16). The degree of injury during such a heat exposure is estimated by following the difference in the plate count between that obtained when the suspension is plated on a rich, nonselective medium, Trypticase soy agar (TSA), and that when it is plated on TSA containing an added 7.0% NaCl (TSAS), a selective medium. The injured cells are sensitive to the added salt and are unable to develop into colonies when plated on TSAS (9).

Baird-Parker agar (BP) can be substituted for TSA in the dual-plating procedure and is the selective medium of choice for the enumeration of uninjured and/or injured cells of *S. aureus*. BP is recommended for use by the Association of Official Analytical Chemists and by the Food and Drug Administration (2, 17). In agreement with Baird-Parker and Davenport (3), we have found that the presence of 1% sodium pyruvate in BP is responsible for the enhanced growth of stressed cells. Similarly, the addition of 1% sodium pyruvate to Vogel-Johnson agar, another selective medium recommended for the enumeration of *S. aureus*, increased recovery of heat-injured cells (Table 1). The proposed mode of action of the sodium pyruvate is via the degradation of the metabolic by-product hydrogen peroxide (H_2O_2), rather than through supplementation of a required nutrient (3).

In an effort to test the hypothesis that sodium pyruvate acted through H_2O_2 degradation, we substituted the enzyme catalase (EC 1.11.1.6)

in place of sodium pyruvate. Catalase addition has been suggested to promote the growth of the lactic bacteria (15) as well as to increase enumeration of *Clostridium perfringens* (S. Harmon and D. Kautter, Abst. Annu. Meet. Am. Soc. Microbiol. 1973, E136, p. 23). Solidified agar plates of the appropriate test medium were spread with 0.1 ml of a 0.02% catalase solution. Catalase added to tempered agar was also effective. This amount of catalase was the minimum amount to give the maximum increased enumeration. The plates were then inoculated as usual and incubated for 48 h. Table 1 presents the results of a typical experiment comparing the effects of added catalase or sodium pyruvate to various media and the influence on the enumeration of injured and uninjured *S. aureus*. As can be seen, the presence of either catalase or sodium pyruvate permits increased enumeration of injured and uninjured cells, often to levels above those obtained using the recommended BP medium. The most dramatic increase in enumeration was evident when TSAS plates were spread with catalase and inoculated with heat-injured cells. The addition of catalase to this selective medium increased enumeration 14,000-fold. These results strongly implicate H_2O_2 as a factor in the failure of injured cells to grow on selective media. Further evidence that catalase increased enumeration via H_2O_2 degradation is shown in Fig. 1. Catalase was heat-inactivated, and injured cells were plated on TSA, TSAS, TSAS with 0.02% catalase, and TSAS with 0.02% heat-inactivated enzyme. The addition of inactive catalase had little beneficial effect on enumeration, whereas addition of active catalase increased enumeration. Work currently in progress suggests that catalase is also effective in the enumeration of freeze-dried *S. aureus*.

TABLE 1. *The effects of catalase or sodium pyruvate on the enumeration of normal and heat-injured Staphylococcus aureus*

Medium[a]	Noninjured cells		Injured cells[b]	
	cfu/ml	% Enumeration[c]	cfu/ml	% Enumeration[c]
BP	3.2×10^9	100	2.7×10^9	100
TSA	2.1×10^9	66	2.2×10^9	82
TSA + catalase	3.3×10^9	>100	2.9×10^9	>100
TSA + sodium pyruvate	3.2×10^9	100	2.9×10^9	>100
TSAS	1.1×10^9	34	2.8×10^4	0.001
TSAS + catalase	1.6×10^9	50	4.0×10^8	15
VJ	3.2×10^9	100	8.4×10^8	31
VJ + catalase	3.4×10^9	>100	2.8×10^9	>100
VJ + sodium pyruvate	3.0×10^9	94	2.6×10^9	96

[a] BP, Baird-Parker agar; TSA, Trypticase soy agar; TSAS, Trypticase soy agar plus 7% NaCl; VJ, Vogel-Johnson agar.

[b] Cells were heated for 20 min at 52°C in 100 mM potassium phosphate buffer.

[c] Percent enumeration was calculated using colony-forming units per milliliter on BP as 100%.

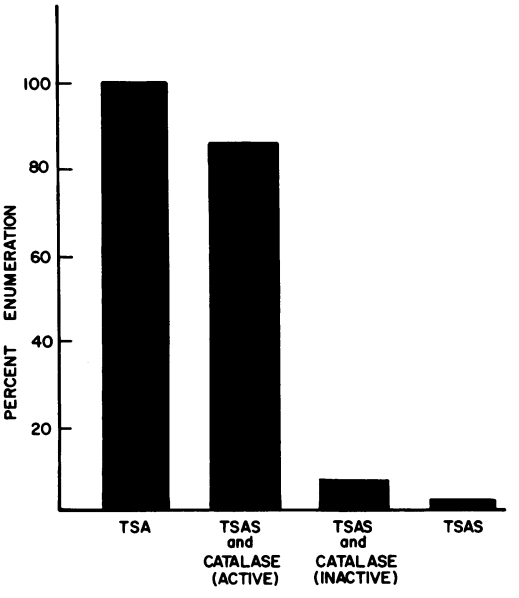

FIG. 1. *Effect of catalase and heat-inactivated catalase on the enumeration of heat-injured Staphylococcus aureus MF-31. Catalase was inactivated at 80°C for 30 min.*

Catalase is produced by *S. aureus* in small amounts during logarithmic growth and in larger quantities during the stationary phase. In young cultures, catalase is attached to the intracellular membrane in an inactive form, becoming activated upon release from the membrane (10, 11). Amin and Olson (1) found that *S. aureus* was relatively sensitive to H_2O_2 and that catalase was instrumental in protecting the cell against this oxidant. Catalase is sensitive to heat and rapidly loses activity at 35°C, at a pH near 7 (18). Amin and Olson (1) found that staphylococcal catalase activity decreased 10- to 20-fold faster at 54.4°C than at 37.8°C, with the thermostability varying with the strain. When catalase activity of whole cells was measured by means of a Clark oxygen electrode (14), we found a decrease in activity between heated and unheated cells. Figure 2 presents the average decrease of four experiments. This decrease was evident when the assay was performed in phosphate buffer alone,

but was more pronounced when 10% NaCl was added to the reaction mixture. The addition of 10% NaCl to the assay mixture using unheated cells also caused a decrease in catalase activity. The results of this assay are consistent with the pattern found in enumeration of heated and unheated cells, as described in Table 1. That is, cells that are unheated and plated on TSA or BP are enumerated more efficiently and also have the most catalase activity (Fig. 2). However, even unheated cells are sensitive to the presence of NaCl, as evidenced by their lower counts on TSAS and also their decreased catalase activity. When plated on TSA, heated cells demonstrate decreased enumeration when compared with unheated cells plated on TSA and also show a lowering of their catalase activity. The most dramatic effects of heating are found when cells are plated or assayed in the presence of NaCl. These effects are most pronounced on TSAS plates, with a corresponding decrease in their catalase activity as shown in Fig. 2. It is problematic that the decrease in catalase activity and the corresponding injury as found on TSAS are greatly different (i.e., 20% and four log cycles). Numerous theories could be proposed to explain this anomaly; however, only further investigation will provide the answers.

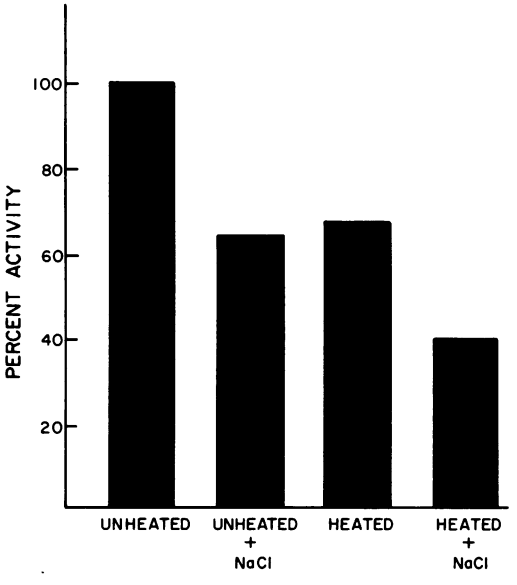

FIG. 2. *Effect of heating and NaCl on catalase activity of cells of Staphylococcus aureus MF-31. Heated or unheated cells, in the presence or absence of 10% NaCl, were assayed for their catalase activity by following the evolution of oxygen, using the Clark oxygen electrode as described by Rørth and Jensen (14). The activity determined for unheated cells in the absence of NaCl was designated as 100%.*

These results are highly indicative of the importance of H_2O_2 and its role in the failure to enumerate injured *S. aureus* on selective media. In an effort to determine whether H_2O_2 affected enumeration of other injured microorganisms, catalase was added to the appropriate selective media, as previously described. Table 2 presents the results of experiments with various gram-negative microorganisms subjected to different injury conditions. As is evident, the presence of catalase permitted increased enumeration in all cases.

These results infer that H_2O_2 accumulation may be a universal phenomenon associated with cellular injury following sublethal stress. The cellular lesions caused by heating, chilling or freezing, and freeze-drying are characterized by an extended lag time, leakage of cellular material (primarily nucleic acids), membrane damage, increased sensitivity to selective media, and increased nutritional requirements (7, 13, 16). Lesions similar to these were found when *E. coli* was inoculated into previously irradiated medium and were attributed to H_2O_2 (5). The presence of H_2O_2 in this irradiated medium was confirmed spectrophotometrically, and the addition of catalase before inoculation completely negated all adverse effects. It has been reported that deoxyribonucleic acid breaks may occur after exposure to a sublethal heat treatment (6, 19). The photooxidation of tryptophan in aqueous solution has been shown to produce numerous biologically active prod-

TABLE 2. *The effects of catalase on the enumeration of injured gram-negative microorganisms*

Injury condition	Organism	CFU/ml		Fold increase
		Medium[a] um[a]	Medium[a] + catalase	
Reduced water activity and storage	*Salmonella typhimurium*	2.4×10^5 (EMBS)	1.1×10^6 (EMBS)	4.6
Heating at 43°C for 2 h	*Pseudomonas fluorescens*	5.8×10^5 (GSA)	2.5×10^6 (GSA)	4.3
Acid injury	*Escherichia coli*	1.3×10^5 (VRBA)	1.2×10^7 (VRBA)	92

[a] EMBS, Eosin methylene blue agar plus 2% NaCl; GSA, glutamate salts agar as described by Gray et al. (8); VRBA, violet red bile agar.

ucts, one of which is H_2O_2 (12). The presence of H_2O_2 in such solution was shown to affect cellular deoxyribonucleic acid, causing increased mutagenicity and selective lethality to recombination-deficient *E. coli* mutants.

Our results, and those of others, strongly suggest an important role of H_2O_2 in the enumeration of microorganisms. The presence of catalase on the surface of selective media, or sodium pyruvate in the media, has been shown to overcome the effects of H_2O_2 and to increase enumeration of injured and normal microorganisms. Enumeration of both gram-positive and gram-negative bacteria was increased in the presence of catalase. We are continuing our studies to further explain these findings.

We thank Gary Adams, Sylvia Pikelis, Preeya Vibulsreth, David McCoy and Kenneth Przybylski for their valuable assistance.

This investigation was supported in part by Food and Drug Administration contract 641-4-220.

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