Effect of Hydrogen Peroxide on the Physiology of *Bifidobacterium thermophilum*

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Hydrogen peroxide was used as a source of OH-free radicals to study their effects on the physiology of *Bifidobacterium thermophilum* (ATCC 25866). H_2O_2 reduced the production of lactate in the absence of iron at $[H_2O_2] = 250-855$ μM; however, lactate production could be fully restored in these cells when they were freed of H_2O_2. Several potential free radical scavengers were not able to abate the effect of H_2O_2 on lactate production. $Fe(OH)_3$ binding by the cells, which was used to detect cellular surface damage, was not affected by $[H_2O_2]$ of up to 830 μM in the absence of exogenous iron. Growth of bifidobacteria could be reduced by $[H_2O_2]$ as low as 41 μM, but growth resumed as the H_2O_2 disappeared from the medium. At high initial H_2O_2 concentrations (600 μM), at which no growth was observed for as long as 48 h, the cells remained viable and resumed growth in the absence of H_2O_2. It was concluded that H_2O_2 was bacteriostatic with respect to *B. thermophilum* at H_2O_2 concentrations used in this study, but whether this applies to other bifidobacterial species and strains must be determined by further research.

Keywords: *Bifidobacteria; hydrogen peroxide; free radicals; probiotics*

INTRODUCTION

Probiotics are microorganisms that are beneficial to human beings and animals when established in their intestinal tracts. Among their health benefits are host resistance to cancer, prevention of pathogen overgrowth in various areas of the organism, lowering of blood cholesterol levels in hypercholesterolemic animals, prevention of diarrheas in infants and adults, and others [e.g., Mital and Garg (1995) and Kailasapathy and Rybka (1997)]. For the most part, probiotics are lactic acid-producing bacteria from the genera *Lactobacillus* and *Bifidobacterium*.

There has recently been much interest in the action of free radicals on the viability of probiotics, especially bifidobacteria, because they have been found to be unstable under a variety of storage conditions (Kailasapathy and Rybka, 1997; Shah, 1997; Micanel et al., 1997; Biavati et al., 1992a). The reasons for this have not been established, but free radical action and low pH have been proposed as some reasons (Shah, 1997; Kailasapathy and Rybka, 1997). Free radicals can arise from a number of sources, such as formation of the superoxide anion via reduction of molecular oxygen or the formation of the OH-free radical from hydrogen peroxide in the presence of iron (Fenton reaction) (Halliwell and Gutteridge, 1985a). One of the better known actions of the OH-free radical is membrane lipid peroxidation (Halliwell and Gutteridge, 1985b). Hydrogen peroxide can appear in the environment from the superoxide anion or be generated by another microorganism. It is thus possible to study the effects of free radicals on microorganisms by introducing hydrogen peroxide and iron into their medium [e.g., Lundrigan et al. (1997)].

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The effects of hydrogen peroxide-generated free radicals on bifodobacteria have not been studied extensively. Bifidobacteria themselves do not produce hydrogen peroxide; however, it can be produced by other organisms such as *Lactobacillus acidophilus* or *Lactobacillus delbrueckii* ssp. *bulgaricus*, with which bifidobacteria may coexist in various food products, such as yogurts. *L. bulgaricus* can produce rather large quantities of hydrogen peroxide when glucose or another fermentable sugar is present in the medium (Kot et al., 1996, 1997).

Our laboratory has investigated the effects of H_2O_2-iron-generated free radicals on the surface properties of both *L. acidophilus* and *L. bulgaricus* by examining the binding of ferric hydroxide to the free radical-damaged bacterial surfaces (Kot et al., 1997). The action of free radicals was associated with an increased level of $Fe(OH)_3$ binding by the bacteria; the most likely reason for such surface alteration was peroxidation of the cell membrane lipid or another structure of cell membranes or cell walls. This phenomenon has not been extensively studied in bifidobacteria.

This paper describes the effects of H_2O_2-iron-generated free radicals on the growth, metabolism, and surface properties of *Bifidobacterium thermophilum*, a bovine rumen organism. This particular bifidobacterial species was used because of our extensive experience with this organism [e.g., Kot and Bezkorovainy (1993) and Kot et al. (1993)]. Its properties closely resemble those of *Bifidobacterium breve*, which is derived from human sources (Kot et al., 1994, 1995).

MATERIALS AND METHODS

Microorganism. *B. thermophilum* (ATCC 25866) was purchased from the American Type Culture Collection (Rockville, MD). It was propagated in the trypticase–phytone–yeast extract (TPY) medium described by Scardovi (1986). Identity of the organism was routinely verified microscopically

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and by the semiquantitative colorimetric determination of fructose-6-phosphate phosphoketolase (F6PPK) in cell sonicates as described by Scardovi (1986). F6PPK activity was thus rated from − to 4+.

For physiological and biochemical studies, the cells were grown in 120-mL bottles for 18 h at 37 °C under anaerobic conditions as previously described (Bezkorovainy, 1984). Turbidity at 610 nm (A_{610}) at that point was 1.2−1.4, and the pH of the suspension was 4.1−4.2. Five milliliters of this suspension contained 4.5 mg of dry bacterial mass. The cells were centrifuged, washed with ice-cold NaCl (0.9%), and suspended in an appropriate volume of 0.1 M 3,3-dimethylglutarate buffer solution at pH 6.5, which also contained 0.4 g of KCl, 8 g of NaCl, and 0.14 g of $CaCl_2$ per liter. Various additions were then made as required to a final cell A_{610} of 1.2.

Ferric Hydroxide Solutions and Binding. Ferric hydroxide solutions (more likely, colloidal suspensions) were prepared as previously described (Kot et al., 1997). Excess hydrogen peroxide content of stock $Fe(OH)_3$ solutions was decreased to undetectable levels by titrating 300 μM H_2O_2 with 10 mM $FeSO_4$. Catalase was not used. Various amounts of H_2O_2 were then added to this solution as required. Binding of $Fe(OH)_3$ by the cells was then performed as previously described (Kot et al., 1997).

Growth Experiments. *B. thermophilum* organisms were grown in bovine milk whey and in the TPY medium in the presence of H_2O_2 concentrations ranging from about 40 to 800 μM. These H_2O_2 concentrations were chosen because *L. bulgaricus* can generate H_2O_2 concentrations of 140−500 μM after incubations with glucose for 10−120 min (Kot et al., 1996). The bovine milk whey medium was prepared in the pH 6.5 3,3-dimethylglutarate buffer solution (see above) and contained 1% bovine milk whey, 2 mg/mL glucose, and 10 μM $FeSO_4$. This was then sterilized by autoclaving. Growth in this medium was sparse, reaching a maximal A_{610} of 0.7 in 24 h. In the TPY medium, growth reached an A_{610} of 1.2−1.4. However, for unknown reasons, H_2O_2 was more stable in the milk whey medium than in the TPY medium. The H_2O_2 stock solution was 3200 μM and was filter-sterilized before use. It should be noted that bifidobacteria require complex biological substances for growth; they will not grow in defined synthetic media. The nature of such growth factors is unknown, though our laboratory has suggested that they might be cystine/cysteine-containing peptides (Poch and Bezkorovainy, 1991). Both the TPY medium of Scardovi (1986) and the milk whey medium (Petschow and Talbott, 1990, 1991) contain such factors.

Cells in the bovine milk whey were permitted to grow in the presence of H_2O_2 for up to 48 h with periodic determinations of A_{610}, while in the TPY medium the growth was continued for 24 h. Following these initial growth studies, the cells were centrifuged and suspended in fresh H_2O_2-free TPY medium to a final A_{610} of 1.2. This was then inoculated into the regular TPY medium and allowed to grow for 24 h, after which time the A_{610} and pH of the suspensions were measured. The presence of F6PPK in the disrupted cells was also determined.

Reagents and Analytical Procedures. Hydrogen peroxide was purchased as a 30% solution from Sigma Chemical Co. (St. Louis, MO). About 30 μL of this was diluted to 50 mL with the 3,3-dimethylglutarate buffer solution at pH 6.5 to give an ≈12 000 μM peroxide solution. The exact concentration of this stock solution was determined colorimetrically via serial dilutions as previously described (Kot et al., 1997). This initial stock solution was diluted further as required.

Bovine milk whey and other chemicals were purchased from Sigma, as were kits for the measurement of L-lactate (No. 826 UV) and ferrous iron (No. 690-A). Hydrogen peroxide was measured by using Sigma kit No. 352 with modification (Kot et al., 1996). Hydrogen peroxide detection limit was ∼25 μM. Radioactive iron was obtained from Du Pont Laboratories (Boston, MA).

Free Radical Scavengers. Free radical scavengers were used in an attempt to diminish the effects of free radicals on lactate production by *B. thermophilum*. They were L-glucose, D-lactate, lactose, dimethyl sulfoxide (DMSO), and *G. biloba* extract. With the exception of *Ginkgo biloba*, which was obtained from a local pharmacy, all scavengers were bought from Sigma. Cells were first incubated with 400−855 μM H_2O_2 and various concentrations of the scavengers (maximum concentrations were 5 mg/mL, 2 mg/mL, 11 mM, and 4 and 0.3 mg/mL, respectively) for 60 min, then glucose (2 mg/mL) was added, and lactate production was measured for another 60 min. Glucose (2 mg/mL) was used as a free radical scavenger in $Fe(OH)_3$ binding experiments.

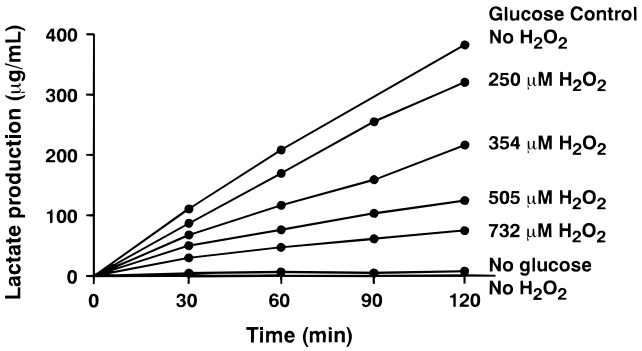

Figure 1. Lactate production by *B. thermophilum* as a function of time and H_2O_2 concentration. The cells were suspended in 0.1 M 3,3-dimethylglutarate buffer solution at pH 6.5 in the presence of 2 mg/mL glucose. Cell density was 0.9 mg of dry cell mass/mL, A_{610} = 1.2. All incubations were carried out at 37 °C.

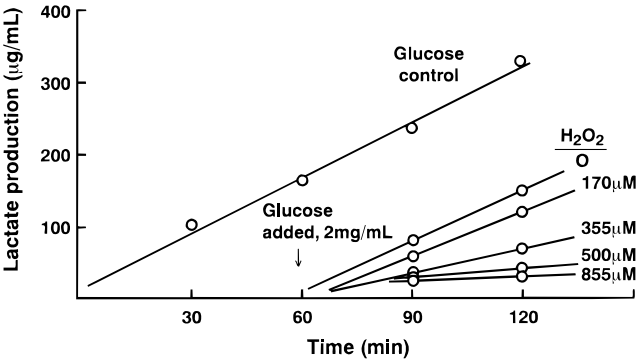

Figure 2. Lactate production by *B. thermophilum* as a function of time and H_2O_2 concentration following preincubation in H_2O_2 for 1 h without glucose, followed by the addition of 2 mg/mL glucose and measurement of lactate for another hour. The control contained 2 mg/mL glucose and no H_2O_2. Cell densities and incubation temperatures were as in Figure 1.

RESULTS

Effect of Hydrogen Peroxide on Lactate Production by *B. thermophilum*. *B. thermophilum*, suspended in the 3,3-dimethylglutarate buffer solution at pH 6.5 (A_{610} = 1.2, 0.9 mg/mL dry cell mass) and in the presence of 2 mg/mL glucose, produced L-lactate in an inverse relationship with H_2O_2 concentration, as shown in Figure 1. There was no loss of H_2O_2 during the experimental period (2 h). Alternatively, the cells were exposed to various concentrations of H_2O_2 for 1 h, followed by addition of glucose and measurement lactate production for an additional hour. The results are depicted in Figure 2, which indicates that lactate production by the organism was initiated at about the same rate as that shown in Figure 1.

The question now arose as to whether the effect of H_2O_2 on lactate production was permanent. The cells were incubated with various concentrations of H_2O_2 in

Table 1. Lactate Production by *B. thermophilum* as a Function of Time following Incubation with Various Concentrations of H_2O_2[a]

H_2O_2 concn (μM)	lactate production (μg/mL)	
	30 min	60 min
0	130	222
340	95	228 (103)[b]
570	86	214 (96)
855	68	180 (81)

[a] The cells were first incubated with H_2O_2 for 1 h in the absence of glucose, then centrifuged, and resuspended in 0.1 M 3,3-dimethylglutarate buffer solution (pH 6.5) in the presence of 2 mg/mL glucose. Cell density was $A_{610} = 1.2$. [b] Values in parentheses are percent of the control.

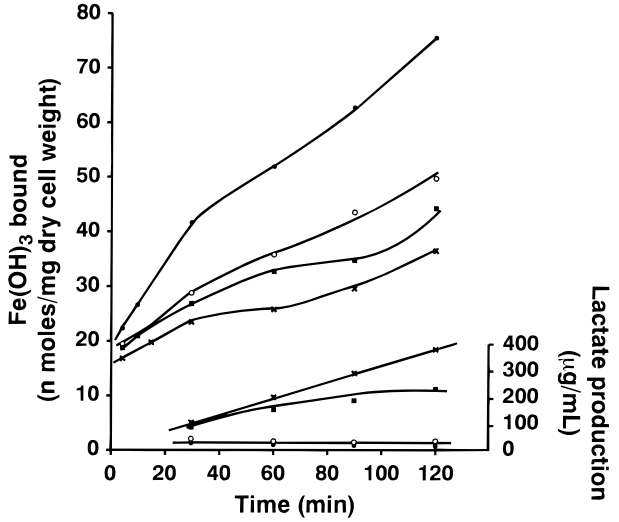

Figure 3. Binding of $Fe(OH)_3$ by *B. thermophilum* as a function of time and in the presence or absence of H_2O_2 and glucose as the free radical scavenger. Hydrogen peroxide, glucose, and $Fe(OH)_3$ concentrations were 287 μM, 2 mM, and 109 μM, respectively. Cell density and incubation temperatures were as in Figure 1. Solid circles indicate binding in the presence of H_2O_2 and in the absence of glucose; open circles, in the absence of both H_2O_2 and glucose; squares, in the presence of both H_2O_2 and glucose; and crosses, in the absence of H_2O_2 and in the presence of glucose. The inset graph indicates lactate production by the above samples as a function of time.

the absence of glucose for 60 min and then centrifuged, and the cell pellets were resuspended in H_2O_2-free 3,3-dimethylglutarate buffer solution at pH 6.5 in the presence of 2 mg/mL glucose. Lactate production was then measured after 30 and 60 min, and the results are shown in Table 1. It is seen that all cell samples were able to produce higher levels of lactate at 60 min after glucose was added. At 855 μM H_2O_2, lactate production was 81% of the level produced without H_2O_2.

Addition of free radical scavengers (see Materials and Methods) did not abate the effects of hydrogen peroxide on lactate production by *B. thermophilum* (data not shown).

Effect of Hydrogen Peroxide on the Binding of $Fe(OH)_3$ by *B. thermophilum*. We have previously shown that the binding of $Fe(OH)_3$ by *B. thermophilum* at 44 °C increases with increasing concentrations of H_2O_2 (Bezkorovainy and Kot, 1998). Figure 3 illustrates this point: At 37 °C, the binding of $Fe(OH)_3$ was highest at an $[H_2O_2] = 287$ μM and lowest when its level was undetectable, but glucose, as a free radical scavenger, was present. Extrapolation of the 5, 10, and 30 min points to 0-time gave similar instantaneous $Fe(OH)_3$ binding values for the four curves shown in Figure 3: 19.3 nmol/mg of dry cell mass when cells were exposed to H_2O_2 only; 17.0 nmol/mg when cells were exposed to both H_2O_2 and glucose; 17.5 nmol/mg when cells were exposed to neither H_2O_2 nor glucose; and 16.5 nmol/mg when cells were exposed to glucose only. Though these 0-time figures are very similar, substantial differences in the levels of $Fe(OH)_3$ binding were observed as incubation was continued for 2 h.

Table 2. Instantaneous (0-Time) Binding of $Fe(OH)_3$ by *B. thermophilum* following Preincubation with Various Concentrations of H_2O_2[a]

H_2O_2 concn (μM)	preincubation time		
	5 min	60 min	90 min
0	11	8.1 (48)[b]	7.2
170	9.2	8.2 (44)	9.2
350	13	7.4 (49)	6.4
500	9.6	6.2 (48)	7.5
830	9.4	4.9 (61)	8.3
0[c]	8.8	− (37)	7.5

[a] In nmol/mg of dry cell mass. The cells were centrifuged after preincubation with H_2O_2 and resuspended in H_2O_2-free buffer solution. The $Fe(OH)_3$ (also H_2O_2-free) was then added to a concentration of 136 μM. 0-Time values were determined by extrapolating binding values obtained at 5−30 min to 0 on the *y*-axis. All incubations were carried out at 37 °C. [b] Values in parentheses were obtained after 120 min incubations. [c] Preincubated with 2 mg/mL glucose without H_2O_2.

Another batch of cells was preincubated with different concentrations of H_2O_2, but in the absence of $Fe(OH)_3$, for 5, 60, and 90 min, and the cells were centrifuged and then reincubated with $Fe(OH)_3$ but in the absence of H_2O_2. This experiment was thus similar to that with lactate production depicted in Table 1. It was anticipated that if the bacterial surface had been altered by the H_2O_2 preincubation, the $Fe(OH)_3$ binding at 0-time would have increased. The results are shown in Table 2. Clearly, there was no increase in $Fe(OH)_3$ binding at 0-time; the binding proceeded as if no preincubation with H_2O_2 had taken place.

Growth of *B. thermophilum* in the Presence of Hydrogen Peroxide. Growth curves of *B. thermophilum* in the bovine milk whey medium are shown in Figure 4. The growth was meager, even in the absence of H_2O_2, reaching a maximum in ~25 h. After 48 h, the pH values of cell suspensions represented by curves 2, 3, 4, and 5 were 5.5, 5.6, 5.9, and 6.3, respectively. Blanks had a pH of 6.3. These were regrown in TPY media containing no H_2O_2, and the results are shown in Table 3. It can be seen that all samples achieved a near maximal growth and that all cultures were 4+ positive for F6PPK activity.

Cells grown in the presence of H_2O_2 in the TPY medium achieved maximum growth levels after 24 h at all H_2O_2 concentrations used, except at 600 μM. However, the magnitude of their lag periods was proportional to initial H_2O_2 concentrations used (Figure 5). The final pH of cultures represented by curves 1−4 was 4.2, that of curve 5 was 4.5, and that of curve 6 was 6.2 (equal to that of the blank). Cultures represented by curves 1−5 were 4+ positive for F6PPK activity. The culture showing no growth (curve 6) was reinoculated into H_2O_2-free TPY medium, and the growth achieved then was maximal as shown in Table 3. This sample was also 4+ positive for F6PPK activity.

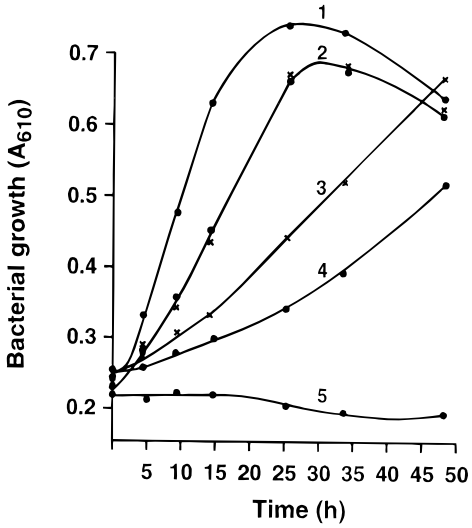

Figure 4. Growth curves of *B. thermophilum* in bovine milk whey medium and at various initial H_2O_2 concentrations. Curve 1 represents growth in the absence of H_2O_2. Curve 2 represents growth in the presence of 41 and 66 μM H_2O_2, and curves 3, 4, and 5 represent growth in the presence of 100, 200, and 400 μM H_2O_2, respectively. The bacteria were grown at 37 °C under anaerobic conditions.

Table 3. Properties of *B. thermophilum* Cultures following Reinoculation of the 48 h (Figure 4) and 24 h (Figure 5) H_2O_2 Cultures into H_2O_2-Free TPY Medium[a]

bacteria initially grown in	initial exposure to H_2O_2 (μM)	initial A_{610}	24 h A_{610}	pH	F6PPK activity[b]
TPY medium	600	0.15	1.4	4.2	4+
whey medium	41	0.14	1.3	4.2	4+
	66	0.11	1.3	4.2	4+
	100	0.11	1.2	4.2	4+
	200	0.11	1.3	4.2	4+
	400	0.11	1.1	4.7	4+
	0 (control)	0.13	1.3	4.2	4+

[a] All readings (except the initial A_{610}) were taken after 24 h of growth at 37 °C under anaerobic conditions. [b] Fructose-6-phosphate phosphoketolase; in cells disrupted by sonication.

Stability of H_2O_2 in the TPY and whey media was investigated in the absence of cells. The results are depicted in Table 4. It is seen that H_2O_2 was less stable in the TPY medium than in the whey medium; no H_2O_2 was detected in the TPY medium after 24 h when the initial H_2O_2 concentrations were 140−800 μM. In the bovine milk whey medium, losses of H_2O_2 were in the range of 63−82%.

DISCUSSION

This paper deals with the effects of H_2O_2 on growth, lactate production, and surface properties of *B. thermophilum*. Bifidobacteria may encounter substantial amounts of H_2O_2 while in storage in fermented milk products or in the gut of humans and animals at inflammation sites, where it is produced in connection with neutrophil and phagocyte action. Hydrogen peroxide acts by producing the highly reactive OH-free radical in the presence of iron or copper (Halliwell and Gutteridge, 1985a). It is thus resonable to assume that the effects of H_2O_2 observed in this study were in fact the effects of the OH-free radical.

Figures 1 and 2 have demonstrated that the metabolism of glucose by *B. thermophilum* was adversely affected by H_2O_2 without the addition of exogenous iron.

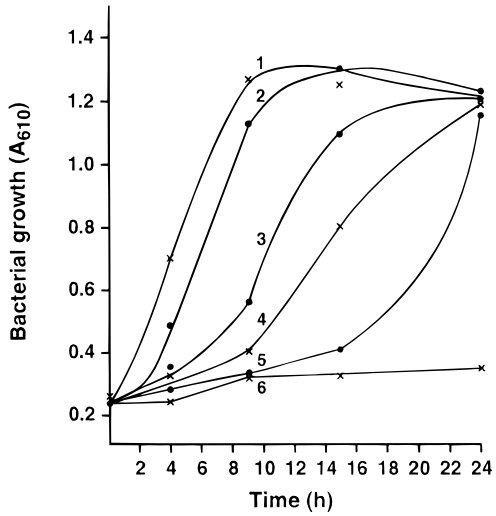

Figure 5. Growth curves of *B. thermophilum* in the TPY extract medium at various initial H_2O_2 concentrations. Curve 1 represents growth in the absence of H_2O_2, and curves 2−6 represent growth in the presence of 100, 200, 300, 400, and 600 μM H_2O_2. Growth conditions were as in Figure 4.

Table 4. Disappearance of H_2O_2 from the Bovine Milk Whey and TPY Media as a Function of Time

medium type	concn of H_2O_2 (μM) at indicated time						
	initial	1 h	2 h	3 h	5 h	24 h	48 h
whey	758	600	580	580	580	328	280
	569	420	390	400	384	184	100
	403	370	340	340	340	120	100
	117	110	110	80	100	40	0
TPY	800	464	380	400	380	0	
	640	372	344	300	20	0	
	463	184	180	140	12	0	
	268	72	64	60	0	0	
	140	40	32	24	0	0	

It is known that H_2O_2 penetrates cell membranes with ease; once inside the cell, it is assumed that it encounters sufficiently large amounts of iron and/or copper to generate OH-free radicals in sufficient quantity to affect lactate production. However, after exposure to rather high initial H_2O_2 concentrations (>800 μM), the cells were able to resume lactate production at nearly normal rates when H_2O_2 was not present in the media. It appears that the effect of H_2O_2 at these levels did not permanently alter the enzymes for lactate production. It is also likely that the OH-free radicals were able to intercept certain bifidus pathway intermediates, thus preventing them from generating lactate.

Free radical scavengers did not abolish the effects of H_2O_2 on lactate production. Most likely, this was due to an inability on part of the scavengers to enter the cell and intercept the OH-free radicals generated therein. It was surprising to see this effect with DMSO, which is supposed to penetrate cellular membranes with ease. On the other hand, *B. thermophilum* does not ferment lactose or L-glucose, and its failure to enter the cell and counteract free radical action was not unexpected.

Cells preincubated with H_2O_2 did not exhibit a greater degree of instantaneous $Fe(OH)_3$ binding than did cells preincubated without H_2O_2 (Table 2). Thus, hydrogen peroxide may not have been able to generate sufficient amounts of the OH-free radical in the medium to affect cellular surface in the absence of exogenous iron. Had cell surface damage occurred as a result of free radical action, the 0-time binding of $Fe(OH)_3$ following prein-

cubation with H_2O_2 for 5−90 min would have been substantially higher than that of the control. However, this was not the case, as shown in Table 2. When both exogenous $Fe(OH)_3$ and H_2O_2 were present, there was an increased level of $Fe(OH)_3$ binding compared to that observed in the absence of H_2O_2 or in the presence of glucose, a free radical scavenger (Figure 3).

Hydrogen peroxide was able to interfere with the normal growth of *B. thermophilum* even at low initial concentrations: 40−60 μM in bovine milk whey and 100 μM in the TPY medium. This can be illustrated by longer lag periods and failure to grow, as indicated in Figures 4 and 5. However, growth resumed as the H_2O_2 disappeared from the medium. Thus, the ability of bifidobacteria to grow following exposure to H_2O_2 at concentrations of up to 800 μM for 48 h was not affected; it was merely bacteriostatic rather than lethal.

In conclusion, this study has shown that H_2O_2 at concentrations of about 250−800 μM was able to abate lactate production by *B. thermophilum* and to decrease its growth rate at concentrations as low as 40 μM using the bovine milk whey medium. However, it did not kill these organisms, and they were able to resume their normal physiologic activity when H_2O_2 was removed from their environment. Hydrogen peroxide was not able to alter the cellular surface without the addition of exogenous iron. The results obtained in this study are suggestive that free radicals may not be the major reason bifidobacteria are lost from fermented milk products upon storage; it is more likely that a low pH is responsible, as previously suggested by Biavati et al. (1992b). Further research should determine whether other bifidobacterial species and strains behave in a similar fashion with respect to H_2O_2.

ABBREVIATIONS USED

A_{610}, turbidity at 610 nm; F6PPK, fructose-6-phosphate phosphoketolase; TPY, trypticase−phytone−yeast extract (medium).

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