Hydrogen Peroxide Production by *Lactobacillus delbrueckii* Subsp. *Lactis* I at 5°C

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ABSTRACT

Cells of *Lactobacillus delbrueckii* subsp. *lactis* I produced hydrogen peroxide at 5°C in sodium phosphate buffer (0.2M, pH 6.5) with or without glucose. However, if the cells were starved by preincubation in buffer alone, glucose or sodium lactate were necessary to cause hydrogen peroxide production at 5°C. Hydrogen peroxide production by nonstarved cells was confirmed to be in part due to a NADH oxidase. The production of hydrogen peroxide by starved cells in buffer plus glucose in the early stage of incubation was associated with the production of a small portion of lactic acid which disappeared upon further incubation. Additional experiments revealed that hydrogen peroxide was produced in buffer containing sodium lactate added in place of glucose. Results suggested the presence of a lactate oxidase in the organism which used D-lactate to produce hydrogen peroxide.

Key Words: *Lactobacillus*, hydrogen peroxide, lactic acid, bacteria

INTRODUCTION

AMONG LACTIC ACID BACTERIA (LAB) THE ABILITY TO PRODUCE hydrogen peroxide is widespread (Anders et al., 1970). In some cultures hydrogen peroxide can accumulate to levels that are autoinhibitory or inhibitory to other bacteria (Anders et al., 1970; Gilliland and Speck, 1974, 1975). Some researchers have suggested hydrogen peroxide production by LAB involves oxidative actions on sugars or related compounds (Dobrogosz and Stone, 1962; Condon, 1987; Grufferty and Condon, 1983; Kot et al., 1996). Others have indicated an NADH oxidase was involved (Anders et al., 1970; Collins and Aramaki, 1980; Higuchi et al., 1993).

Production of hydrogen peroxide by LAB can be beneficial for food preservation and prevention of growth of food borne pathogens (Dahiya and Speck, 1968; Gilliland and Speck, 1969, 1974, 1975, 1977). Hydrogen peroxide producing LAB can inhibit growth of psychrotrophic organisms and pathogens at refrigeration temperatures (Daly et al., 1972; Juffs and Babel, 1975; Martin and Gilliland, 1980; Gilliland and Ewell, 1983). Among the lactobacilli, strains *Lactobacillus delbrueckii* subsp. *lactis* have seemed to produce the highest amounts of hydrogen peroxide at refrigeration temperatures without growing or changing the pH of the medium (Gilliland, 1980). Cells of *Lactobacillus delbrueckii* subsp. *lactis* I, among other strains of *L. delbrueckii* subsp. *lactis* tested, produced sufficient hydrogen peroxide to kill cells of *Escherichia coli* O157:H7 on refrigerated raw chicken meat (Brashears et al., in press). Few reports have been concerned with the conditions, which affect hydrogen peroxide production or the enzyme system(s) of *L. delbrueckii* subsp. lactis involved. The objective of the present study was to determine the enzyme(s) of *L. delbrueckii* subsp. lactis responsible for hydrogen peroxide production at refrigeration temperature and to determine what substrate(s) if any, was needed.

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MATERIAL & METHODS

Sources and maintenance of cultures

Lactobacillus delbrueckii subsp. *lactis* I was obtained from the stock culture collection of the Food Microbiology Laboratory in the Dept. of Animal Science at Oklahoma State Univ. Cultures were maintained by weekly transfers in MRS broth (Difco Laboratories, Detroit, MI) using 1% inocula and 18h incubation at 37°C. They were stored at 5°C between subcultures. Immediately before experimental use, cultures were subcultured at least twice in sterile MRS broth.

Enumeration of bacteria

Lactobacilli were enumerated by the pour plate technique. Appropriate decimal dilutions were prepared with 99 mL dilution blanks containing 0.1% peptone (Difco Laboratories, Detroit, MI) and 0.001% antifoam emulsion (Sigma Chemical Co.) in distilled water. Dilutions were prepared in accordance with procedures in Standard Methods for Examination of Dairy Products (Marshall, 1992). To enumerate lactobacilli, molten MRS agar (lactobacilli MRS broth plus 1.5% agar) at 45°C was poured into the plates with appropriate dilutions. After solidification, plates were placed in plastic bags flushed with CO_2 for 30 sec and sealed. The bags were incubated for 48 h at 37°C. Colonies were counted with the aid of a Quebec Colony Counter.

Production of bacterial cells

Washed cells. Lactobacilli were grown in MRS broth for 16h at 37°C, harvested by centrifugation (16,000 × g for 20 min at 5°C), washed twice in cold physiological saline and resuspended in sodium phosphate buffer (0.2M, pH 6.5) with or without 55.5 mM glucose as required.

Starved bacterial cells. Washed cells of *L. delbrueckii* subsp. *lactis* I resuspended in sodium phosphate buffer (0.2M, pH 6.5) without glucose were incubated for 1h at 5°C with constant agitation on a magnetic stirrer. The cells were recovered by centrifugation (16,000 × g for 20 min at 5°C), washed twice with cold physiological saline and resuspended in cold sodium phosphate (0.2M, pH 6.5).

Cell-free extract

Cell-free extracts of lactobacilli were prepared from starved cells resuspended in sodium phosphate buffer (0.2M pH 6.5) containing 0.1 mM EDTA. The cell suspensions were sonicated at 5 min intervals for 45 min at 4°C. Between intervals they were allowed to cool for 1 min. Cellular debris was removed by centrifugation (16,000 × g for 20 min at 5°C) and the supernatant fluid (cytosolic extract) was passed through a sterile 0.45 µm membrane filter into a sterile test tube. The resulting cell free extract was used for enzymatic assays. It was held in an ice-water bath until assayed (no more than 30 min).

Hydrogen peroxide production

Cells of *L. delbrueckii* subsp. *lactis* I (or cell free extracts) were resuspended in cold phosphate buffer (0.2M, pH 6.5) with or without 55.5 mM glucose as required and incubated for the desired time at 5°C. Following incubation, cells were removed by centrifugation

and the supernatant was assayed for hydrogen peroxide.

Samples were assayed for hydrogen peroxide by placing 5 mL of cell free supernatant into test tubes containing 1 mL of a 0.1% aqueous solution of peroxidase (Horseradish Type VI-A; Sigma Chemical) and 0.1 mL of a 1% aqueous solution of o-dianisidine (Sigma Chemical Co.). A blank was prepared using 5 mL of sodium phosphate buffer instead of the sample supernatant fluid. Tubes were incubated for 10 min at 37°C. The reaction was stopped by adding 0.2 mL of 4N HCl to each test tube. Absorbance reading (A400 nm) of each sample was determined and peroxide content was determined by comparing the A400 nm to a standard curve (Gilliland, 1969).

Protein assay

The protein content of the cellular fraction(s) was determined by the method of the Bradford (1976). Bovine serum albumin (Sigma Chemicals Co.) was used as protein standard.

Lactic acid analysis

Samples were prepared for high performance liquid chromatography (HPLC) analysis by centrifuging 1.0 mL portions in 1.5 mL microcentrifuge vials for 10 min at 12,500 × g (at room temperature). A sample of the supernatant fluid (0.5 mL) was transferred to a clean vial and acidified with an equal volume of 0.01M sulfuric acid. The acidified samples were filtered through 0.2 µm membrane filters directly into 2 mL HPLC autosample vials and capped. Samples were stored at -20°C until analysis was performed. For analysis, frozen tubes were allowed to thaw in tap water at room temperature. Samples were analyzed using a Hewlett Packard 1090 HPLC system equipped with a diode-array detector (Hewlett Packard, Atlanta, GA). The sample was injected into 0.005M H_2SO_4 mobile phase heated to 65°C and separated using a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA). The peaks were detected with a diode array detector at 210 nm. Peak areas determined with known concentrations of lactic acid were used to prepare a standard curve. Lactic acid concentration in the samples was determined by comparing the peak area to the standard curve. Peak purity was monitored by UV scanning techniques as an aid for identifying abnormal wavelength patterns in a single peak.

NADH oxidase assay

Oxidation of NADH was followed spectrophotometrically at $A_{340\,nm}$ with a Beckman DU 75000 spectrophotometer using cuvettes with a 1 cm path. Each cuvette contained 2.25 mL of sodium phosphate buffer (0.2 M, pH 6.5), 0.75 mL of 0.48 µM NADH and 0.5 mL of the cell free extract (3.5 mL total). One unit of NADH oxidase was defined as the µmoles of NADH oxidized/min based on the disappearance of NADH from the reaction mixture. Specific activity was the number of units/mg of protein. Normally, the change of absorbency between 0.2 and 2.0 min was used in calculating the reaction velocity. The velocity was linear up toat least 10 min. (Anders et al., 1970; Collins and Aramaki, 1980).

Statistical analyses

The data which contained a response variable that was dependent on time (Fig. 1–7) were analyzed using an analysis of variance for a split plot over time in a randomized block design. The differences and confidence levels were determined by calculating the least significant difference with SAS® (SAS Institute, Inc., 1985) and significant difference was defined at $P \leq 0.05$.

RESULTS

Growth and hydrogen peroxide production

The growth of *L. delbrueckii* subsp. lactis I was adversely influenced by agitation. A much more pronounced lag phase was observed in the agitated culture than in the static one, and the static culture reached a higher population. Cells harvested from agitated and static cultures at 16 h of incubation were assayed for hydrogen peroxide production. After 1h of static incubation at 5°C, hydrogen peroxide concentrations were 0.42 µg/mL and 0.43 µg/mL for the cells that had been grown under static and agitated conditions respectively. While there was no difference ($P>0.05$) in the total amount of hydrogen peroxide produced, there was a difference ($P<0.05$) in amount of hydrogen peroxide produced per colony formed unit (CFU). Cells grown under agitated conditions produced higher amounts ($P<0.05$) of hydrogen peroxide (0.55 µg/109 CFU) than did those grown under static conditions (0.33 µg/109 CFU).

Effect of glucose on production of hydrogen peroxide

Cells of *L. delbrueckii* subsp. *lactis* I harvested from the stationary phase (12h of incubation) of a statically grown MRS broth culture were washed twice with sodium phosphate buffer (pH 6.5) and resuspended in sodium phosphate containing glucose (55.5 mM). The cell suspension was incubated for 24h at 5°C with constant agitation. No cellular growth was detected; however, hydrogen peroxide production was detected and reached a maximum of 25 µg/10^6 CFU at 18h (Fig. 1). There appeared to be a slight decline in hydrogen peroxide concentration beyond 20h. Accumulation of hydrogen peroxide by cells in sodium phosphate buffer with and without glucose (55.5 mM) was monitored for 55h (Fig. 2). Higher amounts of

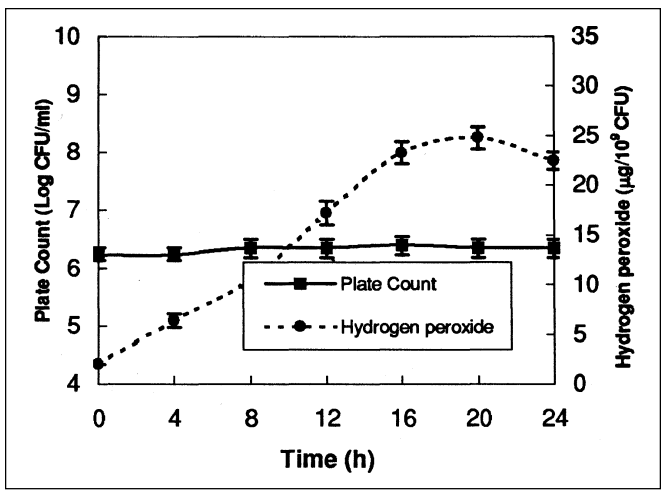

Fig. 1—Hydrogen peroxide production and viable population of *Lactobacillus delbrueckii* subsp. *lactis* I resuspended in buffer containing 55.5 mM glucose with constant agitation at 5°C. Means from 2 independent trials. Vertical bars represent the standard deviation.

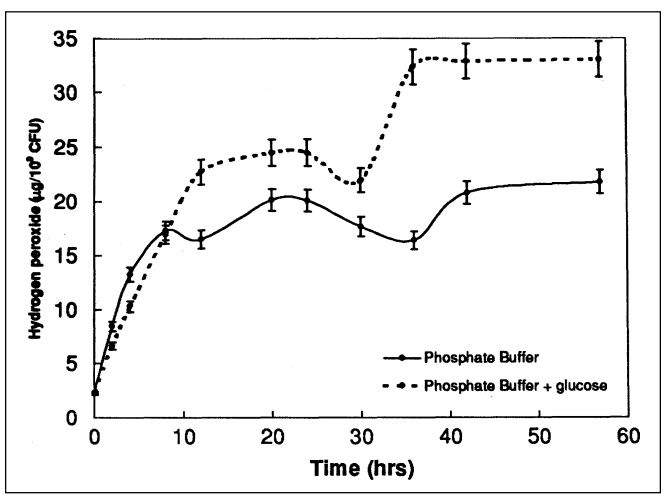

Fig. 2—Effect of glucose (55.5 mM) on hydrogen peroxide production by cells of *Lactobacillus delbrueckii* subsp. *lactis* I at 5°C without agitation. Means from two independent trials. Vertical bars represent the standard deviation. Differences between treatments were significant ($P<0.05$) after 8h.

Table 1—Influence of glucose on hydrogen peroxide production by starved and nonstarved cells of *Lactobacillus delbrueckii* subsp. *lactis* I[a]

Conditions	Glucose	Hydrogen peroxide[b] (μg/10^9 CFU)
Nonstarved	–	6.0[b]
	+	8.6[a]
Starved	–	0.2[d]
	+	4.4[c]

[a]Cells were incubated at 5°C for 3h in sodium phosphate buffer (pH 6.5, 0.2M) with or without 55.5 mM glucose. A control with sodium phosphate buffer without cells at 5°C was included, but no hydrogen peroxide was detected.
[b]Means from two independent trials. Means with same superscript letters are not significantly different (P>0.05).

Table 2—Partial purification of NADH oxidase from *Lactobacillus delbrueckii* subsp. *lactis* I

Fraction	NADH oxidase (nmoles NAD/ min/mL)	Protein (mg/mL)	Specific activity (nmoles NAD/ min/mg protein)	Purification
Cell free extract	97.95[a]	19.1[a]	5.1[b]	1.00[b]
Protamine sulfate	41.10[b]	6.1[b]	6.7[a]	1.30[a]
Dialyzed protamine sulfate extract	4.20[c]	5.6[c]	0.8[c]	0.15[c]

[a]Means from three independent trials. Means with same superscript letters are not significantly different (P>0.05).

hydrogen peroxide (P<0.05) were maintained in the buffer containing glucose than in the buffer alone. There was a slight decline in hydrogen peroxide from 18 to 30h followed by a large increase for cells in the buffer containing glucose. A similar pattern was observed for the cells in buffer alone but the increase was much less pronounced. These data suggested that glucose enhanced the production of hydrogen peroxide, especially after 20h.

Washed cells of *L. delbrueckii* subsp. lactis I were resuspended in phosphate buffer and preincubated for 1h at 5°C, then recovered by centrifugation and resuspended in fresh buffer (starved cells). Only 0.2 μg hydrogen peroxide was produced per 10^9 cells in 3h at 5°C (Table 1). However, if starved cells were resuspended in phosphate buffer containing 55.5 mM glucose 4.4 μg hydrogen peroxide/10^9 cells was produced. This further confirmed the importance of glucose for peroxide production. The amount of hydrogen peroxide produced by preincubated (starved) cells of *L. delbrueckii* subsp. *lactis* I was dependent on the concentration of glucose. Little or no hydrogen peroxide production was observed during 50h incubation at 5°C when 0.05 mM glucose was used. However, larger amounts (P < 0.05) were produced when 0.55, 5.55, and 55.5 mM glucose were added (data not shown) but no differences (P>0.05) were observed in amounts produced in those buffers.

Cell-free extracts of *L. delbrueckii* subsp. *lactis* I from static cultures were assayed for the ability to produce hydrogen peroxide in buffer containing pyruvate (55.5 mM) or different carbohydrates (glucose, sucrose, lactose, maltose, mannose, cellobiose or galactose at 55.5 mM each). Little or no hydrogen peroxide was produced by the cell free extracts under these conditions (data not shown). However, when NADH was added to buffer (without sugar) and the cell free extract, hydrogen peroxide formation which coincided with disappearance of NADH was observed. Such results indicated the presence of NADH oxidase capable of producing hydrogen peroxide.

The NADH oxidase of *L. delbrueckii* subsp. *lactis* I was partially purified (Table 2). The specific activity of the enzyme increased 1.7 times after protamine sulfate treatment. However, the NADH oxidase activity was almost completely lost after dialysis against 0.2M sodium phosphate buffer pH 6.5 for 18h at 4°C. The dialyzed enzyme was reactivated (P<0.05) upon addition of FAD (Table 3). The NADH oxidase activity and hydrogen peroxide formation increased (P<0.05) as the concentration of FAD increased from 0 to 10 μm.

Hydrogen peroxide production by cells using lactate as substrate

Lactic acid was detected during incubation of cells of *L. delbrueckii* subsp. *lactis* I at 5°C in sodium phosphate buffer containing 55.5 mM glucose at day 4 of incubation, but it disappeared by day 5 (Fig. 3). Hydrogen peroxide had reached a maximum level after 1 day followed by a decline, then an increase on day 3. Following day 3, the level of hydrogen peroxide decreased slightly, then remained fairly constant through day 9. The disappearance of lactic acid suggested the involvement of an enzyme(s) other than NADH oxidase in the formation of hydrogen peroxide. A lactate dehydrogenase could have been involved in regenerating NADH so NADH oxidase could continue in the formation of hydrogen peroxide. However, the equilibrium for lactate dehydrogenase is generally considered to be strongly toward lactate. The disappearance of the lactic acid could also have been due to the action of a lactate oxidase which produces hydrogen peroxide during the oxidation of lactate.

Experiments were done to determine if cells of *L. delbrueckii* subsp. *lactis* I would produce hydrogen peroxide at 5°C in buffer supplemented with sodium lactate rather than glucose. The total amount of hydrogen peroxide produced using lactate was higher (P<0.05) than the amount produced using glucose (Fig. 4). In the sample containing glucose as substrate, peroxide production appeared to level between 24 and 48h then increase sharply. In the sample containing sodium lactate, the hydrogen peroxide production did not

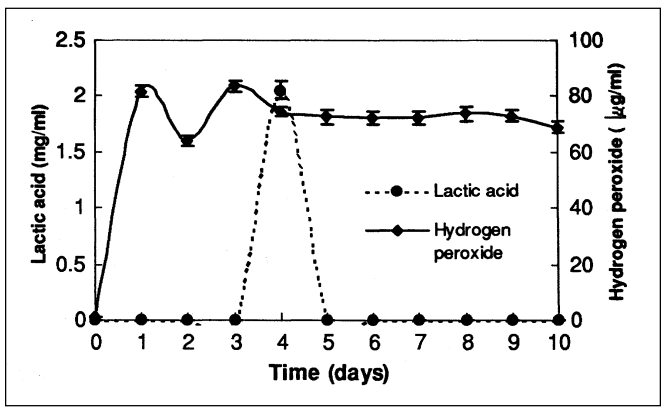

Fig. 3—Lactic acid and hydrogen peroxide formation by washed, starved cells of *Lactobacillus delbrueckii* subsp. *lactis* I in glucose (55.5 mM) sodium phosphate buffer without agitation. Means of two independent trials. Vertical bars represent the standard deviation.

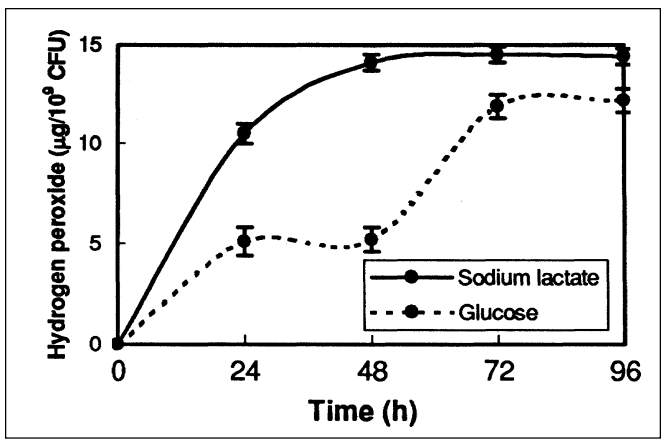

Fig. 4—Hydrogen peroxide production at 5°C by washed, starved cells of *Lactobacillus delbrueckii* subsp. *lactis* I in buffer containing 55.5 mM sodium lactate or 55.5 mM glucose without agitation. Means of three independent trials. Vertical bars represent the standard deviation.

Table 3—Effect of flavin adenine dinucleotide on dialyzed NADH oxidase activity and hydrogen peroxide production

FAD (μM)	NADH oxidase) (nmoles/min/mg)	Hydrogen peroxide[a] (μg/mL)
0	0.8[a]	0.2[a]
1	2.1[b]	0.5[b]
3	7.8[c]	1.4[c]
6	9.7[d]	2.2[d]
10	12.2[e]	3.7[e]

[a–e]Means from three independent trials. Means with same superscript letters are not significantly different (P>0.05).

include a plateau between 24 and 48h.

When starved cells of *L. delbrueckii* subsp. *lactis* I were inoculated into sodium phosphate buffer (pH 6.5, 0.2M) containing 5.55 mM L-, D-, or DL-lactate, hydrogen peroxide production was higher (P<0.05) in the presence of D-lactate than in the presence of DL- or L-lactate (Fig. 5). Little or no hydrogen peroxide was formed in the buffer containing L-lactate and none was formed in the control buffer (i.e. without lactate) during the 24h incubation.

Hydrogen peroxide accumulation by washed starved cells of *L. delbrueckii* subsp. *lactis* I was followed for 6 days in buffer containing DL-lactate at 4 temperatures. After two days incubation more hydrogen peroxide was produced at 25 and 37°C than at 5 or 15°C. After two days, some decreases in hydrogen peroxide concentration were observed at 25 and 37°C, and contrary to this, no decrease in hydrogen peroxide production was detected at 5 and 15°C. Peroxide production was associated with decreases in lactate concentrations at all four temperatures.

DISCUSSION

AGITATION OF CULTURES OF *L. DELBRUECKII* SUBSP. *LACTIS* I during growth did not influence the amount of growth, although it appeared to slow growth. However, when agitated, the cultures produced higher amounts of hydrogen peroxide/CFU than when grown statically. This could have been in part due to relatively shorter lengths of chains of cells in the agitated culture than in the static culture. Note that hydrogen peroxide was produced even under static conditions which would have had a lower concentration of oxygen than in agitated cultures. Wheater et al. (1952) reported that hydrogen peroxide was produced by *L. lactis* from glucose when cells were growing under aerobic or anaerobic conditions. Collins and Aramaki (1980) also reported that hydrogen peroxide was produced under static conditions by *L. acidophilus*, but higher amounts of hydrogen peroxide were produced when the culture was agitated. Hydrogen peroxide has an inhibitory effect towards some lactobacilli (Gilliland and Speck, 1969; Anders et al., 1970; Grufferty and Condon, 1983). In order for *L. delbrueckii* subsp. *lactis* I to have grown without inhibition from the hydrogen peroxide it may have a scavenger system intracellularly to eliminate some of the hydrogen peroxide. Lactobacilli may be able to grow under aerobic conditions in a glucose medium if a flavin respiratory system formed by a NADH oxidase and NADH peroxidase is present (Gregory and Fridovich, 1974; Whittenbury, 1964). Archibald and Fridovich (1981) reported that manganese within *L. plantarum* eliminated oxygen in a manner analogous to that in which a peroxidase eliminates hydrogen peroxide at the expense of some reductant, such as, NAD(P)H.

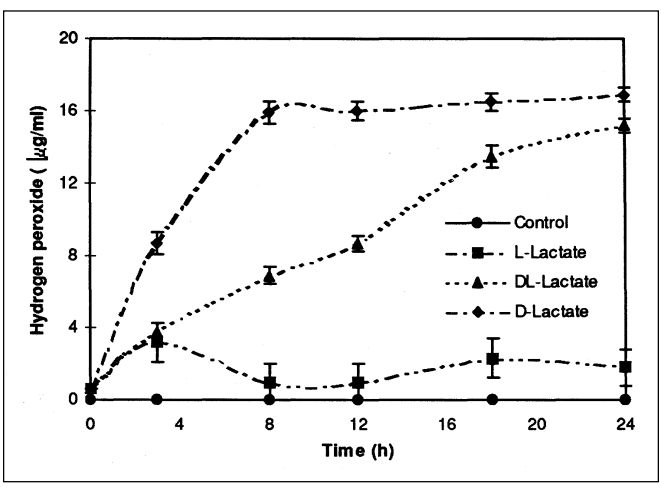

Fig. 5—Effect of L-, D- and DL- lactate (55.5 mM) in phosphate buffer on hydrogen peroxide production by washed, starved cells of *Lactobacillus delbrueckii* subsp. *lactis* I without agitation. The control was phosphate buffer without lactate. Means of two independent trials. Vertical bars represent the standard deviation.

Lactobacillus delbrueckii subsp. *lactis* I produced and accumulated hydrogen peroxide at 5°C in sodium phosphate buffer containing 55.5 mM glucose. More hydrogen peroxide was produced when the mixture was agitated as reported by Iwamoto et al. (1979a, b) and Collins and Aramaki (1980). No growth of *L. delbrueckii* subsp. *lactis* I was observed under these conditions, but the cells evidently remained metabolically active. Exposure of *L. mesenteroides* cells to air induced the loss of glycolytic activity, however, the cells remained metabolically active as evidenced by production of hydrogen peroxide (Ito et al., 1983). Wheater et al. (1952) reported that hydrogen peroxide production was associated with living, but not necessarily with multiplying LAB.

Measurement of hydrogen peroxide over time showed a plateau during incubation at 5°C followed by increased formation of hydrogen peroxide. This suggested that two substrates or more than one enzymatic system was involved. A similar plateau in accumulation of hydrogen peroxide had been reported by Price and Lee (1970) using cells of *L. plantarum* which produces hydrogen peroxide by more than one enzymatic system.

The possibility of hydrogen peroxide production in sodium phosphate buffer without added sugar was suggested in preliminary experiments in which washed cells from MRS broth were used to measure hydrogen peroxide production. However experiments in which starved cells (i.e. washed cells preincubated in buffer then recovered for assay) confirmed that glucose or other suitable substrate was required. The lack of hydrogen peroxide production by starved cells when introduced to fresh phosphate buffer likely was due to depletion of intracellular substrate(s) for peroxide production. The minimum concentration of glucose required to produce appreciable amounts of hydrogen peroxide was 0.55 mM. A reduction (P<0.05) in hydrogen peroxide production was detected with lower concentrations of glucose indicating the requirement for the carbohydrate.

Production of hydrogen peroxide was not observed when cell free extracts of starved cells were added to buffer containing only pyruvate or various sugars. However, if hydrogen peroxide was produced using pyruvate, a spontaneous chemical reaction may have occurred between the two compounds that would have destroyed any hydrogen peroxide formed (Gunsalus and Umbreit, 1945). Glucose, galactose and other substrates may promote O_2 utilization by whole cells generating NADH, (the substrate for NADH oxidase) but the NADH generating systems were lost when cell free extracts were prepared (Grufferty and Condon, 1983). This may explain the inability of cell free extracts to utilize carbohydrate sources for peroxide formation. The NADH oxidases are usually cytosolic enzymes (Grufferty and Condon, 1983) and for this reason accumulation of hydrogen peroxide was observed when the cell free extract was added to buffer containing NADH. The loss of NADH oxidase activity in cell free extracts after dialysis suggested the loss of a cofactor(s). Activity of NADH oxidase was recovered when the dialyzed enzyme was incubated with different concentrations of FAD. This effect has long been recognized (Hoskins et al., 1962; Collins and Ara-

maki 1980).

The reduction of pyruvate to lactate by lactate dehydrogenase (LDH) includes NAD regeneration which would be required for continued glycolysis. When NADH oxidase activity competes for NADH to produce hydrogen peroxide, LDH activity may be reduced. Since lactic acid was detected during formation of hydrogen peroxide at 5°C, the possibility exists that LDH was active at least when NADH was in excess. For this reason, when samples were stirred for long times at 5°C, inhibition of the LDH enzyme could have occurred and no further production of lactic acid was detected. There has long been evidence of the toxic effects of oxygen and metabolites formed in the presence of oxygen on G3P-dehydrogenase and it is probable that oxidation of sulfhydryl groups to disulfide groups adversely affected enzymes such as lactate dehydrogenase (Haugaard, 1968). The disappearance of the lactic acid that was produced suggested the possible existence of a lactate oxidase. Our results showed that cells of *L. delbrueckii* subsp. *lactis* I incubated in buffer containing sodium lactate produced hydrogen peroxide indicating the possible presence of a lactate oxidase. This enzyme, L-lactate oxidase (L-lactate: oxygen reductase E.C.1.1.3.2), has been reported in some LAB such as *Pediococcus* sp (Mizutani et al., 1983) and *S. faecalis* (London, 1968; Esder et al., 1979). Starved cells of *L. delbrueckii* subsp. *lactis* I produced hydrogen peroxide when D- and DL-lactate were used in the buffer media. The lack of appreciable production of peroxide on L-lactate suggested that the enzyme was specific for D-lactate. Further evidence for a lactate oxidase enzyme was indicated when cells of *L. delbrueckii* subsp. *lactis* I produced hydrogen peroxide at four different temperatures (5, 15, 25 and 37°C) on DL-lactate which was coupled with the consumption of DL-lactate at all temperatures.

Because of the potential application of peroxide production at refrigeration temperature to preserve food products there is need to understand the mechanism involved in its production. It may thus be possible to utilize selected strains and/or specific conditions to enhance use of *L. delbrueckii* subsp. *lactis* in food preservation. Knowing the primary enzyme(s) involved could enable the enhancement of production of hydrogen peroxide. If confirmed that lactate oxidase is involved, then combination of cells of *L. delbrueckii* subsp. *lactis* and low levels of sodium lactate could provide an economical way to help preserve of refrigerated foods. The peroxide thus produced also could be inhibitory to spoilage and/or pathogenic organisms.

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