HYDROGEN PEROXIDE PRODUCTION BY *LACTOBACILLUS DELBRUCKII* SSP. *LACTIS* AT REFRIGERATION TEMPERATURES

By

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TEMPERATURES

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TABLE OF CONTENTS

Chapter		Page
I	INTRODUCTION	1
II	REVIEW OF LITERATURE	5
	Biopreservation of foods by lactic acid bacteria	5
	Hydrogen peroxide production	7
	H_2O forming NADH oxidase	10
	H_2O_2 forming NADH oxidase	11
	Pyruvate oxidase	14
	α-Glycerolphosphate oxidase	16
	L-Lactate oxidase	17
	Diaphorase	18
	Oxygen scavenger systems	18
	NADH peroxidase	19
	Superoxide Dismutase and Manganesse	20
	Pseudocatalases and heme-catalases	21
	Mode of action	22
	Bacteriostatic effect	22
	Bactericidal effect	23
	Effect on lactic flora	23
	Effect on non lactic flora	24
	REFERENCES	27
III	FACTORS INFLUENCING HYDROGEN PEROXIDE PRODUCTION IN *LACTOBACILLUS DELBRUCKII* SSP. *LACTIS* I	36
	ABSTRACT	36
	INTRODUCTION	36
	MATERIAL AND METHODS	37
	Sources and maintenance of cultures	37
	Enumeration of bacteria	38
	Production of bacterial cells	38
	Cell-free extract	39
	Hydrogen peroxide production	39
	Protein	40
	Lactic acid analysis	40

Chapter Page

NADH oxidase 41
Statistical analyses 41
RESULTS 41
Growth and hydrogen peroxide production 41
Effect of glucose on the production of hydrogen peroxide 42
Hydrogen peroxide production in agitated and static resting cell suspensions 43
Hydrogen peroxide production by cell-free extracts 43
Hydrogen peroxide production by resting cells using lactate as substrate 44
DISCUSSION 46
REFERENCES 63

APPENDIXES 66
APPENDIX A-- identification of *Lactobacillus delbruckii ssp. lactis* I, spectrogram of NADH oxidase activity and chromatogram of lactic acid 66
APPENDIX B-- Factors affecting cell growth and hydrogen peroxide production in *Lactobacillus delbrukii* ssp. *lactis* I 70
APPENDIX C-- Statistical analyses 78

LIST OF TABLES

Table		Page
A	Enzymes involved in reactions between lactic acid bacteria and oxygen or oxygen metabolites	26
1	Influence of glucose on hydrogen peroxide production by starved and nonstarved cells of *Lactobacillus delbruckii* spp. *lactis* I	51
2	Partial purification of NADH oxidase from *Lactobacillus delbruckii* ssp. *lactis* I	52
3	Effect of Flavin Adenin Dinucleotide on dialyzed NADH oxidase	53
A1	Identification of the three strains of *Lactobacillus delbruckii* ssp. *lactis* by their pattern of fermented carbohydrates	67
B1	Effect of different carbohydrates sources on the production of hydrogen peroxide in preincubated resting cell of *Lactobacillus delbruckii* ssp. *lactis* I	74
B2	Effect of the concentration of cells on the accumulation of hydrogen peroxide	75
B3	Effect of hydrogen peroxide on survival of *Lactobacillus delbruckii* ssp. *lactis* I	76
B4	Effect of pH in hydrogen peroxide production by *Lactobacillus delbruckii* ssp. *lactis* I	77
C1	Analysis of variance of table 1 - Influence of glucose on hydrogen peroxide productionby starved and nonstarved cells of *Lactobacillus delbruckii* ssp. *lactis* I	79
C2	Analysis of variance of table 2 - Partial purification of NADH oxidase from *Lactobacillus delbruckii* ssp. *lactis* I (variable specific activity)	79

Table Page

C3 Analysis of variance of table 3 - Effect of Flavin Adenin Dinucleotide on dialyzed NADH oxidase (variable NADH)..... 80

C4 Analysis of variance of figure 1 - Influence of static and agitated conditions on the growth of *Lactobacillus delbruckii* ssp. *lactis* I..... 80

C5 Analysis of variance of figure 2 - Hydrogen peroxide production and viable population and hydrogen peroxide accumulation in buffer containing 55.5 mM glucose with constant agitation at 5°C (var plate count)..... 81

C6 Analysis of variance of figure 3 - Effect of glucose on hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* ssp. *lactis* I incubated at 5°C..... 81

C7 Analysis of variance of figure 4 - Effect of glucose on hydrogen peroxide production under agitated conditions at 5°C..... 82

C8 Analysis of variance of figure 5 - Hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* spp. *lactis* I at 5°C in phosphate buffer containing 55.5 mM glucose under agitated and static conditions during five hour of incubation..... 82

C9 Analysis of variance of figure 6 - Lactic acid detected by HPLC after incubation of *Lactobacillus delbruckii* ssp. *lactis* I in glucose sodium phosphate buffer..... 83

C10 Analysis of variance of figure 7 - Effect of sodium lactate (55.5 mM) and glucose (55.5 mM) on the production of hydrogen peroxide at 5°C with constant agitation..... 83

C11 Analysis of variance of figure 8 - Effect of L-, D- and DL- lactate in hydrogen peroxide production by resting cells of *L. delbruckii* spp. *lactis* I..... 84

C12 Analysis of variance of figure 9 - Effect of temperature on the production of hydrogen peroxide (A) and on the consumption of DL-lactate (B) by *L. delbruckii* spp. *lactis* I in DL lactate containing buffer at 5°C..... 85

LIST OF FIGURES

Figure		Page
1	Influence of static and agitated conditions on the growth of *Lactobacillus delbruckii* ssp. *lactis* I.....	54
2	Hydrogen peroxide production and viable population and hydrogen peroxide accumulation in buffer containing 55.5 mM glucose with constant agitation at 5°C.....	55
3	Effect of glucose on hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* ssp. *lactis* I incubated at 5°C.....	56
4	Effect of glucose on hydrogen peroxide production under agitated conditions at 5°C.....	57
5	Hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* spp. *lactis* I at 5°C in phosphate buffer containing 55.5 mM glucose under agitated and static conditions during five hour of incubation.....	58
6	Lactic acid detected by HPLC after incubation of *Lactobacillus delbruckii* ssp. *lactis* I in glucose sodium phosphate buffer.....	59
7	Effect of sodium lactate (55.5 mM) and glucose (55.5 mM) on the production of hydrogen peroxide at 5°C with constant agitation..	60
8	Effect of L-, D- and DL- lactate in hydrogen peroxide production by resting cells of *L. delbruckii* spp. *lactis* I.....	61
9	Effect of temperature on the production of hydrogen peroxide (A) and on the consumption of DL-lactate (B) by *L. delbruckii* spp. *lactis* I in DL lactate containing buffer at 5°C.....	62
A1	NADH comsuption by NADH oxidase from a cell free extract of *Lactobacillus delbruckii* ssp. *lactis* I.....	68

Figure Page

A2 Chromatogram of lactic acid from preincubated cells of *Lactobacillus delbruckii* ssp. *lactis* I resuspended in buffer containing 55.5 mM glucose..... 69

B1 Effect of $MnSO_4$ on the growth of *Lactobacillus delbruckii* ssp. *lactis* I under static and agitated conditions..... 71

B2 Growth of *Lactobacillus delbruckii* ssp. *lactis* I in agitated MRS broth containing glucose or lactose at pH 6.5..... 72

B3 Hydrogen peroxide production by cells harvested at different times from static MRS broth cultures at 37°C..... 73

CHAPTER I

INTRODUCTION

The lactic acid bacteria (LAB) constitute a large group of widely distributed gram-positive, rod or coccus shaped microorganisms which share several properties such as lack of hemes and cyanide-sensitive respiration, production of lactate, presence of flavin-linked oxidases, catalase, and peroxidase. Because of the lack of catalase and peroxidase, they frequently accumulate hydrogen peroxide. Lactic acid bacteria include the genera *Streptococcus*, *Pediococcus*, *Leuconostoc, Lactobacillus*, and *Bifidobacterium*. Of the LAB, group N streptococci and homofermentative *Lactobacillus* ssp. are the most frequently utilized genera in food products. They are characterized by the almost exclusive metabolic conversion of glucose to lactic acid. The homofermentative LAB produce more than 85% lactate as end product, and the heterofermentative LAB, produce lactate, ethanol, and carbon dioxide, among others as end products (Kandler, 1983). The metabolic pathway and type of optical isomers of lactate produced are distinctive for many individual species, either D or L, or a mixture of both (Batt, 1986). Aside from their fundamental roles in formation of organoleptic and rheological characteristics during the transformation of milk to products such as, cheese and yogurt, LAB are used and studied for their ability to inhibit unwanted bacteria, and thus for increasing the shelf-life of foods. It is known that the decrease of pH in itself is a factor of fundamental importance in controlling undesirable microorganisms in foods. However, the capacity of LAB to effectively inhibit other species not only results from their ability to lower the pH, but on the nature of the organic acids they produce. Furthermore, LAB can produce a variety of antimicrobial compounds such as, bacteriocins, diacetyl, acetaldehyde, D isomers of amino acids and hydrogen peroxide,

which may help to enhance their inhibitory activity (Piard and Desmazeaud, 1991). Of the possible oxygen metabolites only H_2O_2 is known to accumulate to a significant extent, in cultures of certain LAB (Condon, 1983). Hydrogen peroxide can generate cytotoxic oxygen species such as the hydroxyl radical, which is a powerful oxidant (Halliwell, 1978; Repine et al, 1981). It has been reported that H_2O_2 producing LAB can inhibit the 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). At refrigeration temperatures the amount of inhibition produced by different strains of lactobacilli is related to the amount of hydrogen peroxide they are capable of producing (Gilliland, 1980).

Hydrogen peroxide production by LAB usually occurs by direct reduction of atmospheric oxygen catalyzed by a small number of flavoprotein oxidase enzymes, such as, H_2O forming NADH oxidase, H_2O_2 forming NADH oxidase, pyruvate oxidase, α-glycerolphosphate oxidase, lactate oxidase, and NAD-independent lactic dehydrogenase (Götz et al, 1980a; Condon, 1987; Esders et al, 1979; Kandler, 1983). Through these oxidase enzymes and NADH peroxidase, which is a hydrogen peroxide scavenger, O_2 and H_2O_2 can accept electrons from sugar metabolism, and thus have a sparing effect on the use of metabolic intermediates, such as pyruvate or acetaldehyde as electron acceptors (Condon, 1987). Considerable variation of H_2O_2 production among strains of LAB is expected to be found, for instance, lactobacilli produce higher amount of H_2O_2 than streptococci (Gilliland and Speck, 1969). Among the lactobacilli strains *Lactobacillus lactis* produce the highest amount of H_2O_2 at refrigeration temperatures without growing or changing the pH of the medium (Gilliland, 1980). Leuconostocs and lactic streptococci have been reported to exert antagonistic action towards psychrotrophic flora in refrigerated meats (Daly et al, 1972; Reddy et al, 1970), however, at refrigerated temperature they grow slowly and produce some acid during refrigerated storage (Gilliland, 1980). Finally,

catalase-negative strains of *P. pentosaceus* have been reported to produce H_2O_2 but not at refrigerated temperature (Dobrogoz and Stone, 1962; Gilliland, 1980).

It is important to note that no relationship has been reported with regard to the preference or requirement for aerobic or anaerobic conditions for the production of detectable amounts of H_2O_2 (Wheather et al, 1952). In some cultures exposed to oxygen, hydrogen peroxide accumulates to autoinhibitory levels or levels inhibitory to other bacteria (Grufferty and Condon, 1983). Whereas, accumulation to autoinhibitory levels is widespread among LAB (Dobrogosz and Stone, 1962; Dahiya and Speck, 1968; Gilliland and Speck, 1969), it is not universal, some LAB produce H_2O_2 without any accumulation (Fukui, 1961; Collins and Aramaki, 1980; Murphy and Condon, 1984). This is thought to be due to a greater overall activity of systems within the bacteria cells that might destroy or eliminate H_2O_2 than those which produce it (Condon, 1987).

Several factors play an important role in H_2O_2 production such as, medium composition, metabolic activity, aerobic/anaerobic conditions, age and size of the inoculum. Hydrogen peroxide accumulation has been observed when LAB grow aerobically in milk, broth cultures, and in buffered cell suspensions at the expense of various substrates, such as, tryptone, casein hydrolysate (Bruhn and Collins, 1970), carbohydrates or substrates related to carbohydrate catabolism (Grufferty and Condon, 1983; Gunsalus and Umbreit, 1945; Dobrogosz and Stone, 1962; Brown and VanDemark, 1968; Dahiya and Speck, 1968). Oxygen can be used advantageously while streptococci utilize glucose, fructose, galactose, lactose, glycerol, α -glycerolphosphate, α-ketoglutarate, ethanol, succinate, butyrate (Cogan et al, 1989; Condon, 1983; Jacobs and VanDermark, 1960); some lactobacilli utilize glucose, maltose, mannitol, galactose, glycerol, pyruvate, lactate, fructose, sorbitol, α-glycerolphosphate, fructose 1, 6-diphosphate, gluconate, and ethanol (Strittmatter, 1959a; Fukui, 1961; Condon, 1983; Kot et al, 1996; Higuchi et al, 1992). Leuconostocs utilize glucose, fructose and gluconate (Condon, 1983), and certain pediococci utilize glucose and glycerol (Dobrogosz and Stone,

1962). Under this conditions high amounts of oxygen are taken up and H_2O_2 is excreted. However, several strains are inhibited in aerated cultures because the formation of H_2O_2 (Gilliland and Speck, 1969; Anders et al, 1970; Grufferty and Condon, 1983). The ability to grow aerobically must be distinguished from the ability to survive exposure to O_2. Resting cells of aerobically/anaerobically grown cells can be employed in aerobic/anaerobic conditions to produce hydrogen peroxide. Large numbers of *L. bulgaricus* are required to produce enough H_2O_2 in refrigerated milk to retard the growth of psycrotrophic bacteria (Juffs and Babel, 1975). Manganese content in growth media is important as a defense mechanism against oxygen toxicity when LAB lack superoxide dismutase system (SOD), manganese serves to scavenge O_2^- and thus protects the cell from the direct and indirect toxic effect of this radical. While some LAB that contain high levels of Mn^{2+} are unable to grow aerobically, they remained viable for several days when exposed to air. Therefore, Mn^{2+} would scavenge at expense of some reductant, such as NAD(P)H (Archibald and Fridovich, 1981a, 1981b). Production of H_2O_2 is deemed to be beneficial for food preservation and prevention of growth of pathogens (Dahiya and Speck, 1968).

Renewed interest in these aspects of the metabolism of LAB and their potential use to improve food safety has occurred. *Lactobacillus delbruckii* ssp. *lactis*, a microorganism used in the manufacture of some cultured dairy products, produce sufficient hydrogen peroxide at refrigerated temperatures to exert inhibitory action toward some undesirable microorganism in foods. As an example it has been demonstrated that this organism produces sufficient hydrogen peroxide to inactivate *Escherichia coli* O157:H7 at 7°C on fresh raw poultry meat (Brashears et al, in press). There are few reports concerning the circumstances, which affect H_2O_2 production and the enzyme system that is involved. The primary purpose of this study was to identified the enzyme system involve in hydrogen peroxide production by *Lactobacillus delbruckii* ssp. *lactis* at refrigeration temperature as well as the factors influencing the activity of the enzyme.

CHAPTER II

LITERATURE REVIEW

Biopreservation of foods by Lactic Acid Bacteria

The ability to preserve food by means of fermentation is an ancient and well-accepted form of biotechnology. One has only to view the wide range of fermented milks, breads, wines, pickles, meats and cheeses available to the consumer to recognize their valuable contribution to our daily diet. It is estimated that 25% of the European diet and 60% of the diet in many developing countries consist of fermented foods (Stiles, 1996). Hurst (1973) reviewed the preservation of foods by antagonistic actions produced by growth of desirable microorganisms. The growth of LAB in milk, sauerkraut, and vacuum packaged meats was reported as examples of protective, antagonistic growth. Members of LAB which include the genera *Lactococcus*, *Leuconostoc*, *Streptococcus, Pediococus,* and *Lactobacillus*, play an important role in these fermentations. In addition to providing an effective form of natural preservation, they beneficially influence the flavor, texture and nutritional attributes of the cultured products enhancing the variety of foods available to us. It has been showed that lactobacilli, N streptococci and Lecuconostoc species exert antagonistic action toward food-borne pathogens in associative cultures (Hurst, 1973; Reddy et al, 1970; Gilliland and Speck, 1974, 1975, 1977, Gilliland, 1980; Martin and Gilliland, 1980; Gilliland and Ewell, 1983; Abdel-bar and Harris, 1984).

It is possible that the effect of several factors acting together is responsible of the whole inhibitory action of the starter culture. Reduction of pH and removal of large amount of carbohydrates by fermentation are the primary preserving actions that these bacteria

provide to a fermented food. For a long time it has been known that certain LAB also produce antimicrobial compounds such as, diacetyl, acetaldheyde, D isomer of amino acids, bacteriocins and hydrogen peroxide (Piard and Desmazeaud, 1991). Hydrogen peroxide is known to accumulate to a significant extent, in cultures of some LAB incubated under normal aerobic conditions (Condon, 1983). The antimicrobial activity of hydrogen peroxide is well recognized and documented (Daeschel, 1989). Among the lactobacilli strains *Lactobacillus lactis* produce the highest amount of H_2O_2 at refrigeration temperatures without growing or changing the pH of the medium (Gilliland, 1980). Leuconostoc and lactic streptococci have been reported to exert antagonistic action towards psychrotrophic flora in refrigerated meats (Daly et al, 1972; Reddy et al, 1970), however, at refrigerated temperature they will grow slowly and produce some acid during refrigerated storage (Gilliland, 1980). Catalase negative strains of *P. pentosaceus* have been showed to produce H_2O_2 but not at refrigerated temperature (Dobrogoz and Stone, 1962; Gilliland, 1980). *Lactobacillus bulgaricus* produced higher amounts of hydrogen peroxide than did streptococci in milk incubated at 32 and 45 °C respectively (Gilliland and Speck, 1969). Various species of lactobacilli have been shown to produce enough H_2O_2 (Premi and Bottazzi, 1972) to increase the lag phase of *Pseudomonas* species (Price and Lee, 1970). Large numbers of *L. bulgaricus* are required to produce enough H_2O_2 in refrigerated milk to retard the growth of psycrotrophic bacteria (Juffs and Babel, 1975). Dahiya and Speck (1968) showed that *L. lactis* had higher inhibitory activity against *S. aureus* than *L. bulgaricus.* Moreover, *L. lactis* have been shown to produce antagonistic action toward the growth of psychrotophs in raw milk at 5 °C (Gilliland and Ewell, 1983). Resting cells of *Lactobacillus lactis* I among other strains of *L. lactis* has been proven to produce sufficient amounts of hydrogen peroxide to be inhibitory toward *Escherichia coli* O157:H7 on refrigerated raw chicken meat (Brashears et al, in press).

Hydrogen peroxide production

Lactic acid bacteria are called aerotolerant, facultative anaerobes, or micro-aerophilic, and they catabolize sugars via fermentation, where electron transport chains do not intervene, but in subsequent oxido-reduction steps nicotinamide adenine dinucleotide oxidized form (NAD^+) participates, undergoing cycling oxidation and reduction. When growing in the absence of oxygen, LAB rely mainly on lactic dehydrogenase (LDH), acetaldehyde dehydrogenase and alcohol dehydrogenase, to regenerate NAD^+ needed for the dehydrogenation reactions of sugar metabolism (Condon, 1987). In the presence of oxygen, however, H_2O and H_2O_2 forming NADH oxidases and NADH peroxidase are alternative mechanisms of NAD^+ regeneration. This mechanism can have a sparing effect on pyruvate, acetyl-CoA and acetaldehyde and can alter the spectrum of fermentation end products. When oxygen is available, the specific activities of NADH oxidases and acetate kinase (in heterofermentative strains) are high and those of phosphate acetyl transferase and alcohol dehydrogenase are low, thus facilitating acetate synthesis and high-energy phosphate conservation (Condon, 1987). Accumulation of products other than lactate from sugars by homofermentative LAB requires an alternative mechanism to lactate dehydrogenase (LDH) to oxidaze NADH, or an alternative source of pyruvate which does not involve reduction of NAD^+. Different strains of LAB under aerobic conditions could oxidize carbohydrates and a number of other substances related to carbohydrate metabolism with considerable rates of oxygen consumption, although the levels of the activity and the nature of the substrates preferentially attacked varied with different strains (Strittmater, 1959a). Hydrogen peroxide accumulation has been observed when LAB grew aerobically in milk, broth cultures, and buffered cell suspensions at the expense of various substrates, such as, tryptone, casein hydrolysate, carbohydrates or substrates related to carbohydrate catabolism (Bruhn and Collins, 1970; Grufferty and Condon, 1983; Gunsalus and Umbreit, 1945; Dobrogosz and Stone, 1962; Brown and VanDemark, 1968; Dahiya and

Speck, 1968). Oxygen can be used advantageously while streptococci utilize glucose, fructose, galactose, lactose, glycerol, α-glycerolphosphate, α-ketoglutarate, ethanol, succinate, butyrate (Cogan et al, 1989; Condon, 1983; Jacobs and VanDermark, 1960); some lactobacilli utilize glucose, maltose, mannitol, galactose, glycerol, pyruvate, lactate, fructose, sorbitol, α-glycerolphosphate, fructose 1, 6-diphosphate, gluconate, and ethanol (Strittmatter, 1959a; Fukui, 1969; Condon, 1983; Kot et al, 1996; Higuchi et al, 1992). Leuconostocs are able to utilize glucose, fructose and gluconate (Condon, 1983), and certain pediococci utilize glucose and glycerol (Dobrogosz and Stone, 1962). However, growth of several strains of LAB are inhibited in aerated cultures because the formation of H_2O_2 (Gilliland and Speck, 1969; Anders et al, 1970; Grufferty and Condon, 1983).

Aeration is not always inhibitory for LAB, and the growth of several LAB strains is markedly stimulated in aerated cultures (Condon, 1987; Cogan, 1989). However, a considerable part of carbohydrate metabolism of most LAB is affected by the presence of oxygen. Oxygen enters through the cell membrane as dioxygen molecules in aqueous solution. Dioxygen molecules are not very reactive but in the reducing atmosphere inside microbial cell they can acquire one or two additional electrons and become much more reactive as superoxide anions and hydrogen peroxide molecules. The simultaneous presence of hydrogen peroxide and superoxide anions can be the origin of hydroxyl radical formation and single oxygen molecules, (Gregory and Fridovich, 1974; Halliwell, 1978; Repine et al, 1981). Aerobic growth of LAB thus leads to the formation of three principal derivatives of oxygen: H_2O_2, O_2^-, $OH\cdot$, which are vectors of "oxygen toxicity" (Condon, 1983). Production and accumulation of such compounds may result in a toxic condition for microbial cells unless they are equipped with adequate protective systems which convert the toxic metabolites into harmless compounds (Condon, 1987).

The LAB have the ability to generate hydrogen peroxide during growth by several different mechanisms (Table A). It is generally believed that synthesis of H_2O_2 is

conducted by flavoprotein oxidases, which catalyze a $2e^-$, reduction to O_2. There are at least four flavoprotein oxidases reported in LAB. The first one, H_2O forming NADH oxidase catalyses the reduction of O_2 to H_2O. The second one, H_2O_2 forming NADH oxidase leads the reduction of O_2 to H_2O_2. To assay NADH oxidase enzymes in LAB, oxidation of NADH is detected and the product H_2O_2, is not usually quantified. The difficulty in distinguishing between H_2O and H_2O_2 forming enzymes is enlarged by the presence of NADH peroxidase, which is a scavenger of hydrogen peroxide. In many extracts, the activity of a H_2O_2 forming NADH oxidase plus that of excess NADH peroxidase is similar to the activity of H_2O forming NADH oxidase. The reduction of hydrogen peroxide to water can be catalyzed by NADH peroxidase at the expenses of NADH regenerating NAD^+. The third enzyme, pyruvate oxidase catalyzes the decarboxylation of pyruvate with the formation of acetyl phosphate and the reduction of O_2 to H_2O_2. The fourth enzyme is α-glycerolphosphate oxidase flavoprotein involved in the aerobic metabolism of glycerol and production of H_2O_2. In addition, lactate oxidase and NAD-independent lactate dehydrogenase are responsible of the enzymatic oxidation of lactate releasing H_2O_2 as a reaction product. In some lactobacilli the coexistence of two, three, four or more flavoproteins has been reported (Dolin, 1955; Higuchi et al, 1993; Premi and Bottazzi, 1972; Murphy and Condon, 1984; Götz et al, 1980a).

Accumulation of hydrogen peroxide in growth media can occur because lactobacilli do not possess the catalase enzyme (Daeschel, 1989). The ability to grow aerobically must be distinguished from the ability to survive exposure to O_2, that is, some LAB will survive exposure to oxygen atmospheres without growing but remaining metabolically active. The homofermentative LAB in general do no possess a catalase activity and only NADH peroxidase and superoxide dismutase play an important role as a defense mechanism against oxygen toxicity. When LAB lack superoxide dismutase system (SOD), manganese can serve to scavenge O_2^- and thus to protect the cell against the direct and indirect toxic

effect of this radical (Archibald and Fridovich, 1981a, 1981b).

H_2O forming NADH oxidase

Reduced nicotinamide adenine dinucleotide oxidase corresponding to H_2O-forming oxidase (NADH:H_2O oxidase) has been demonstrated in many LAB. It is cytoplasmic, defensive against O_2 toxicity, and does not form H_2O_2 as an intermediate during the oxidation activity (Condon, 1987). The enzyme can be induced by oxygen, H_2O_2 and in media containing carbon sources, such as, D-gluconate, D-glucose, D-mannitol, (Fukui, 1961; Murphy and Condon; 1984; Condon, 1987; Tseng and Montville, 1990), or tryptone broth (Bruhn and Collins, 1970). The activity of NADH:H_2O oxidase can be stimulated by FAD and/or thiol in some LAB (Brown and VanDemark, 1968), except for the NADH oxidase found in *L. mesenteroides* (Koike et al, 1985). Respiration in lactobacilli is a function of both carbon source and culture age (Iwamoto et al, 1979a, 1979b). The NADH:H_2O oxidase enzyme was completely purified to a crystalline form and a model was proposed to explain H_2O formation in *L. plantarum* (Fukui, 1961; Murphy and Condon, 1984; Tseng and Montville, 1990; Iwamoto et al, 1979a). It also has been purified from other LAB, for example, *L. casei* (Walker and Kilgour, 1965a, 1965b; Brown and VanDemark, 1968), *S. faecalis* (Dolin, 1955; Hoskins et al, 1962; Schmidt et al, 1986; Ross and Claiborne, 1992), *S. mutans* (Higuchi et al, 1993, 1994), *S. agalacatiae* (Mickelson, 1967), *S. diacetilactis* (Speckman and Collins, 1968; Bruhn and Collins, 1970), *L. citrovorum* (Speckman and Collins, 1968), and *L. mesenteroides* (Koike et al, 1985). Production of acetoin and diacetyl by *S. diacetilactis* in casein hydrolysate medium was attributed to the action NADH:H_2O oxidase enabling the organism to divert some pyruvate from the formation of lactic acid (Speckman and Collins, 1968). This diverting mechanism has been reported to function in different strain of streptococci. Higuchi et al

(1993) identified a H_2O forming NADH oxidase in *S. mutans* as a monomer and activity independent of exogenously added flavin, with an isoelectric point of 4.8 and optimal activity between pH 7.0 and 7.5. The molecular mass of this enzyme (50 kD) is slightly higher than the molecular mass (14 kD) reported for the enzyme of *L. plantarum* by Fukui (1961). The oxidizing activity of this enzyme has been detected in the extracts of heterolactic fermenters (Bruhn and Collins, 1970; Kawai et al, 1971). *Streptococcus agalactiae* posses an NADH:H_2O oxidase that is cyanide sensitive, suggesting a metallo-component involved in electron transport to oxygen (Mickelson, 1967). Koike et al (1985) reported an NADH:H_2O oxidase which utilizes ferricyanide, cytochrome c or H_2O_2 as oxidants. Cysteine was necessarily for stabilization of the enzyme at all stages of purification, but it was not required for maximum activity.

H_2O_2 forming NADH oxidase

Reduced nicotinamide adenine dinucleotide oxidase forming H_2O_2 (NADH:H_2O_2 oxidase) plays an important role in the regulation of aerobic metabolism, through the regeneration of NAD^+ from NADH derived from glucose oxidation or substances related to carbohydrate metabolism. It is rather unstable and can cause cellular damage (Higuchi et al, 1993). The enzyme activity of NADH oxidase forming H_2O_2 has been described for many LAB and apparently represents a highly active flavoprotein or metallo-flavoprotein specialized for hydrogen transport from reduced pyridine nucleotide to oxygen. It is apparently a cytosolic enzyme and its activity is influenced by the presence of FAD (Collins and Aramaki, 1980; Anders et al, 1970). A relationship between the quantity of hydrogen peroxide produced and total flavin content among LAB species has been shown. This relationship is direct, which means that when higher flavin content was found also higher amount of hydrogen peroxide was produced (Seeley and del Rio Estrada, 1951). Oxygen may induce the enzyme activity in some cases (Cogan et al, 1989; Higuchi et al, 1993) but

presence of glucose in cell free extracts partially overrides the induction by O_2. This enzyme was first reported in *S. faecalis* by Seeley and del Rio Estrada (1951). Dolin (1955) partially purified it from anaerobically grown cells of *S. faecalis* and found that the reaction goes rapidly to completion with the uptake of 1/2 mole O_2/mole NADH.

The activity of NADH:H_2O_2 oxidase enzyme has been studied, partially purified and characterized from several LAB species, such as *L. delbrueckii* ssp *bulgaricus* (Kot et al, 1996), *L. acidophilus* (Collins and Aramaki, 1980; Klebanoff and Belding, 1974), *L. lactis* (Wheather et al, 1952; Premi and Bottazzi, 1972), *L. jugurti, L. helveticus* (Premi and Bottazzi, 1972), *L. casei* (Strittmatter, 1959b), *L. plantarum* (Götz et al, 1980b), *L. hilgardii* (Rodriguez and Manca de Nadra, 1995), *L. sanfrancisco* (Stolz et al, 1995a, 1995b), *S. cremoris*, *S. lactis* (Anders et al, 1970; Grufferty and Condon, 1983; Cogan et al, 1989), *S. mutants* (Higuchi et al, 1994), *S. mitis* (Klebanoff and Belding, 1974), *L. brevis*, *L. buchneri*, *L. cellobiosus*, *S. faecium*, *L. mesenteroides*, *P. pentosaceus*, *P. acidilactici*, and *P. urinari-equi* (Whitenbury, 1964).

Conditions under which hydrogen peroxide is produced by LAB may vary considerably among species (Kot et al, 1995a). Some lactobacilli formed detectable H_2O_2 and some did not, regardless of their preference or requirement for aerobic or anaerobic conditions. (Kot et al, 1995a, 1996; Collins and Aramaki, 1980; Cogan et al, 1989). Other LAB require both oxygen and carbohydrates or substances related to carbohydrate metabolism in order to produce H_2O_2. *L. delbruekii* ssp *bulgaricus* and *S. lactis* required both aerobic conditions and presence of glucose to accumulate H_2O_2 (Kot et al, 1995a; Anders et al, 1970). Moreover, in *L. delbruekii* ssp *bulgaricus* H_2O_2 production depend on the operation of the glycolysis pathway (Kot et al, 1996). In contrast, *L. acidophilus* produced H_2O_2 only in small amounts and without requirement of a carbon source (Kot et al, 1995a). Resting cells of *L. lactis* produced H_2O_2 from glucose whether they were grown in the shaker or under an anaerobic seal (Wheater et al, 1952). In this case,

NADH:H_2O_2 oxidase activity was associated with living but not necessarily multiplying cells and H_2O_2 was produced even when only traces of oxygen were present (Wheater et al, 1952). *Streptococcus lactis* cells utilized glucose, galactose or components of yeast extracts as direct reductants of O_2, but cell-free extracts were completely inactivated in the assays. It is, therefore, probable that glucose, galactose and other media ingredients promote oxygen utilization in whole cells by generating NADH, the substrate for NADH oxidase, but NADH generating systems are lost in the formation of cell free extracts (Grufferty and Condon, 1983). The only oxygen uptake registered with cell free extracts was at the expenses of NADH and formed H_2O_2 as product. A similar activity has been observed in other LAB (Anders et al, 1970; Strittmatter, 1959a; Dolin, 1953; Fukui, 1961; Walker and Kilgour, 1965b). Some lactobacilli require high number of cells to produce H_2O_2 at 4°C (Collins and Aramaki, 1980). Several strains of *L. acidophilus* produce enough H_2O_2 in milk to be autoinhibitory and some did not (Collin and Aramaki, 1980). The quantity of hydrogen peroxide accumulated in cell suspensions in milk was always less than that found in buffered cell suspensions suggesting a possible reactions between H_2O_2 and milk components.

Accumulation of hydrogen peroxide by *L. lactis* has been studied and results did not show hydrogen peroxide splitting activity. Moreover, it was difficult to understand, from this point of view, why *L. lactis* was 25 times more resistant to H_2O_2 (being inhibited by 100-200 µg/ml of H_2O_2) than *S. aureus* (Wheater et al, 1952). *Lactobacillus casei* posses an NADH oxidase that is partially inhibited by cyanide and azide. These agents can inhibit a number of non-hemin oxidative enzymes including several flavin oxidase systems which requires FAD, as well as non-oxidative metal-activated enzymes that may involve a metal component (Strittmatter, 1959b). Higuchi et al (1993) found an induced NADH:H_2O_2 oxidase from aerobically grown *S. mutans*. It has a unique tetramer structure with four identical monomers which made it different from other NADH:H_2O_2

enzymes. Its molecular mass is about 56 kD and required FAD for full activity. In contrast, NADH:H_2O_2 oxidase from *L. mesenteroides* does not require FAD for full activity (Higuchi et al, 1993). Cultures of *S. faecalis* grown aerobically and transferred to a fresh medium under aerobic conditions do not form free H_2O_2 in the medium regardless of the riboflavin content. Cultures grown anaerobically and transferred to a fresh medium under aerobic conditions form considerable amounts of free H_2O_2 (Seeley and del Rio Estrada, 1951).

Pyruvate oxidase

Lactobacillus delbrucki can generate H_2O_2 by a pyruvate oxidase system. It was first demonstrated in *L. delbruckii* (Lipmann, 1940; Hager et al, 1954; Lloyd et al, 1978; Condon, 1987), other LAB such as *L. casei* (Strittmatter, 1959b), *L. plantarum* (Götz et al, 1980b; Sedewitz et al, 1984a, 1984b; Murphy and Condon, 1984), *S. faecalis* (Dolin and Gunsalus, 1951), *S. mutan* (Carlsson and Kujuala, 1985; Lucey and Condon, 1986), *S. salivarius*, *S. mitis*, *S. sanguis* (Carlsson and Kujuala, 1985), and *Leuconostoc* ssp. (Lucey and Condon, 1986), also posess this enzyme. The enzyme is cytoplasmic and was first purified by Hager et al (1954) who showed that it required phosphate as a cosubstrate and FAD, TPP and Mg^{2+} as cofactors (Götz et al, 1980b; Murphy and Condon, 1984). Pyruvate oxidase in cell-free extract of *L. plantarum* (Götz et al, 1980b) did not produce O_2^- as an intermediate and was entirely dependent upon inorganic phosphate (Götz et al, 1980b; Sedewitz et al, 1984a, 1984b; Murphy and Condon, 1984). Under aerobic conditions the specific activity of pyruvate oxidase was five to sixfold greater with lactose as the sugar source than with glucose (Sedewitz et al, 1984b). The pyruvate oxidase of *L. plantarum* was isolated from the soluble fraction. In crude cell free extracts and during the purification steps, the enzyme showed no dependence upon cofactors, such as, CoA,

FMN, FAD or TTP. In contrast, pyruvate oxidase from *L. delbrueckii* required both cofactors TPP and FAD. In addition to O_2 several other oxidants (methylene blue, ferricyanide, neo-tetrazolium) could serve as electron acceptors for the pyruvate oxidase in *L. delbrueckii.* It has a DPN-linked system that requires P, FAD, TPP, enzyme, pyruvate and ferricyanide. During purification of pyruvate oxidase, the ability to catalyze pyruvate dismutation was lost. This activity could be restored by addition of fractions having high lactic acid oxidation activity. Hager et al (1954) proposed a model where the activity of pyruvate oxidase was linked to the activity of a lactate oxidase. Once pyruvate oxidase was reduced it could be oxidized by oxygen or other electron acceptors, such as, methylene blue, ferricyanide, neotetrazolium and riboflavin. Riboflavin in the dismutation reaction acted as an electron carrier between the two flavin enzymes. Whereas, on the lactic acid side, leuco-riboflavin reduced the lactic-flavoprotein, this eventually reduced pyruvate to lactate and reentered the dismutation cycle. In aerated cultures of *L. plantarum* oxidation of pyruvate *via* pyruvate oxidase and acetate kinase could be an alternative pathway for acetate production and NADH oxidase will provide for NAD^+ regeneration (Bobillo and Marshall, 1992).

Streptococcus faecalis oxidized glucose to acetate and CO_2, under aerobic conditions, without accumulation of hydrogen peroxide (O'Kane, 1950). Dolin and Gunsalus (1951) reported that under aerobic conditions cell suspensions of *S. faecalis* convert virtually all the pyruvate to acetate and CO_2. However, with a cell free extract, the products were predominantly acetoin with about 30% of the pyruvate undergoing oxidation. This results showed that in cell free extracts pyruvate dismutation was lost. They reported that extracts which no longer take up oxygen catalyzed the dismutation of pyruvate under conditions which furnished a suitable acceptor for the acetyl generated in the process. Under aerobic conditions hydrogen peroxide was excreted by *S. sanguis* and *S. mintis* but not by *S. mutants* or by *S. salivar. Streptococcus sanguis* and *S. mitis* have pyruvate oxidase activity dependent on TPP, FAD, orthophosphate, and produce H_2O_2 and acetyl-

phosphate. Whereas, the pyruvate oxidizing activity of *S. mutants* and *S. salivarius* was dependent on TPP, CoA, and NAD. The latter two species may have a pyruvate dehydrogenase, which reduced NAD to NADH (Carlsson and Kujuala, 1985).

α-Glycerolphosphate oxidase

The reaction catalyzed by α-glycerolphosphate oxidase flavoprotein occurs in those LAB which are involved with aerobic metabolism of glycerol as a dependent growth substrate. A variety of enzymes oxidizing α-glycerolphosphate have been described in the literature. They are of three general types: i) linked to nicotinamide adenine dinucleotide (NAD^+) reduction; ii) linked to cytocromes; or iii) connected directly to oxygen without the intervention of NAD^+ or cytochromes. This latter type, α-glycerolphosphate oxidase, has been found in streptococci and has been prepared from aerobically grown cells of *S. faecalis* (Gunsalus and Umbreit, 1945; Jacobs and Vandermark, 1960), *S. faecium* (Koditschek and Umbreit, 1969), *L. casei* (Strittmater, 1959b) and *P. pentosaceus* (Dobrogosz and Stone, 1962). Gunsalus and Umbreit (1945) studied the mechanism of glycerol oxidation by *S. faecalis*, and found that glycerol reacts with ATP and is converted into the natural L α-glycerol phosphate before oxidation. It was suggested that this α-glycerolphosphate oxidizing system was linked directly to oxygen via a flavoprotein mediated system with H_2O_2 as one of the products (Jacobs and VanDermark 1960). Dobrogosz and Stone (1962) suggested a tentative pathway of glycerol metabolism in *P. pentosaceus* which took into account the same reactions to form L-α-glycerol phosphate and H_2O_2 by *S. faecalis*. This pathway included the conversion of L-α-glycerol phosphate to pyruvate and the production of lactic acid, acetic acid, acetoin and CO_2 in a molar ration 1:1:1:3. The α-glycerolphosphate oxidase of *S. faecium* was similar to the enzyme of *S. faecalis* in that it was a nonparticulate flavoprotein, with FAD as the probable prosthetic group, and was involved in direct electron transfer from L-α-glycerolphosphate to oxygen.

Hydrogen peroxide formed in stoichiometric concentration did not inhibit the activity of cell-free α-glycerolphosphate oxidase, although its accumulation in whole cells was related to inhibition of further glycerol oxidation.

L-Lactate oxidase

Although lactate is the end product of lactic acid fermentation it can be further metabolized to acetate and CO_2 under aerobic conditions by stereospecific NAD-independent, flavin containing lactate dehydrogenase or lactate oxidase. Lactate oxidase activity was reported in *L. delbrueckii* when Hager et al (1954) studied pyruvate oxidation in this strain. Lactate oxidation also has been observed in *L. casei, L. arabinosus* and *L. plantarum*; methylene blue was used to indicate that a lactate dehydrogenase (LDH) was involved in this process (Strittmatter, 1959a). Götz et al (1980a) reported a lactate oxidase enzyme in *L. plantarum* however during the reaction of this enzyme no hydrogen peroxide was demonstrable. London (1968) found lactate oxidase activity in *S. faecium*, which use O_2 as electron acceptor. Twenty one strains of lactobacilli were found to oxidize lactate by Kandler (1983). They were divided into two groups. The first one, had a flavin containing L-lactate oxidase which used O_2 as electron acceptor to produce H_2O_2 and pyruvate. The *in vivo* dissimilation of pyruvate in this group remains to be further elucidated. Organism included in this group were *L. curvatus, L. sake, L. acidophilus, L. bulgaricus* and *L. lactis*. This type of lactate oxidase was also described in *Tetrahymena pyriformis* (Eichel and Rem, 1962), *Aerococcus viridans* (Duncan et al, 1989), *L. plantarum* (Götz et al, 1980b; Sedewitz et al, 1984a, 1984b), *S. faecalis* (Esder et al, 1979) and some species of *Pediococcus* (Mizutani et al, 1983). The second enzyme (NAD independent D-LDH) exhibited only a very low rate of lactate oxidation to acetate and CO_2, the oxidation was increased tenfold by the addition of methylene blue yielding H_2O_2 and pyruvate, the accumulation of which lead further degradation of pyruvate (Kandler, 1983). The addition

of dichlorophenolindophenol (DCPIP) resulted in accumulation of pyruvate and $DCPIP_{red}$. The *in vivo* electron carrier reacting with artificial redox substances and the mechanism of pyruvate degradation following lactate oxidation remain to be elucidated. Strains included in the second group were *L. plantarum*, *L. casei*, *L. coryniformis*, and *S. faecium.* A lactate oxidase that catalyzes the overall oxidation and decarboxylation of lactate to acetate and CO_2 with the reduction of O_2 to H_2O is produced by *Micobacterium smegmatis* (Lockridge et al, 1972).

Diaphorase

The term diaphorase is used to designate a flavoprotein enzyme capable of catalyzing the oxidation of a reduced pyridine nucleotide by artificial or model electron acceptors, such as, organic dyes (methylene blue and iodophenol) or by inorganic compounds such as ferricyanide, but not capable of reacting directly with oxygen (Walker and Kilgour, 1965a). This situation may mean either that the physiological acceptor is not yet recognized or that the diaphorase is an altered form of a physiologically functional electron transport (Dolin and Wood, 1960). A soluble, reduced diphosphopyridine nucleotide-specific diaphorase has been highly purified from extracts of *S. faecalis* (Dolin and Wood, 1960). Representatives of almost all the known classes of flavoprotein oxidants have been tested as electron acceptors; however, the only oxidants found were ferricyanide, 2,6-dichlorophenolindophenol (DCPIP) and various quinones. Evidence was present that the diaphorase forms a complex with reduced diphosphopyridine nucleotide.

Oxygen scavenger systems

Although aeration as a way to steer metabolic fluxes could have advantages because of its simple application and efficiency, there are many undesirable side effects in LAB. As

a consequence of chemical and enzymatic reactions involving oxygen, toxic derivatives may be generated such as, superoxide and hydrogen peroxide (Condon, 1987). These may in fact be so deleterious to lactic acid bacteria that some species can not grow at all in the presence of oxygen (Archibald and Fridovich, 1981a). Hydrogen peroxide can liberate DNA bases, cause DNA strand breakage and alter the chemical composition of the bases (Haugaard, 1968; Ananthaswamy and Eisenstark, 1977). However, various detoxification systems have been found to operate in LAB and several of these have been now genetically characterized (De Vos, 1996). Peroxidases and catalases usually detoxify hydrogen peroxide. Free oxygen radicals can be detoxified in LAB by high internal levels of Mn^{2+} which are present in various species or by the activity of superoxide dismutase which may have different metal cofactors (Archibald and Fridovich, 1981a; Parker and Blake, 1988).

NADH peroxidase

Many LAB have the ability to beneficalli utilize oxygen and to dispose of the H_2O_2 which may be formed (Whittenbury, 1964). Peroxidases and catalases usually detoxify hydrogen peroxide with the exception of several isolated cases (Whittenbury, 1964; Kono and Fridovich, 1983). Lactic acid bacteria lack catalase because they cannot synthesize cytochromes. Thus, LAB can rid themselves of H_2O_2 formed, only by their NADH-peroxidase system. It is known that *S. faecalis* contains at least five different peroxidases and diaphorase like NADH which have been partially purified (Dolin, 1955, 1957; Dolin and Wood, 1960). The existence of an adaptive peroxidase system in cells of *S. faecalis* has been reported by Seely and Vandemark (1951). Aerated cultures of *S. faecalis* developed a mechanism for utilization of H_2O_2 through riboflavin. A relationship between the riboflavin content of growing cells of *S. faecalis* and their ability to decompose peroxide in the presence of oxidable substrates was found (Seely and Vandemark, 1951). The enzyme was associated with the membrane fraction and was thought to form part of a

rudimentary electron transport chain, which reduced O_2 to water and promoted a weak proton extrusion. It was presumed that O_2 was an intermediate in formation H_2O_2. *Streptococcus cremoris* and *S. lactis* also possess and NADH peroxidase activity which displayed different levels of activity. However, neither of these enzymes was sufficiently active to remove all the H_2O_2 formed by NADH:H_2O_2 oxidase reaction (Anders et al, 1970). Some LAB growing aerobically +/or in media containing hematin, beef infusion broth and yeast extract broth produced NADH peroxidase (Seeley and Vandemark, 1951). In *L. plantarum* cultures NADH peroxidase was induced by O_2 and catabolite depressed by glucose.

Superoxide Dismutase and Manganesse

Most LAB can deal with oxygen radicals by either superoxide dismutase (SOD) or high internal Mn^{2+} concentrations. Superoxide dismutase can remove oxygen radicals by catalyzing the reaction showed in Table A. In prokaryotes three types of SOD can be distinguished depending in the metal cofactor contained (Cu-Zn, Fe, or Mn). A single organism can have two SOD genes; the corresponding enzymes differ in their metal cofactor and in their expression pattern in response to oxygen (Sanders et al, 1995). In an analysis of the stress response of *Lactococcus lactis* ssp. *lactis*, formerly *Streptococcus lactis*, three proteins that were induced under low pH cultures conditions were detected. One of these was identified as the lactococcal superoxide dismutase (SodA) by N-terminal aminoacid sequence analysis (Sanders et al, 1995). It has been suggested that dismutation of O_2^- may also be a physiologically important source of H_2O_2. Hyperbaric O_2 or agents which increase O_2 uptake such as plumbagin, will promote extensive O_2^- production which can be converted to H_2O_2 by SOD or high internal manganese concentrations (Condon 1987). A widespread distribution of SOD or non-enzymatic manganese dismutation system

among lactobacilli has been reported (Archibald and Fridovich, 1981a). Low activity of SOD has been reported in *L. plantarum* (Götz et al, 1980a, 1980b). Superoxide dismutase was found to be induced by oxygen in *S. faecalis* and in *E. coli*, and cells which contained the high induced level of superoxide dismutase were more resistant towards hyperbaric oxygen (Gregory and Fridovich, 1973). Superoxide dismutase may have other physiological functions besides dismutation of O_2^-, but is also possible that even low levels of O_2^- are so reactive that an active dismutation system is an important safeguard in aerobic environments. The interesting question of whether aerobically grown cells of *L. plantarum* contains superoxide dismutase to protect the organism from the deteriorating effect of O_2^- was recently studied. It was shown by Götz et al (1980a), and confirmed by Archibald and Fridovich (1981b), that *L. plantarum* does not contain superoxide dismutase. Instead Mn^{2+} is responsible for the catalytic scavenging of O_2^-. The reaction of Mn^{2+} with O_2^- in the presence of pyrophosphate or phosphate is well known from experiments with chloroplast and model systems. Archibald and Fridovich (1982) showed the reaction catalyzed by Mn^{2+} (Table A). *Lactobacillus plantarum* requires Mn^{2+} for growth and accumulates up to 25 mM intracellularly (Archibald and Fridovich, 1981b). The replacement of superoxide dismutase by Mn^{2+} as an O_2^- scavanger is a unique feature of LAB and the very high manganese requirement and content of lactobacilli may reflect this function rather the manganese requirement of several enzymes which are saturated at much lower concentrations (Kandler, 1983).

Pseudocatalases and heme-catalases

Both non-heme catalases termed pseudocatalases and heme-catalases have been reported in a limited number of LAB, including lactobacilli and pediococci that occur on meat (Hammes et al, 1990). Pseudocatalase was first observed in pediococci and soon after in lactobacilli, leuconostoc and streptococci (Kono and Fridovich, 1983). Kono and

Fridovich (1983) isolated and characterized the pseudocatalase of *L. plantarum*, it was composed of six subunits of equal size associated by noncovalent forces. Manganese was the only metal contained, and the strain do not accumulate H_2O_2. *Lactobacillus plantarum* strains ATCC 14917 and ATCC 8014, do not possess the pseudocatalase activity (Kono and Fridovich, 1983).

Mode of action

Although the toxicity of hydrogen peroxide has been demonstrated, its mode of action has not been as clearly elucidated. Two types may be distinguished, one leading to a bacteriostatic effect, the other being bactericidal.

Bacteriostatic effect

The oxidation of sulfhydryl compounds may have a bacteriostatic or bactericidal effect, depending on whether or not the reaction is reversible when the cell returns to anaerobic conditions (Piard and Desmazeaud, 1991). The denaturation effect of oxygen and its metabolites on G3P-dehydrogenase has been showed and its probable that oxidation of sulfhydryl to disulfide affects a number of enzymes (lactate dehydrogenase, alcohol dehydrogenase) or coenzymes with sulfhydryl groups CoA (Haugaard, 1968; Piard and Desmazeaud, 1991). The hypothesis that oxygen toxicity may result from the peroxidation of membrane lipids, has been reviewed by Harley et al (1978). The hypothesis would explain the increased membrane permeability caused by H_2O_2, O_2^-, $OH\cdot$, and has been supported by the finding that the degree of membrane lipid peroxidation was related to a loss of viability of *E. coli* (Harley et al, 1978). Once cell envelopes are damaged by the above mentioned metabolites, the presence of molecular oxygen may reinforce the damage,

perhaps by a direct effect at the level of the affected membrane sites (Piard and Desmazeaud, 1991).

Bactericidal effect

Free radicals and hydrogen peroxide can damage bacterial nucleic acids leading to reversible or irreversible alteration. Anathaswamy and Eisenstark (1977) demonstrated the modification of DNA by hydrogen peroxide showing that *E. coli* mutants lacking DNA repair systems were more sensitive to H_2O_2 than strains with intact repair systems. Hydrogen peroxide apparently causes breaks in the carbon phosphate backbone of DNA, releasing nucleotides and preventing chromosome replication (Freese et al, 1967). Hydroxyl radicals can attack the methyl group of thymine, thereby damaging DNA. Active molecular oxygen can also react with guanidine and cause breaks in one strand of DNA (Piard and Desmazeaud, 1991). Gram-negative bacteria are better protected from the toxic effect of oxygen metabolites because of the presence of an outer lipopolysaccharide layer. This envelope traps active molecular oxygen. Lactic acid bacteria have an additional handicap in the resistance to molecular oxygen because they lack membrane carotenoids. Active molecular oxygen thus diffuses through the peptidoglycan layer rapidly and reacts with sensitive membrane sites (Piard and Desmazeaud, 1991).

Effect on lactic flora

Concerning to lactococci, there is an apparent consensus that hydrogen peroxide is the major inhibitor (Condon, 1987). Autoinhibition by H_2O_2, however, is not universal among lactococci. In oxygen insensitive strains, there is adequate balance between the

activities of NADH:H_2O_2 oxidase and NADH peroxidase preventing accumulation of hydrogen peroxide. In some strains, reduction of oxygen to water by an NADH:H_2O oxidase enables some bacteria to remain unaffected by aerobiosis (Piard and Desmazeaud, 1991). Although inhibition is occasionally observed in lactobacilli in aerobic conditions, the modes of action remains to be established. It is generally accepted that hydrogen peroxide is not responsible for this inhibition (Gregory and Fridovich, 1974). Some authors have advanced the notion that the absence of SOD in lactobacilli could enable the formation of hydroxyl radicals via a reaction between superoxide anion and hydrogen peroxide. However, it has been reported that LAB lacking SOD had a dismutation activity resulting from their high Mn^{2+} levels (Archibald and Fridovich, 1981a, 1981b, 1982). These systems protect them from the superoxide anion and prevents the formation of hydroxyl radicals. Recent data suggest that LAB can adapt to oxygen. Lactococci sensitive to hydrogen peroxide and exposed to sublethal concentration of the compound became able to growth in presence of lethal concentrations of hydrogen peroxide (Condon, 1987).

Effect on non lactic flora

It is of interest that the catalase positive character of unwanted gram-negative bacteria does not render them particularity tolerant to oxygen metabolites. Price and Lee (1970) concluded that hydrogen peroxide was responsible for the inhibition of *Pseudomonas*, *Bacillus* and *Proteus* by *L. plantarum.* Dahiya and Speck (1968) reported that H_2O_2 produced by lactobacillus inhibited *Staphylococcus aureus* and the optimal H_2O_2 production was obtained at 5°C. The question of whether the inhibition of LAB, which coincides with H_2O_2 accumulation, is caused directly by H_2O_2 or indirectly by a metabolite of H_2O_2 remains (Condon, 1987). Inhibition of LAB by aeration at normal atmospheric pressures is invariably prevented by catalase. However, it has been suggested

that H_2O_2 can react with O_2^- to form hydroxyl radical $OH^\cdot$, and that the later is the direct inhibitor of O_2 sensitive cells (Gregory and Fridovich, 1974). Although the feasibility is not universally accepted, formation of $OH^\cdot$ from H_2O_2 and O_2 under physiological conditions has been demonstrated. If $OH^\cdot$ is the direct toxic metabolite, it would have to be effective at concentration much lower than those of O_2, which accumulates in aeration-inhibited cultures of LAB (Condon, 1987). Direct inhibition by H_2O_2 rather than $OH^\cdot$ remains the most likely explanation for O_2 sensitivity (Condon, 1987).

The inhibitory effects of H_2O_2 of LAB may be potentiated in natural environments, such as milk or saliva; concentrations which are not of themselves inhibitory, may become so, because of the presence of lactoperoxidase and thiocyanate in a reaction catalyzed by lactoperoxidase as follows:

$$SCN^- + H_2O_2 \text{ ----------> } OSCN^- + H_2O$$

Further oxidation products formed in the presence of excess hydrogen peroxide (O_2SCN^- and O_3SCN^-) may also be involved in the inhibition caused by the lactoperoxidase thiocyanate-H_2O_2 system but most attention has been focused on $OSCN^-$ (Condon, 1987). The mode of action of hypothiocyanite ($OSCN^-$) is complex and not fully understood. The overall effect on LAB is generally bacteriostatic whereas many Gram-negative bacteria are rapidly killed in its presence. A number of different physiological effects have been noted, principally leakage of K^+ and amino acids, inhibition of uptake of carbohydrates, lactate, amino acids and the inhibition of specific glycolysis enzymes such as aldolases, hexokinase and glyceraldehyde-3-phosphate dehydrogenase (Condon, 1987). Streptococci resistant to hypothiocyanite have an NADH:$OSCN^-$ oxidoreductase, which reduces $OSCN^-$ to the inert SCN^-. In biological media H_2O_2 readily forms complexes with many compounds, including carbonyls (glyoxal, glycolaldehyde), amino acids and thymine. The inhibitory effect of H_2O_2 may be partially or wholly due to the peroxide

complexes rather than free H_2O_2 (Watson and Schubert, 1969).

TABLE A

ENZYMES INVOLVED IN REACTIONS BETWEEN LACTIC ACID BACTERIA AND OXYGEN OR OXYGEN METABOLITES[1].

Reactions and catalyzing enzymes:
NADH:H_2O_2 oxidase
$NADH + H^+ + O_2$----------> $NAD^+ + H_2O_2$
NADH:H_2O oxidase
$2NADH + 2H^+ + O_2$----------> $NAD^+ + 2\ H_2O$
Pyruvate oxidase
Pyruvate + Pi^-+O_2---------- -->acetyl-P+ CO_2 + H_2O_2
TPP, FAD
α-Glycerolphosphate oxidase
α-Glycerolphosphate+O_2---------->dihydroxyacetone + Pi + H_2O_2
L-Lactate oxidase
Lactate + O_2----------> pyruvate + H_2O_2
NAD-independent D-LDH
Lactate + O_2----------> pyruvate + H_2O_2
Oxygen scavengers systems:
NADH peroxidase
$NADH + H^+ + H_2O_2$----------> $2H_2O + NAD^+$
Mn^{2+}
$O_2^- + 2H^+$----------> $H_2O_2 + O_2$
SOD
$2O_2^- + 2H^+$----------> $H_2O_2 + O_2$

[1]Modified from Piard and Desmazeaud (1991). TPP, Thyamine Pyrophosphate; FAD, Flavin Adenine Dinucleotide; GP, Glycerophosphate; SOD, Superoxide Dismutase.

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CHAPTER III

FACTORS INFLUENCING HYDROGEN PEROXIDE PRODUCTION IN *LACTOBACILLUS DELBRUCKII* SSP. *LACTIS* I

ABSTRACT

Resting cells of *Lactobacillus delbruckii* ssp. *lactis* I produced hydrogen peroxide at 5°C in sodium phosphate buffer (0.2 M, pH 6.5) with or without glucose. 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 was associated with the production of a small portion of lactic acid which disappeared upon further incubation. Further experiments revealed that hydrogen peroxide production was obtained in buffer containing added sodium lactate without glucose. These and other results suggested the presence of a lactate oxidase enzyme which used D-lactate to produce hydrogen peroxide.

INTRODUCTION

Among lactic acid bacteria (LAB) the ability to produce hydrogen peroxide is widespread (Anders et al, 1970). Generation of hydrogen peroxide may occur by several mechanisms (Collins and Aramaki, 1980). In some cultures exposed to oxygen, hydrogen peroxide can accumulate to autoinhibitory levels or levels inhibitory to other bacteria (Anders et al, 1970; Gilliland and Speck, 1974, 1975). Some authors have suggested

hydrogen peroxide production by the lactic acid bacteria involves oxidative actions on sugars or related compounds (Dobrogosz and Stone, 1962; Condon, 1987; Grufferty and Condon, 1983; Kot et al, 1996). Others have included an NADH oxidase as part of this process (Anders et al, 1970; Collins and Aramaki, 1980; Higuchi et al, 1993).

Production of hydrogen peroxide is deemed to be beneficial for food preservation and prevention of growth of food borne pathogens (Dahiya and Speck, 1968; Gilliland and Speck, 1969, 1974, 1975, 1977). It has been reported that hydrogen peroxide producing LAB can inhibit the 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 delbruckii* ssp. *lactis* produce the highest amount of hydrogen peroxide at refrigeration temperatures without growing or changing the pH of the medium (Gilliland, 1980). Resting cells of *Lactobacillus delbruckii* spp *lactis* I among other strains of *L. delbruckii* spp *lactis* have been proven to produce sufficient amounts of hydrogen peroxide to be inhibitory toward *Escherichia coli* O157:H7 on refrigerated raw chicken meat (Brashears et al, in press). Few reports have been concerned with the circumstances, which affect hydrogen peroxide production, and the enzyme system(s) of *L. delbruckii* spp. *lactis* that is involved. The present study provides results about hydrogen peroxide production by *L. delbruckii* spp. *lactis* I and some preliminary findings about the enzyme system(s) involved.

MATERIAL AND METHODS

Sources and maintenance of cultures

The strain of *L. delbruckii* spp. *lactis* was obtained from the stock culture collection of the Food Microbiology Laboratory in the Department of Animal Science at Oklahoma State University. Cultures were maintained by weekly transfers in MRS broth

(Difco Laboratories, Detroit, MI) using 1% inocula and 18 hours incubation at 37°C. They were stored at 5°C between subcultures. Before experimental use, cultures were subcultured twice in sterile MRS broth.

Enumeration of bacteria

Microorganisms were enumerated by a 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 the 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 hours at 37°C. Colonies were counted with the aid of the Quebec Colony Counter.

Production of bacterial cells

Washed cells. Lactobacilli were grown in MRS broth for 16 hr at 37°C, harvested by centrifugation (16,000 x g for 200 min at 5°C), washed twice in cold physiological saline solution and resuspended in sodium phosphate buffer (0.2 M, pH 6.5) with or without 55.5 mM glucose as required.

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

Cell-free extract

Cell-free extracts of lactobacilli were prepared from starved cells resuspended in sodium phosphate buffer (0.2 M 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 x 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. delbruckii* spp. *lactis* I (or cell free extracts) were resuspended in cold phosphate buffer (0.2 M, pH 6.5) with and without 55.5 mM glucose as desired and incubated for the desired time at 5°C. Following incubation, the cells were removed by centrifugation and the supernatant was assayed for hydrogen peroxide.

Samples were assayed for hydrogen peroxide by placing 5 ml of the cell free supernatant into test tubes containing 1 ml of a 0.1 % aqueous solution of peroxidase (Sigma Chemical) and 0.1 ml of a 1 % aqueous solution of o-dianisidine (Sigma Chemical Co.). A blank was prepared containing 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 a 4 N HCl to each test tube. Absorbance reading (A_{400} nm) of each sample was determined and peroxide content was determined by comparing the A_{400} nm to a standard curve (Gilliland, 1969).

Protein assay

The protein content of the cell 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 Pressure Liquid Chromatography (HPLC) analysis by centrifuging 1.0 ml portions in 1.5 ml microcentrifuge vials for 10 min at 12,500 x 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 0.01 M sulfuric acid. The acidified samples were filtered through 0.2 μm membrane filters directly into 2 ml HPLC autosample vials and capped. These 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, Georgia). The sample was injected into 0.005 M H_2SO_4 mobile phase heated to 65°C and separated using a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, California). The peaks were detected with a diode array detector at 210 nm. Peak areas obtained using known concentrations of lactic acid were used to prepare a standard curve. Lactic acid concentration in the samples was determined using the standard curve. Peak purity was monitored by UV scanning techniques as an aid for identifying abnormal wavelength patterns present in a single peak.

NADH oxidase assay

Oxidation of NADH was followed spectrophotometrically at A_{340} nm with a Beckman DU 75000 spectrophotometer with cuvettes having a 1 cm length 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 define as the µmoles of NADH oxidized per minute based on the disappearance of NADH from the reaction mixture. Specific activity was the number of units per milligram of protein. Normally, the change of absorbancy between 0.2 and 2.0 min was used in calculating the reaction velocity. The velocity was linear up to at 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 (figures 1 through 9) 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® (1985).

RESULTS

Growth and hydrogen peroxide production

The growth of *L. delbruckii* spp. *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 one reached a higher population than did the agitated one (FIG. 1). Cells harvested from agitated and static cultures at 16 hr of incubation were assayed for

hydrogen peroxide production. After 1 hr of 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 significant difference ($P>0.05$) in the total amount of hydrogen peroxide produced, there was a significant difference ($P< 0.05$) in the amount of hydrogen peroxide produced per colony formed units (CFU). Cells that were grown under agitated conditions produced higher amounts ($P<0.05$) of hydrogen peroxide (0.55 $\mu g/10^9$ CFU) than did those grown under static conditions (0.33 $\mu g/10^9$ CFU).

Effect of glucose on the production of hydrogen peroxide

Cells of *L. delbruckii* spp. *lactis* I harvested from the stationary phase (12 hr 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 24 hr at 5°C with constant agitation. No cellular growth was detected; however, hydrogen peroxide production was detected and reached a maximum of 25 $\mu g/10^6$ CFU at 18 hr (FIG. 2). There appeared to be a slight decline in hydrogen peroxide concentration beyond 20 hr. The accumulation of hydrogen peroxide by resting cells in sodium phosphate buffer with and without glucose (55.5 mM) was monitored for 55 hr (FIG. 3). Higher amounts of 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 30 hr followed by a large increase for the 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 beyond twenty hours.

In another series of experiments, the amount of hydrogen peroxide formed by resting cells of *L. delbruckii* spp. *lactis* I at 5°C was significantly less ($P<0.05$) in the

buffer without glucose than in the presence of glucose (Table 1). When washed cells were resuspended in phosphate buffer and preincubated for 1 hr at 5°C, then recovered by centrifugation and resuspended in fresh buffer (starved cells), little or no hydrogen peroxide was produced in 3 hr at 5°C. However, if starved cells were resuspended in phosphate buffer containing 55.5 mM glucose hydrogen peroxide was produced. Comparison of the amounts of hydrogen peroxide produced in the latter two samples confirmed the importance of glucose (Table 1).

The amount of hydrogen peroxide produced by preincubated (starved) cells of *L. delbruckii* spp. *lactis* I was significantly ($P<0.05$) dependent on the concentration of glucose in the buffer (FIG. 4). Significantly less ($P<0.05$) hydrogen peroxide production was observed when 0.05 mM of glucose was used compared to 0.55, 5.55, and 55.5 mM glucose. No significant differences ($P>0.05$) were observed in the amounts of hydrogen peroxide produced in buffer containing 5.5, and 55.5 mM of glucose.

Hydrogen peroxide production in agitated and static resting cell suspensions

More ($P<0.05$) hydrogen peroxide production by starved cells was obtained in the phosphate buffer containing glucose when it was agitated continuously compared to that when the buffer was not agitated (FIG. 5). Hydrogen peroxide accumulated under both conditions, however, the amount in the static buffer did not reach the level in the agitated buffer during 5 hr of incubation.

Hydrogen peroxide production by cell-free extracts

Cell-free extracts of *L. delbruckii* spp. *lactis* I from static cultures were assayed for the ability to produce hydrogen peroxide in buffer containing pyruvate (55.5mM) or different carbohydrates (Glucose, Sucrose, Lactose, Maltose, Mannose, Cellobiose or

Galactose 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 and the cell free extract, hydrogen peroxide formation and NADH disappearance were observed. Such results indicated the presence of NADH oxidase enzyme capable of producing hydrogen peroxide.

The NADH oxidase of *L. delbruckii* spp. *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 0.2 M sodium phosphate buffer pH 6.5 for 18 h at 4°C. The dialyzed enzyme was significantly ($P<0.05$) reactivated upon addition of a variety of concentrations of FAD (Table 3). The NADH oxidase activity increased significantly ($P<0.05$) at each higher level of FAD concentration up to 10 μm. Similar significant ($P<0.05$) increases were observed for hydrogen peroxide production.

Hydrogen peroxide production by resting cells using lactate as substrate

Lactic acid was detected during incubation of *L. delbruckii* spp. *lactis* I resting cells at 5°C in sodium phosphate buffer containing 55.5 mM glucose using HPLC after 3 days of incubation, but it disappeared following 5 days (FIG. 6). 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 remain fairly constant through day 9. The pH in the buffer solution changed just slightly from 6.50 to 6.39. The disappearance of lactic acid suggested the involvement of other enzymes in the formation of hydrogen peroxide, a lactate dehydrogenase could have been implicated in regenerating NADH so NADH oxidase could continue in the formation of hydrogen peroxide. However, the disappearance of the lactic acid suggested another possibility. Experiments were done to determine if resting cell of *L. delbruckii* spp. *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 significantly higher ($P<0.05$) than the amount produced using glucose (FIG-7). In the sample containing glucose as substrate, peroxide production appeared to level off between 24 and 48 hours then increase dramatically. In the sample containing sodium lactate, the hydrogen peroxide production did not include such a plateau between 24 and 48 hours.

When starved cells of *L. delbruckii* spp. *lactis* I were inoculated into sodium phosphate buffer (pH 6.5, 0.2 M) containing 5.55 mM L-, D-, or DL-lactate, hydrogen peroxide production was significantly higher ($P<0.05$) in the presence of D-lactate than in the presence of DL- or L-lactate (FIG. 8). Little or no hydrogen peroxide was formed in the buffer containing L-lactate and none was formed in the control buffer during the 24 hr incubation period.

Hydrogen peroxide accumulation by washed starved cells of *L. delbruckii* spp. *lactis* I was followed for 6 days in buffer containing DL-lactate at four different temperatures (FIG. 9A). After two days of incubation it was observed that more hydrogen peroxide was produced at 25 and 37°C than at 5 or 15°C. After the second day of incubation, decreases in hydrogen peroxide concentration were observed at 25 and 37°C, contrary to this, no decrease in hydrogen peroxide production was detected at 5 and 15°C. However, a change in hydrogen peroxide production velocity was observed. A slight plateau in the production appeared between days 2 and 4 and followed by accumulation of hydrogen peroxide again until the sixth day. Decreases in lactate concentrations were detected in the media incubated at all four temperatures (FIG. 9B). The consumption of lactate at 25°C was significantly higher ($P<0.05$) than at any other temperature. Slow lactate utilization was observed in media at incubation temperatures of 5, 15, 37°C and lactate was not completely depleted from such a media.

DISCUSSION

Agitation of cultures of *L. delbruckii* spp. *lactis* I during growth did not significantly influence the amount of growth obtained. However, when agitated, the cultures produced higher amounts of hydrogen peroxide per CFU than when grown statically. It is important to 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 even when cells were growing in agitated or anaerobic conditions. Collins and Aramaki (1980) also reported that hydrogen peroxide was produced in static conditions by *L. acidophilus*, but higher amounts of hydrogen peroxide were produced in agitated conditions by the same microorganism. 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. delbruckii* spp. *lactis* I to have grown without significant inhibition from the hydrogen peroxide formed it could possess a scavenger system to eliminate some of the hydrogen peroxide formed intracellularly. It has been mentioned that lactobacilli may be able to grow in 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). It has been also reported that Mn^{2+} afforded oxygen tolerance to *L. plantarum*. Archibald and Fridovich (1981a) 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. Moreover, they mentioned that high internal concentrations of Mn^{2+} were required for this system to work, but two exceptions have been reported, a very high oxygen sensitivity and absence of superoxide dismutase system. In our study resting cells obtained from MRS broth supplemented with Mn^{2+} did not produce higher amounts of hydrogen peroxide (data no shown), this may be due to the scavenger effect that the

manganese produced. It appears that *L. delbruckii* spp. *lactis* I did not posses a very active NADH peroxidase system since increased accumulation of hydrogen peroxide did not occur in the presence of added Mn^{2+}.

Lactobacillus delbruckii spp. *lactis* I was able to produce and accumulate 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 it was reported by Iwamoto et al (1979a, 1979b), and Collins and Aramaki (1980). No growth of *L. delbruckii* spp. *lactis* I was observed under this conditions, but the cells evidently remained metabolically active. No significant change in pH was registered in the medium indicating that the peroxide production was not dependent on the cells obtaining energy from fermentation. Exposure of *L. mesenteroides* cells to air induced the loss of their glycolytic activity, however, the cells remained metabolically active since they produced hydrogen peroxide (Ito et al, 1983). Wheater et al (1952) reported that hydrogen peroxide production was associated with living, but not necessarily with multiplying cells of the lactic acid bacteria.

Measurement of hydrogen peroxide over time showed a plateau during the time of incubation, and then after a short period of time, hydrogen peroxide formation increased (FIG. 7). This behavior suggests that two substrates or more than one enzymatic system was involved in the production of hydrogen peroxide. A similar plateau in the accumulation of hydrogen peroxide was previously reported by Price and Lee (1970) using resting cells of *L. plantarum* in 1% peptone broth at 30°C. It is known that *L. plantarum* produce hydrogen peroxide by different enzymatic systems.

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 as resting cells 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 indeed 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 significant reduction ($P<0.05$) in hydrogen peroxide production was detected with lower concentrations of glucose indicating the need for the carbohydrate. Aeration increased the amount of hydrogen peroxide produced, nevertheless, hydrogen peroxide was produced without any aeration, as was previously stated.

Production of hydrogen peroxide was not observed when starved cells were added to buffer containing only pyruvate or NADH. Two reasons may account for these results. First, if hydrogen peroxide was produced using pyruvate, a spontaneous chemical reaction may have occurred between these two compounds that would have destroyed any hydrogen peroxide formed and thus it would not be detected (Gunsalus and Umbreit, 1945). Second, NADH may not have been able to enter the cell and the NADH oxidases are usually cytosolic enzymes (Grufferty and Condon, 1983), for this reason no reaction could be detected. Accumulation of hydrogen peroxide was dependent on the source of oxidizable substrate.

It has been reported that glucose, galactose and other substrates promotes O_2 utilization by whole cells generating NADH, which is 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. Inactivation of NADH oxidase in cell free extract after dialysis suggests the lost of cofactors required for activity of the enzyme. Activity of NADH oxidase was recovered when the enzyme was incubated with different concentration of FAD. This effect was previously reported by Hoskins et al (1962) and Collins and Aramaki (1980).

The reduction of pyruvate to lactate by lactate dehydrogenase (LDH) allows for NAD regeneration which is 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 is in excess. For this reason, when samples were stirred for a long periods of time at 5°C, inhibition of the LDH enzyme could have occurred and no further production of lactic acid was detected. There is evidence of the toxic effect of oxygen and its metabolites on G3P-dehydrogenase and it is probable that oxidation of sulfhydryl groups to disulfide groups adversely affects a number of enzymes such as lactate dehydrogenase (Haugaard, 1968). The disappearance of the lactic acid that was produced suggested the possible existence of a lactate oxidase. Results showed that resting cells of *L. delbruckii* spp. *lactis* I incubated in buffer containing sodium lactate produced hydrogen peroxide indicating the possible presence of a lactate oxidase. Lactate oxidase utilizes lactate and at the same time is able to produce hydrogen peroxide and pyruvate. 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 resting cells of *L. delbruckii* spp. *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 suggests that the enzyme is specific for D-lactate. Further evidence for a lactate oxidase enzyme being involved was indicated when resting cells of *L. delbruckii* spp. *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. However, it was difficult to demonstrate the activity of lactate oxidase in cell free extract by measuring hydrogen peroxide production. This activity was also lost during dialysis and could not be recovered.

Hydrogen peroxide as a metabolic by-product of lactobacilli is an important subject for theoretical and practical study. Because of it application to preserve food products it appeared to be important to clarify the mechanism involved in its production. By understanding the mechanism it may be possible to utilize selected strains and/or specific conditions to enhance the use of *L. delbruckii* spp. *lactis* in food preservation. To achieve

this, it is important to characterize the lactate dehydrogenase, NADH oxidase and lactate oxidase, and to study the relation among them for the production of hydrogen peroxide. If these three enzymes work together it may explain the continuous production of hydrogen peroxide by *L. delbruckii* spp. *lactis* at refrigeration temperatures.

Table-1. Influence of glucose on hydrogen peroxide production by starved and nonstarved cells of *Lactobacillus delbruckii* spp. *lactis* I[1].

Type of cells	Glucose	Hydrogen peroxide[2] ($\mu g/10^9$ CFU)
Nonstarved	-	6.0^b
	+	8.6^a
Starved	-	0.2^d
	+	4.4^c

[1]Cells were incubated at 5°C for 3 hours in sodium phosphate buffer (pH 6.5, 0.2 M) with and without 55.5 mM glucose as indicated. A control with sodium phosphate buffer without cells at 5°C was also included, but no hydrogen peroxide was detected.

[2]Each data point represents the mean from two independent trials. Means with same letter in superscripts are not significantly different (P>0.05).

Table-2. Partial purification of NADH oxidase from *Lactobacillus delbruckii* ssp. *lactis* I.

Fraction	NADH oxidase[1] (nmoles/min/ml)	Protein[1] (mg/ml)	Specific activity[1] (nmoles/min/mg)	Purification[1]
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]

[1]Each data point represents the mean from three independent trials. Means with same letter in superscripts are not significantly different (P>0.05).

Table-3. Effect of Flavin Adenin Dinucleotide on dialyzed NADH oxidase.

FAD (μM)	NADH oxidase (nmoles/min/mg)	Hydrogen peroxide[1] (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]

[1]Each data point represents the mean from three independent trials. Means with same letter in superscripts are not significantly different (P>0.05).

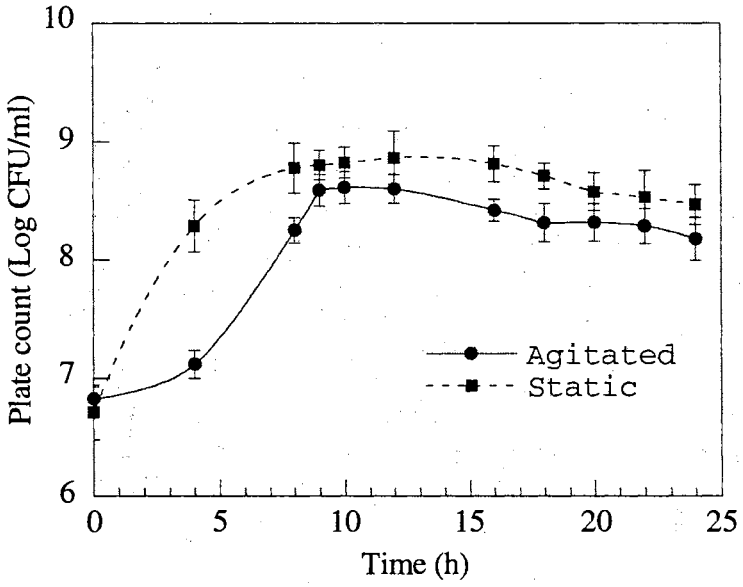

FIG-1. Influence of static and agitated conditions on the growth of *Lactobacillus delbruckii* ssp. *lactis* I. Each data point represents the mean from two independent trials. The vertical bars represent the standard deviation.

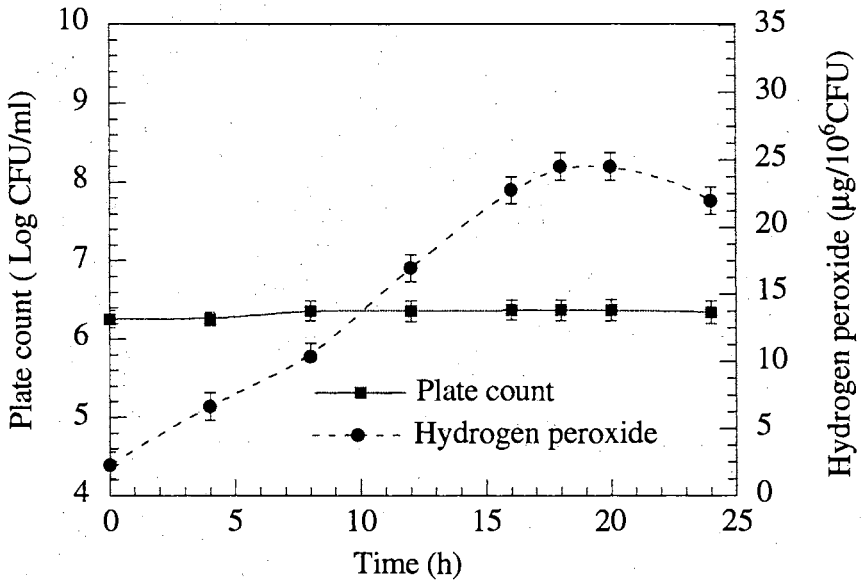

FIG-2. Hydrogen peroxide production and viable population and hydrogen peroxide accumulation in buffer containing 55.5 mM glucose with constant agitation at 5°C. Each data point is the mean from two independent trials. The vertical bars represent the standard deviation. No preincubated cells were used.

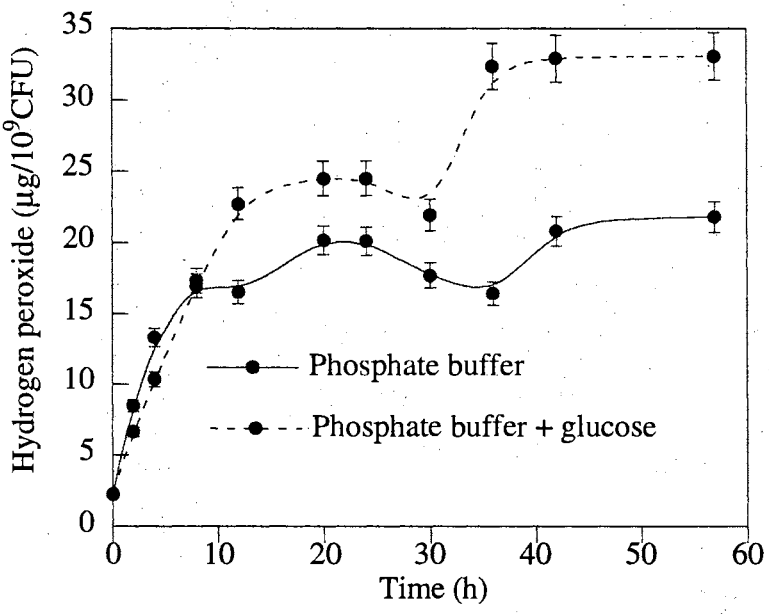

FIG-3. Effect of glucose on hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* ssp. *lactis* I incubated at 5°C. Each data point represents the mean from two independent trials. The vertical bars represent the standard deviation. Significant differences ($P<0.05$) between treatments were observed after 8 hr of incubation period. No preincubated cells were used.

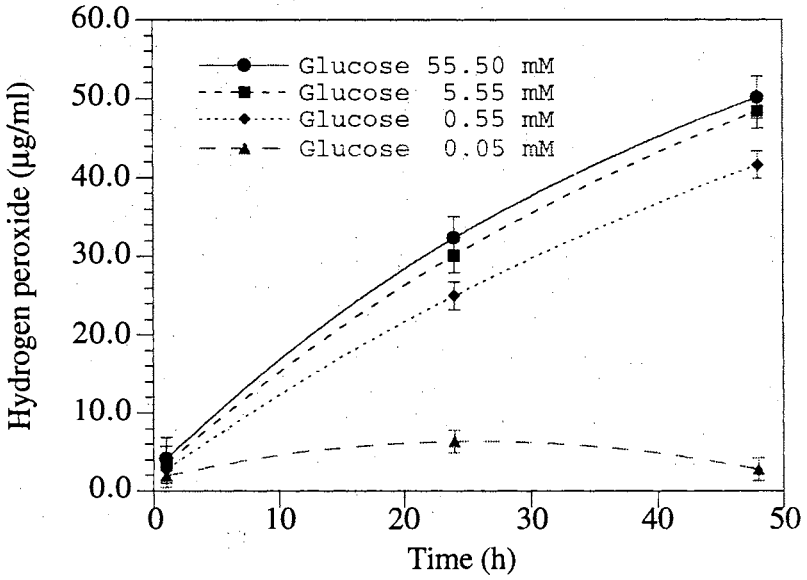

FIG-4. Effect of glucose on hydrogen peroxide production under agitated conditions at 5°C. Each data point represents the mean from three independent trials. Preincubated (starved) cells were used for this experiment.

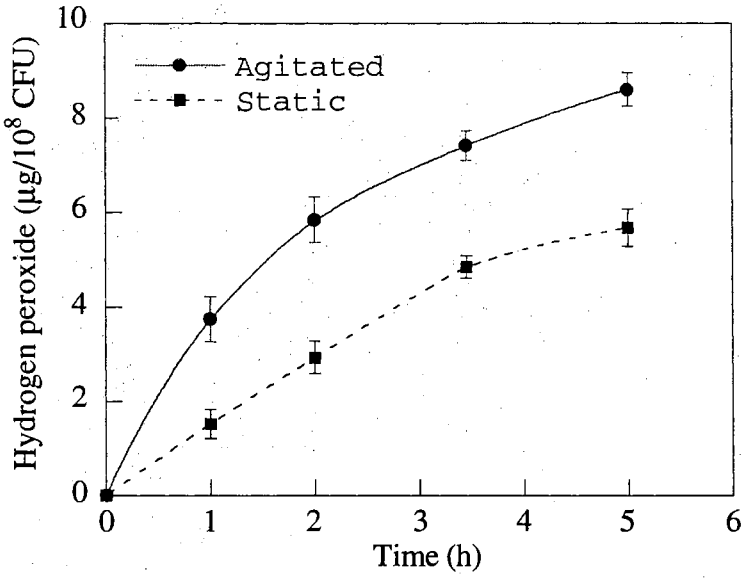

FIG-5. Hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* spp. *lactis* I at 5°C in phosphate buffer containing 55.5 mM glucose under agitated and static conditions during five hour of incubation. Each data point represents the mean from three independent trials. The vertical bars represent the standard deviation. Significant differences ($P<0.05$) were observed between treatments. Preincubated (starved) cells were used for this experiment.

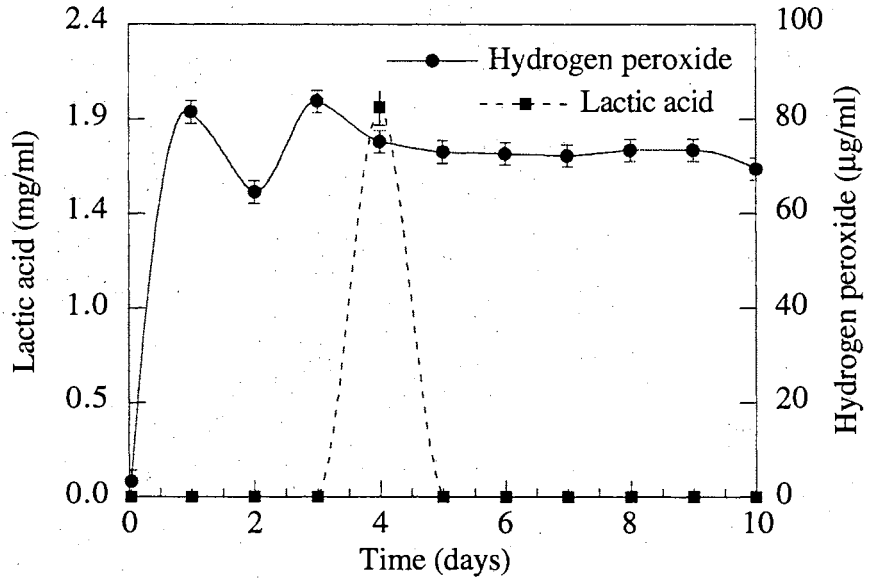

FIG-6. Lactic acid detected by HPLC after incubation of *Lactobacillus delbruckii* ssp. *lactis* I in glucose sodium phosphate buffer. Each value is the mean of two independent trials. The vertical bars represent the standard deviation. Preincubated cells (starved) were used for this experiment.

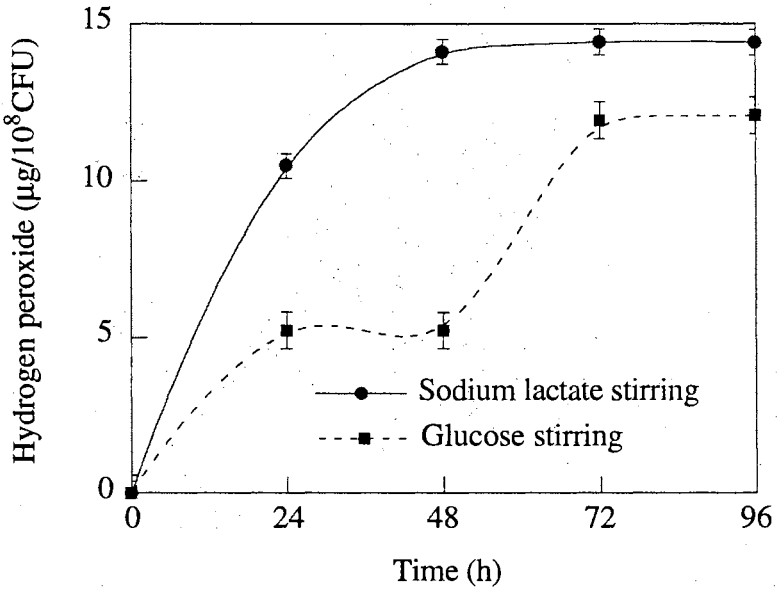

FIG-7. Effect of sodium lactate (55.5 mM) and glucose (55.5 mM) on the production of hydrogen peroxide at 5°C with constant agitation. Each value is the mean of three independent trials. The vertical bars represent the standard deviation. Preincubated (starved) cells were used for this experiment.

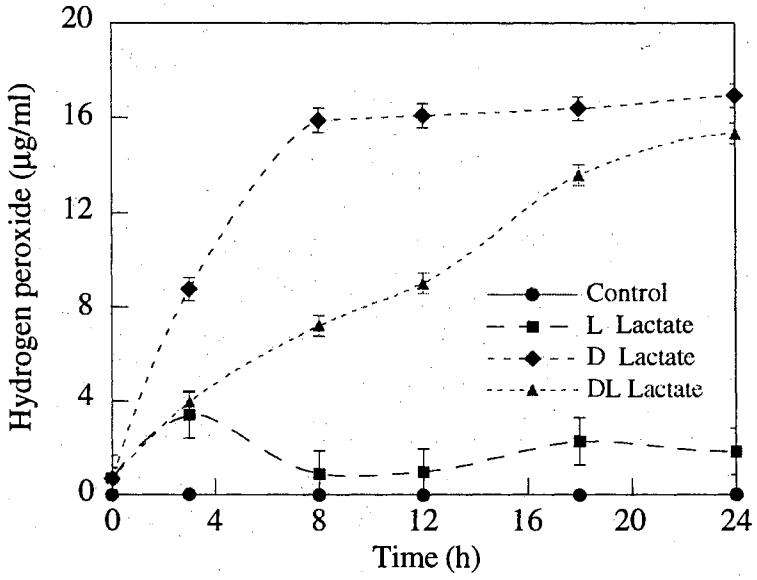

FIG-8. Effect of L-, D- and DL- lactate in hydrogen peroxide production by resting cells of *L. delbruckii* spp. *lactis* I. Each value is the mean of two independent trials. The vertical bars represent the standard deviation. Preincubated (starved) cells were used for this experiment.

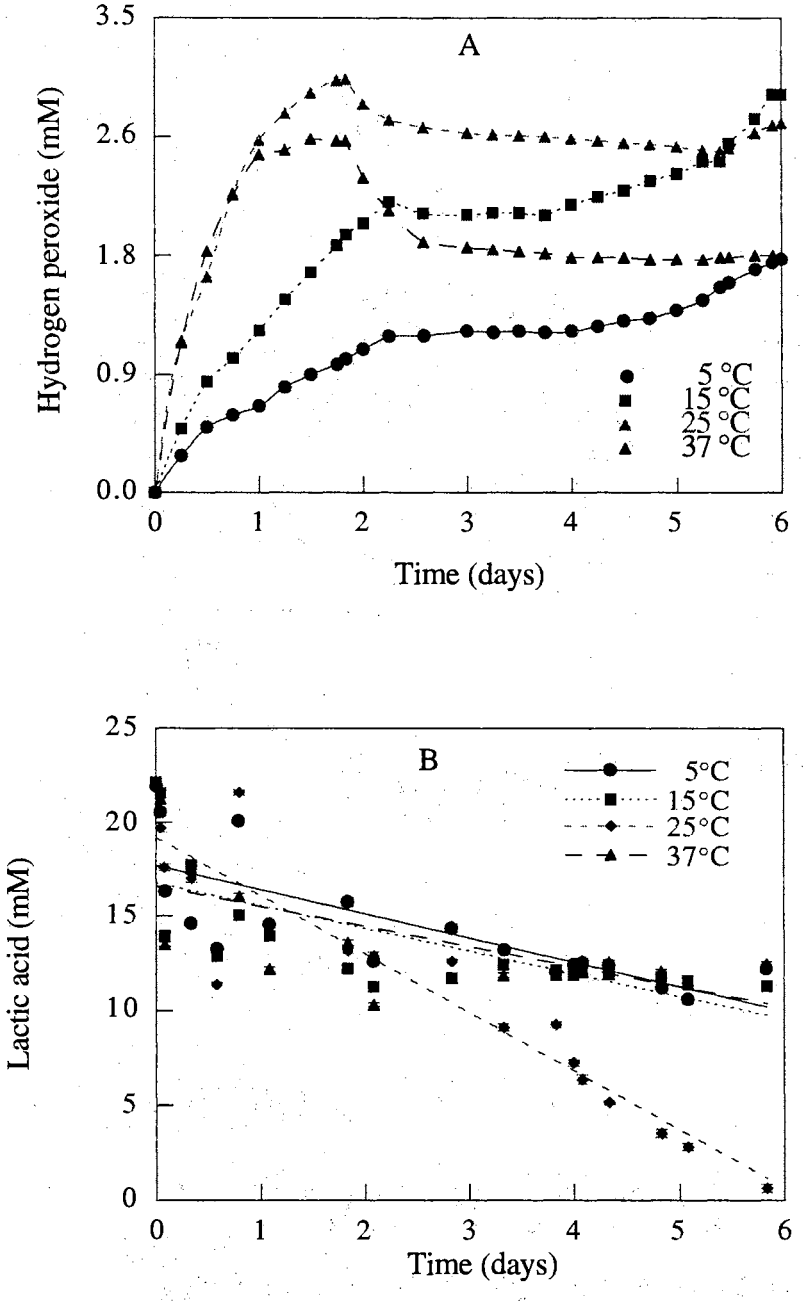

FIG-9. Effect of temperature on the production of hydrogen peroxide (A) and on the consumption of DL-lactate (B) by *L. delbruckii* spp. *lactis* I in DL lactate containing buffer at 5°C. Each value is the mean of two independent trials. Error bars are omitted for clarity. Preincubated (starved) cells were used for this experiment.

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APPENDIX A

IDENTIFICATION OF *LACTOBACILLUS DELBRUCKII SSP. LACTIS* I, SPECTROGRAM OF NADH OXIDASE ACTIVITY AND CHROMATOGRAM OF LACTIC ACID

Table-A1. Identification of the three strains of *Lactobacillus delbruckii* ssp. *lactis* by their pattern of fermented carbohydrates[1].

Carbohydrate	Bergey's[2]	*L. delbruckii* spp. *lactis* I
Galactose	d	+/-
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
Mannitol	-	-
Amygdaline	+	-
Esculin	+	+
Salicin	+	+/-
Cellobiose	d	+
Maltose	+	+
Lactose	+	+
Melibiose	-	-
Sucrose	+	+
Trehalose	+	+
D-Raffinose	-	-
Ribose	-	-
Rhamnose	-	-
Arabinose	-	-
Melezitose	-	-
Sorbitol	-	-
Xylose	-	-
Grow @ 15°C	-	-
Grow @ 45°C	+	+

[1]Based in the API 50 CH system (bioMerieux sa, France). All three strains were Gram positive catalase negative rods.

[2]Reactions that are listed for *L. delbruckii* ssp. *lactis* in the 8th edition of Bergey's Manual of Determinative Bacteriology. Symbols: +, 90 % or more strains positive; -, 90 % or more strains positive; d=11-89 % strains positive.

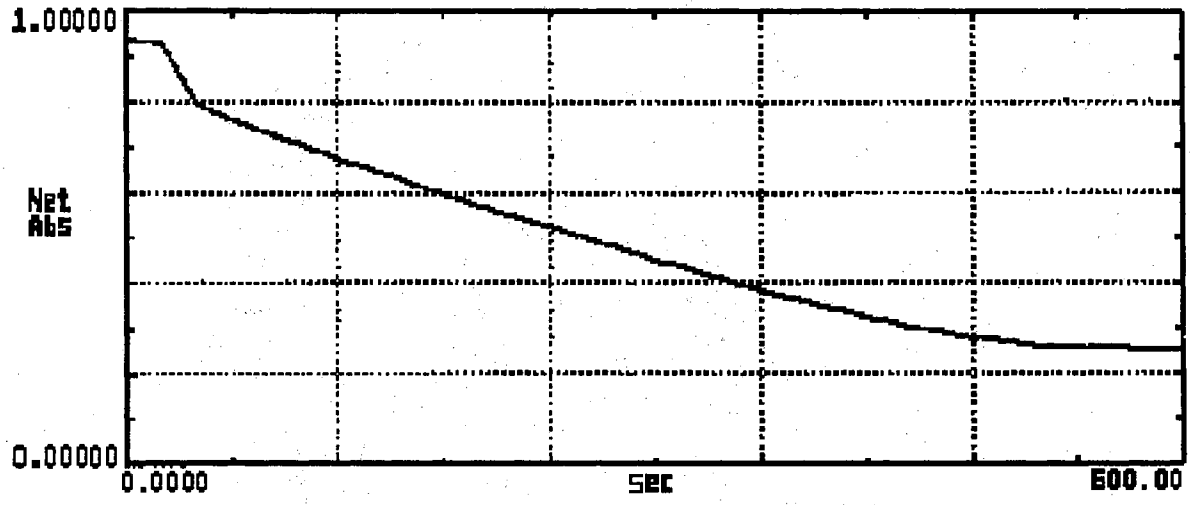

FIG-A1. NADH comsuption by NADH oxidase from a cell free extract of *Lactobacillus delbruckii* ssp. *lactis* I. Preincubated (starved) cells were used to obtain the cell free extract.

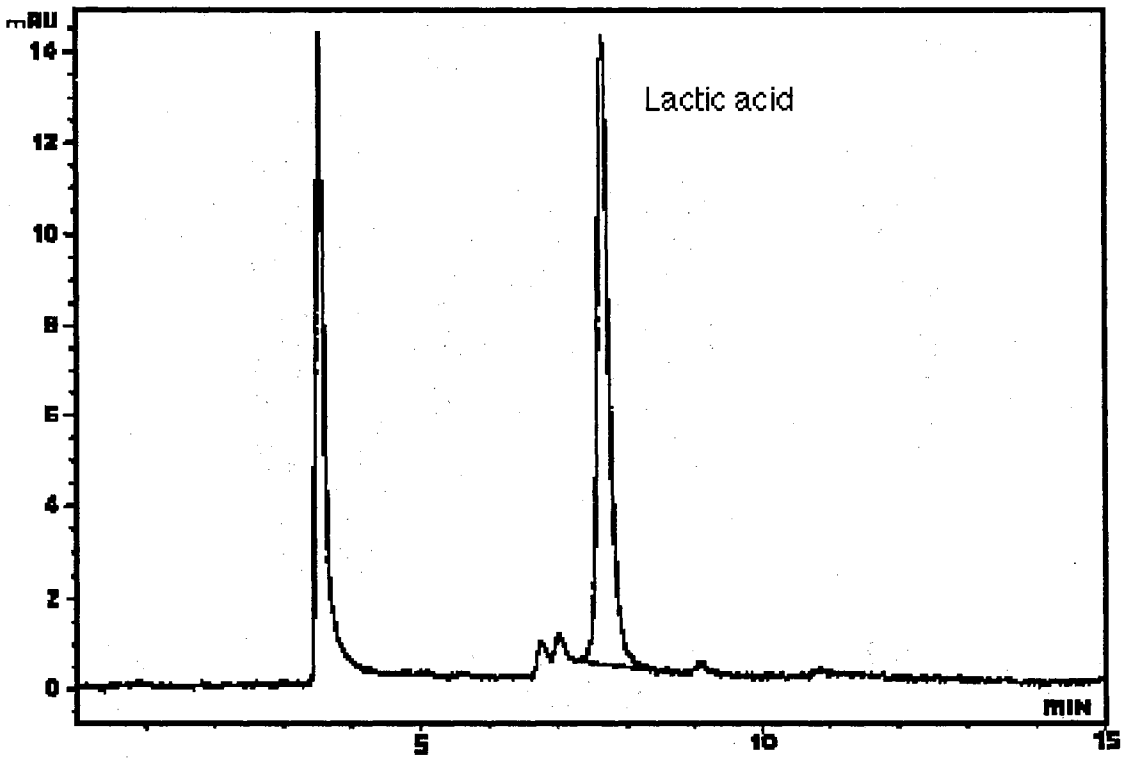

FIG-A2. Chromatogram of lactic acid from preincubated cells of *Lactobacillus delbruckii* ssp. *lactis* I resuspended in buffer containing 55.5 mM glucose.

APPENDIX B

FACTORS AFFECTING CELL GROWTH AND HYDROGEN PEROXIDE PRODUCTION IN *LACTOBACILLUS DELBRUKII* SSP. *LACTIS* I

Effect of manganese on the growth of *Lactobacillus delbruckii* ssp. *lactis* I

When MRS broth was supplemented with 1.4 mM of $MnSO_4$ (4.4 times the amount normally found in MRS broth) slower growth of the microorganism was observed under both static and agitated conditions compared to static growth in unsupplemented broth (FIG. B1). However, the agitated culture supplemented with $MnSO_4$ reached the same maximum population as did the static control culture. The static culture supplemented with $MnSO_4$, however, reached a slower maximum population than did the static control.

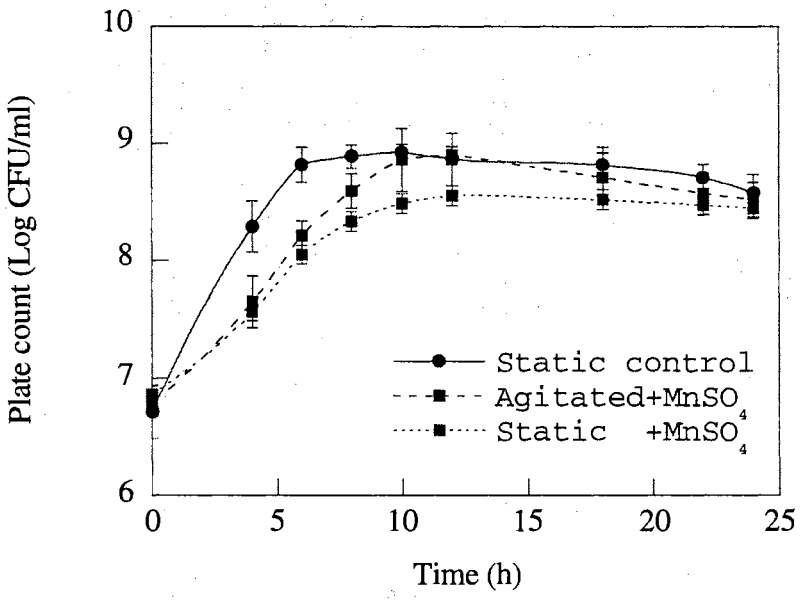

FIG-B1. Effect of $MnSO_4$ on the growth of *Lactobacillus delbruckii* ssp. *lactis* I under static and agitated conditions. Each data point represents the mean from two independent trials. The vertical bars represent the standard deviation. No significant differences ($P>0.05$) in maximum population among treatments were found.

Effect of glucose and lactose on the growth of *Lactobacillus delbruckii* ssp. *lactis* I

The ability of *L. delbruckii* spp. *lactis* I to grow in a modified MRS broth containing lactose (111 mM) instead of glucose as the sugar source was compared with its ability to grow in a normal MRS broth containing glucose (111 mM) when both media were maintained at pH 6.5 (FIG. B2). There was no significant differences ($P>0.05$) in cell numbers during the log phase of growth. There also was no significant difference ($P>0.05$) in the maximum population achieved in the two media, however, the death phase was more pronounced in the culture medium containing lactose.

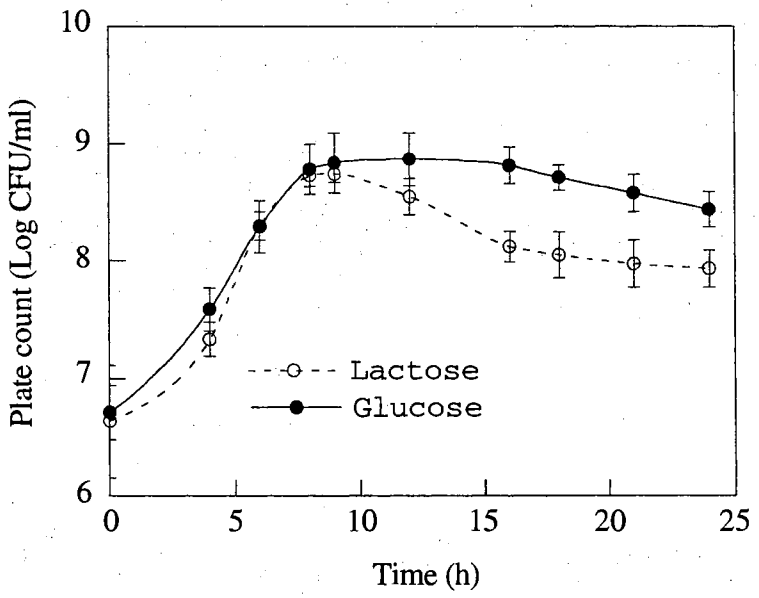

FIG-B2. Growth of *Lactobacillus delbruckii* ssp. *lactis* I in agitated MRS broth containing glucose or lactose at pH 6.5. Each data point is the mean from two independent trials. The vertical bars represent the standard deviation. Significant differences ($P<0.05$) between treatments was observed.

Effect of the time of the harvest of cells from MRS broth on the hydrogen peroxide production

No significant differences (P>0.05) were observed in the amount of hydrogen peroxide produced from cells harvested from the stationary phase after 14, 16 and 18 hr of incubation.

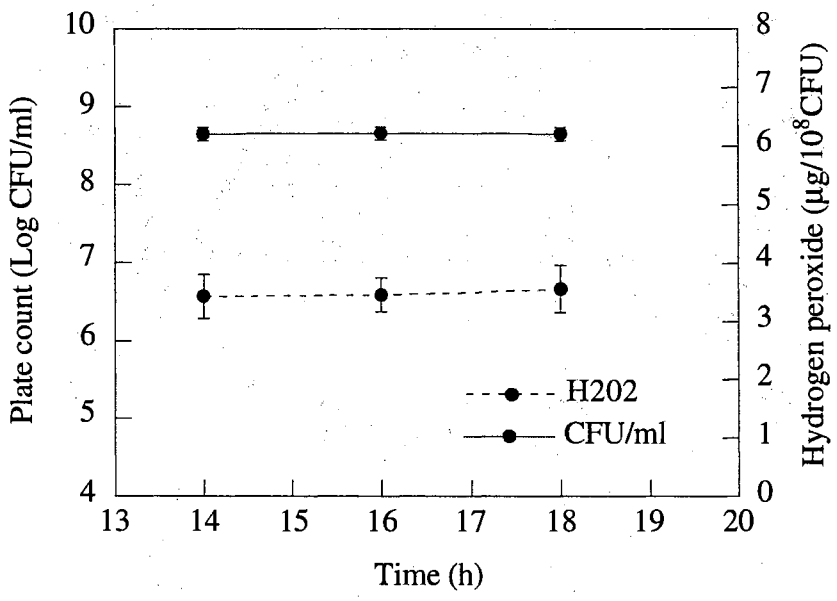

FIG-B3. Hydrogen peroxide production by cells harvested at different times from static MRS broth cultures at 37°C. Cells were incubated at 5°C for 24 hours in sodium phosphate buffer (pH 6.5, 0.2 M). Preincubated (starved) cells were used in this experiment.

The effect of carbohydrates on the production of hydrogen peroxide

Starved cells of *L. delbruckii* spp. *lactis* I (i.e. washed cells preincubated in phosphate buffer) were used to measure the influence of various carbohydrates on hydrogen peroxide production in sodium phosphate buffer solutions (pH 6.5, 0.2 M) at 5°C (Table B1). The carbohydrates used for this experiment were selected based in the ability of *L. delbruckii* spp. *lactis* to ferment the individual carbohydrates. Significantly more ($P<0.05$) hydrogen peroxide was formed in buffer containing each sugar than in the buffer alone.

Table-B1. Effect of different carbohydrates sources on the production of hydrogen peroxide in preincubated resting cell of *Lactobacillus delbruckii* ssp. *lactis* I[1].

Carbohydrate	Hydrogen peroxide[1] ($\mu g/10^8$CFU)
Lactose	2.24[a]
Cellobiose	1.78[b]
Glucose	1.77[b]
Sucrose	1.74[b]
Mannose	1.61[c]
Maltose	1.38[d]
Galactose	0.58[e]
Buffer	0.12[f]

[1]Each data point represents the mean from two independent trials. Means with same letter in superscripts are not significantly different ($P>0.05$). Cells were incubated at 5°C for 1 hour in sodium phosphate buffer (pH 6.5, 0.2 M). Preincubated (starved) cells were used in this experiment.

The effect of the cell concentration on the production of hydrogen peroxide

The number of *L. delbruckii* spp. *lactis* I influenced the accumulation of hydrogen peroxide (Table B2). Cell numbers in the range from 1.4 to 4.3 x 10^8 CFU/ml produces similar amounts of hydrogen peroxide. After 48 h of incubation, the hydrogen peroxide accumulated remained constant (80 μg/ml). Concentrations of cells from 8.5 to 1.7 x 10^9 CFU/ml produced up to 160 μg/ml. A cell number of 2.6 x 10^9 CFU/ml produced a maximum of 172 μg/ml of hydrogen peroxide.

Table-B2. Effect of the concentration of cells on the accumulation of hydrogen peroxide.

Cell number (10^9CFU/ml)	Hydrogen peroxide[1] (μg/ml)	Hydrogen peroxide[1] (μg/10^7 CFU)
0.14	75 [c]	5.3 [a]
0.43	80 [c]	1.9 [b]
0.86	136 [b]	1.8 [b]
1.72	140 [b]	0.77 [c]
2.58	172 [a]	0.68 [c]

[1]Each data point represents the mean from two independent trials. Means with same letter in superscripts are not significantly different (P>0.05). Cells were incubated at 5°C for 4 days in sodium phosphate buffer (pH 6.5, 0.2 M). Preincubated (starved) cells were used in this experiment.

Effect of hydrogen peroxide on the survival of *Lactobacillus delbruckii* ssp. *lactis* I

Cells of *L. delbruckii* spp. *lactis* I from the late stationary phase were exposed to different concentrations of hydrogen peroxide (Table B3). No significant differences ($P>0.05$) in CFU/ml were detected at hydrogen peroxide concentrations below 80 µg/ml during the 24 hr period at 5°C. However, the sensitivity of the cells increased at hydrogen peroxide concentrations above 85 µg/ml.

Table-B3. Effect of hydrogen peroxide on survival of *Lactobacillus delbruckii* ssp. *lactis* I.

Hydrogen peroxide (µg/ml)	Survival of *L. delbruckii* spp. *lactis* I[1] (%)
0	100[a]
8	100[a]
16	98.5[ab]
24	98.5[ab]
32	98.3[ab]
40	98.3[ab]
48	97.6[ab]
56	97.2[ab]
64	96.0[b]
72	95.8[b]
80	95.8[b]
85	85.8[c]
90	85.4[c]
95	85.4[c]
100	80.0[d]
110	72.5[e]
120	71.3[e]

[1]Each data point represents the mean from two independent trials. Means with same letter in superscripts are not significantly different ($P>0.05$). Cells were incubated at 5°C for 24 hours in sodium phosphate buffer (pH 6.5, 0.2 M). No preincubated cells were used in this experiment.

Effect of pH on the hydrogen peroxide production

The pH did not affect significantly ($P<0.05$) the hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* ssp. *lactis* I.

Table-B4. Effect of pH in hydrogen peroxide production by *Lactobacillus delbruckii* ssp. *lactis* I.

pH	Hydrogen peroxide[1] (µg/ml)
5.5	4.4[a]
6.0	5.1[a]
6.5	4.9[a]
7.0	5.0[a]

[1]Each data point represents the mean from three independent trials. Means with same letter in superscripts are not significantly different ($P>0.05$). Cells were incubated at 5°C for 24 hours in sodium phosphate buffer (pH 6.5, 0.2 M). No preincubated cells were used in this experiment.

APPENDIX C

STATISTICAL ANALYSES

Table C1 - Analysis of variance of table 1 - Influence of glucose on hydrogen peroxide productionby starved and nonstarved cells of *Lactobacillus delbruckii* ssp. *lactis* I.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.08262112	0.08262112	3.40	0.1623
Treatment	3	76.32176838	25.44058946	1047.75	0.0001
Error	3	0.07284337	0.02428112		
Total	7	76.47723287			

$LSD_{0.05}$ treatment = 0.4959

Table C2 - Analysis of variance of table 2 - Partial purification of NADH oxidase from *Lactobacillus delbruckii* ssp. *lactis* I (variable specific activity).

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	2	0.04015556	0.02007778	1.90	0.2632
Treatment	2	57.55555556	28.77777778	2720.59	0.0001
Error	4	0.04231111	0.01057778		
Total	8	57.63802222			

$LSD_{0.05}$ treatment = 0.2332

Table C3 - Analysis of variance of table 3 - Effect of Flavin Adenin Dinucleotide on dialyzed NADH oxidase (variable NADH).

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	2	0.04497333	0.02248667	1.06	0.3916
Treatment	4	288.09850667	72.02462667	3383.56	0.0001
Error	8	0.17029333	0.02128667		
Total	14	288.31377333			

$LSD_{0.05}$ treatment = 0.2747

Table C4 - Analysis of variance of figure 1 - Influence of static and agitated conditions on the growth of *Lactobacillus delbruckii* ssp. *lactis* I.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.04778409	0.04778409	0.10	0.8011
Treatment	1	1.21445682	1.21445682	2.65	0.3507
Error (a)	1	0.45818409	0.45818409		
Time	10	14.31367273	1.43136727	43.24	0.0001
Time*Treatment	10	0.96771818	0.09677181	2.92	0.0197
Error (b)	20	0.66208182	0.03310409		
Total	43	17.66389773			

$LSD_{0.05}$ treatment = 2.5932

Table C5 - Analysis of variance of figure 2 - Hydrogen peroxide production and viable population and hydrogen peroxide accumulation in buffer containing 55.5 mM glucose with constant agitation at 5°C (var plate count).

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.28861008	0.28861008	130.58	0.0001
Treatment	5	0.00029942	0.00005988	0.03	0.9994
Error	5	0.01105142	0.00221028		
Total	11	0.29996092			

$LSD_{0.05}$ treatment = 0.1209

Table C6 - Analysis of variance of figure 3 - Effect of glucose on hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* ssp. *lactis* I incubated at 5°C.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	2.10328182	2.10328182	53.11	0.0868
Treatment	1	259.42694545	259.42694545	6551.19	0.0079
Error (a)	1	0.03960000	0.03960000		
Time	10	2584.90169091	258.49016909	35332.61	0.0001
Time*Treatment	10	375.91515455	37.59151545	5138.32	0.0001
Error (b)	20	0.14631818	0.00731591		
Total	43	3222.53299091			

$LSD_{0.05}$ treatment = 0.7624

Table C7 - Analysis of variance of figure 4 - Effect of glucose on hydrogen peroxide production under agitated conditions at 5°C.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	2	0.01211667	0.00605833	0.95	0.4059
Treatment	3	3730.54663056	1243.51554352	99999.99	0.0001
Error (a)	6	0.01819444	0.00605833		
Time	2	6545.07661667	3272.53830833	99999.99	0.0001
Time*Treatment	6	2143.41176111	357.23529352	56282.15	0.0001
Error (b)	16	0.10155556	0.00634722		
Total	35	12419.16687500			

$LSD_{0.05}$ treatment = 0.0635

Table C8 - Analysis of variance of figure 5 - Hydrogen peroxide production by resting cells of *Lactobacillus delbruckii* spp. *lactis* I at 5°C in phosphate buffer containing 55.5 mM glucose under agitated and static conditions during five hour of incubation.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.49612500	0.49612500	2.51	0.3587
Treatment	1	22.36612500	22.36612500	112.96	0.0597
Error (a)	1	0.19800500	0.19800500		
Time	4	129.69523000	32.42380750	171.22	0.0001
Time*Treatment	4	5.87885000	1.46971250	7.76	0.0074
Error (b)	8	1.51492000	0.18936500		
Total	16	160.14925500			

$LSD_{0.05}$ treatment = 2.5285

Table C9 - Analysis of variance of figure 6 - Lactic acid detected by HPLC after incubation of *Lactobacillus delbruckii* ssp. *lactis* I in glucose sodium phosphate buffer.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.01272005	0.01272005	30.32	0.0003
Treatment	10	8.31500800	0.83150080	1981.91	0.0001
Error	10	0.00419545	0.00041954		
Total	21	8.33192350			

$LSD_{0.05}$ treatment = 0.0456

Table C10 - Analysis of variance of figure 7 - Effect of sodium lactate (55.5 mM) and glucose (55.5 mM) on the production of hydrogen peroxide at 5°C with constant agitation.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	2	0.00772667	0.00386333	0.26	0.7920
Treatment	1	107.27643000	107.27643000	7292.76	0.0001
Error (a)	2	0.02942000	0.01471000		
Time	4	708.82554667	177.20638667	32102.61	0.0001
Time*Treatment	4	70.24205333	17.56051333	3181.25	0.0001
Error (b)	16	0.08832000	0.00552000		
Total	29	886.46949667			

$LSD_{0.05}$ treatment = 0.1906

Table C11 - Analysis of variance of figure 8 - Effect of L-, D- and DL- lactate in hydrogen peroxide production by resting cells of *L. delbruckii* spp. *lactis* I.

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.02434502	0.02434502	7.18	0.0751
Treatment	3	89.94503506	29.98167835	8842.65	0.0001
Error (a)	3	0.01017173	0.00339058		
Time	5	29.18457144	5.83691429	2601.95	0.0001
Time*Treatment	15	32.32638431	2.15509229	960.68	0.0001
Error (b)	20	0.04486575	0.00224329		
Total	47	151.53537331			

$LSD_{0.05}$ treatment = 0.0757

Table C12 - Analysis of variance of figure 9 - Effect of temperature on the production of hydrogen peroxide (A) and on the consumption of DL-lactate (B) by *L. delbruckii* spp. *lactis* I in DL lactate containing buffer at 5°C.

A

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	0.04631745	0.04631745	2.45	0.2157
Treatment	3	53.61852568	17.87284189	944.54	0.0001
Error (a)	3	0.05676672	0.01892224		
Time	26	45.09324140	1.73435544	808.62	0.0001
Time*Treatment	78	19.30848219	0.24754464	115.41	0.0001
Error (b)	104	0.22306233	0.00214483		
Total	215	118.34639577			

$LSD_{0.05}$ treatment = 0.0842

B

Source	DF	Sum of squares	Mean square	F Value	Pr>F
Replication	1	2.87698136	2.87698136	616.28	0.0001
Treatment	3	189.63983094	63.21327698	13540.95	0.0001
Error (a)	3	0.01400492	0.00466831		
Time	17	1990.80956400	117.10644494	3837.32	0.0001
Time*Treatment	51	554.29619906	10.86855292	356.14	0.0001
Error (b)	68	2.07520672	0.03051775		
Total	143	2739.71178700			

$LSD_{0.05}$ treatment = 0.0513

VITA

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Candidate for the Degree of

Doctor of Philosophy

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