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Growth and End Product Formation of Two Psychrotrophic *Lactobacillus* spp. and *Brochothrix thermosphacta* ATCC 11509^T at Different pH Values and Temperatures

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Lactobacillus viridescens, *Lactobacillus* sp. strain 173 (homofermentative), and *Brochothrix thermosphacta* ATCC 11509^T were studied at different pH values and temperatures in aerobic and anaerobic batch cultures. The growth rates were higher in aerobic than in anaerobic cultures. *L. viridescens* grew faster at pH 5.8 than at pH 6.3, whereas the opposite was true for *B. thermosphacta*. *Lactobacillus* sp. strain 173 was inhibited in air or at 8°C in anaerobic culture. *B. thermosphacta* did not grow in anaerobic culture at pH 5.3. The following variations in growth yields were found in the different environments studied: *Lactobacillus* sp. strain 173, 23 to 25 g (dry weight) per mol of glucose consumed; *L. viridescens*, 11 to 23 g/mol; *B. thermosphacta*, 16 to 38 g/mol. In air, *L. viridescens* produced D-lactic acid, ethanol, and acetic acid, whereas no acetic acid was produced anaerobically. Acetic acid and ethanol together constituted 41 to 48% of the total product yield irrespective of pH and temperature. *Lactobacillus* sp. strain 173 produced a racemic mixture of D- and L-lactic acid at pH 6.3, whereas the proportion of L-lactic acid was higher than that of D-lactic acid at pH 5.3. In air, product formation of *B. thermosphacta* varied from a domination of L-lactic acid to increasing yields of acetoin, acetic acid, 2,3-butanediol and isovaleric acid. The effect of pH and temperature on product formation was difficult to separate from the effect of O_2 availability in aerobic cultures. However, it was indicated that more 2,3-butanediol and less acetoin were produced with a decreasing temperature. In anaerobic cultures, L-lactic acid and small amounts of formic acid were produced by *B. thermosphacta*, irrespective of the temperature.

Bacterial growth on meat and meat products is affected by factors such as pH, temperature, and gas atmosphere. In different environments, different organisms will dominate, and to understand both how spoilage arises and how to prevent it, it is important to know in what way significant bacteria are affected.

Lactobacillus spp. and *Brochothrix thermosphacta* significantly influence the quality of meat and meat products. On vacuum- or gas-packed meat and meat products, *Lactobacillus* spp. often dominate the microflora (1, 2, 4, 9). *B. thermosphacta* has on several occasions been found together with *Lactobacillus* spp. (9, 13, 16).

The physiology of *B. thermosphacta* has been studied by several authors (6, 8, 10), whereas *Lactobacillus* spp. from meat or meat products have been given much less attention. This can perhaps be explained by the fact that it is at present impossible to identify the majority of the *Lactobacillus* spp. found on meat or meat products since their characteristics do not conform with any described, approved species of lactobacillus (4, 11). Thus, it is difficult to choose representative test strains. However, *Lactobacillus viridescens* has been isolated from meat (2) and meat products (4) and is associated with green discolorations (12).

The present work studies the influence of different pH values and temperatures on the growth of one homofermentative and one heterofermentative *Lactobacillus* sp. and the *B. thermosphacta* type strain.

MATERIALS AND METHODS

Organisms. The test organisms used were *L. viridescens* SMRICC174, *Lactobacillus* sp. strain SMRICC173, and *B. thermosphacta* ATCC 11509^T. *L. viridescens* 174 was characterized by the following: acid formation from cellobiose, fructose, glucose, maltose, mannose, ribose, and sucrose but not from amygdalin, arabinose, galactose, lactose, mannitol, melezitose, raffinose, rhamnose, trehalose, and xylose; no hydrolysis of arginine; and gas production from glucose. *Lactobacillus* sp. strain 173 was characterized by the following: acid formation from fructose,

galactose, glucose, mannose, ribose, sucrose, and trehalose but not from amygdalin, arabinose, cellobiose, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, and xylose; hydrolysis of arginine; and no gas production from glucose.

Medium. The growth medium (YTGT) had the following composition (g/liter): yeast extract (Difco Laboratories, Detroit, Mich.), 24.0; glucose, 20.0; tryptone (Difco), 5.0; sodium acetate, 1.0; KH_2PO_4, 0.70; $MgSO_4 \cdot 7H_2O$, 0.52; $Na_2HPO_4 \cdot 2H_2O$, 0.22; silicone antifoaming agent (30% wt/wt; BDH Chemicals Ltd., Poole, England), 0.1; $MnSO_4 \cdot H_2O$, 0.06; thiaminehydrochloride, 0.001. The glucose was autoclaved separately.

Growth experiments. The cultures were performed batchwise in a 1-liter fermentor at controlled pH and temperature. The flow rate of the gas was 17 liters/h, and the stirring rate was 400 rpm. The medium was sparged with the applied gas for 20 min before inoculation and then throughout the cultivation. The inoculum was subcultured twice before the fermentor was inoculated to a concentration of 4×10^6 cells per ml.

Batch cultures were performed aerobically or anaerobically (5% CO_2 plus 95% N_2) at different pH values (5.3, 5.8, and 6.3) and temperatures (8, 15, and 25°C). The initial pH was adjusted with 2 M HCl. A constant pH during the growth was maintained by automatic titration (Radiometer PHM62 + TTT60 titrator, Copenhagen, Denmark) with 1 M NaOH.

Analysis. Growth was followed by viable count (pour plate technique with APT agar [BBL Microbiology Systems, Cockeysville, Md.] and incubation at 25°C for 3 days), determination of dry weight (3), and absorbance at 620 nm. Samples were taken out once every half hour during the daytime.

Samples (two of 15 ml each) withdrawn from the fermentor were centrifuged (2,500 × *g* for 20 min) at room temperature, and the supernatant liquid was stored at −40°C until determination was carried out. The concentrations of acetic acid, ethanol, formic acid, glucose, D-lactic acid, and L-lactic acid were determined enzymatically (Boehringer Mannheim, GmbH-Biochemica, Mannheim, Federal Republic of Germany). The concentrations of acetoin, isobutyric acid, isovaleric acid, and 2,3-butanediol were, after ether extraction (8), determined on a gas chromatograph (Varian 400; Varian Associates, Inc., Palo Alto, Calif.). A 3-ft (ca. 91-cm) glass column (Chromosorb WAW, 80–100 mesh and 10% diethylene glycol adiapate; Ohio Valley Specialty Chemical Inc., Marietta, Ohio) and a flame ionization detector were used. The flow rate in the column was 40 ml of N_2 per min, and the flow rate in the detector was 30 ml of H_2 per min plus 190 ml of air per min. The temperature in the column was raised from 90 to 140°C (6°C/min). The injector temperature was 150°C, and the detector temperature was 200°C (the method was described in a personal communication by C. Hibbard, Meat Research Institute, Langford, United Kingdom). The presence of hydrogen peroxide was detected with Perid strips (detection limit, 5 mg/liter; Boehringer Mannheim).

Calculations. The calculation of the maximum specific growth rate (μ_{max}) was done according to Pirt (14) and by using the absorbance at 620 nm as a measure of cell mass. The overall growth yield (Y_x = gram [dry weight] produced per mole of glucose consumed) and the overall product yield (Y_p = mole of product formed per mole of glucose consumed) were calculated.

RESULTS

Effect of pH. The highest specific growth rate (μ_{max}) of *L. viridescens* was found at pH 5.8 in aerobic culture (0.50 h^{-1}, Table 1). The μ_{max} values obtained in aerobic cultures were higher than those in corresponding anaerobic cultures. The growth yield (Y_x) was about 22 g/mol with minor variations at the different pH values applied, except at pH 5.3 in anaerobic culture where a much lower cell mass was detected (Table 1). *L. viridescens* produced the same type of end products irrespective of pH but with slightly varying yields (Table 1). D-Lactic acid dominated the end products. Ethanol was produced aerobically as well as anaerobically, whereas acetic acid was found only in aerobic culture. The heterofermentative end products (other than lactate) constituted 41 to 48% of the total product yield. Hydrogen peroxide was produced in aerobic cultures.

Lactobacillus sp. strain 173 was inhibited in air at a low final dry weight (inhibited culture, 0.4 g/liter; noninhibited culture, 2.6 to 3.0 g/liter). The μ_{max} was higher at pH 6.3 than at pH 5.3 (Table 1). The Y_x was about 24 g/mol (Table 1). *Lactobacillus* sp. strain 173 produced a racemic mixture of lactic acid at pH 6.3, whereas the proportion of L-lactic acid was slightly higher at pH 5.3 (Table 1).

The highest μ_{max} of *B. thermosphacta* was found at pH 6.3 in air (0.49 h^{-1}, Table 1). The μ_{max} values obtained in aerobic cultures were higher than those in the corresponding anaerobic cultures. *B. thermosphacta* did not grow at pH 5.3 in anaerobic culture. At pH 5.3, the aerobic growth was terminated before all glucose was consumed, and the μ_{max} was 40% lower than that at pH 6.3. The Y_x decreased from 27 to 16 g/mol when the pH was decreased from 6.3 to 5.3 in aerobic culture (Table 1). The product formation of *B. thermosphacta* in air varied with the pH (Table 1). The yield of L-lactic acid decreased from 1.4 mol/mol at pH 6.3 to 0.8 mol/mol at pH 5.3. In addition to L-lactic acid, small yields of ethanol, acetic acid, acetoin, 2,3-butanediol, isovaleric acid, isobutyric acid, and formic acid were also found.

Effect of temperature. The μ_{max} values of *L. viridescens* were higher in aerobic cultures than in corresponding anaerobic cultures (Table 2). The growth yield varied between 18 and 23 g/mol, and the lowest yield was found at 8°C in anaerobic culture (Table 2). *L. viridescens* produced D-lactic acid as a dominating end product irrespective of temperature (Table 2). The heterofermentative end products constituted 41 to 48% of the total product yield. *Lactobacillus* sp.

TABLE 1. Growth and end product formation in aerobic and anaerobic cultures of *L. viridescens* SMRICC 174, *Lactobacillus* sp. strain SMRICC 173, and *B. thermosphacta* ATCC 11509^T at different pH values[a]

Organism	Gas atmosphere	pH	Maximum specific growth rate (h^{-1})	Growth yield (g [dry wt] per mol of glucose consumed)	Product yield (mol of product per mol of glucose consumed)[b]								
					D-Lactic acid	L-Lactic acid	Ethanol	Acetic acid	Acetoin	2,3-Butanediol	Isovaleric acid	Isobutyric acid	Formic acid
L. viridescens	Aerobic	6.3[c]	0.46	23	0.88	0	0.58	0.24	0	0	0	0	0
	Aerobic	5.8[c]	0.50	22	0.96	0	0.60	0.24	0	0	0	0	0
	Aerobic	5.3[c]	0.35	20	1.01	0	0.70	0.15	0	0	0	0	0
	Anaerobic	6.3[c]	0.39	23	1.13	0	0.86	0	0	0	0	0	0
	Anaerobic	5.3	0.30	11	1.01	0	0.70	0	NA	NA	NA	NA	0
Lactobacillus sp. strain 173	Aerobic	6.3	Inhibited growth										
	Anaerobic	6.3	0.37	23	0.88	0.86	0	0	0	0	0	0	0
	Anaerobic	5.3	0.24	25	0.76	0.98	0	NA	NA	NA	NA	NA	NA
B. thermosphacta	Aerobic	6.3[c]	0.49	27	0	1.42	0.08	0.12	tr	0.04	tr	tr	0.08
	Aerobic	5.8	0.45	20	0	1.06	0.04	0.15	0.02	0.06	tr	tr	0.01
	Aerobic	5.3[c,d]	0.20	16	0	0.76	0	0.24	0.16	0.06	tr	tr	0
	Anaerobic	6.3[c]	0.17	22	0	1.52	0.12	0	0	0	tr	tr	0.08
	Anaerobic	5.3[c]	No growth										

[a] Cultures were performed at 25°C in a complex medium with 2% glucose.
[b] 0, Not detected; NA, not analyzed; tr, trace amounts below 0.01 mol of product per mol of glucose consumed.
[c] Mean values from two cultures are reported.
[d] Only 13.2 g of glucose per liter consumed.

TABLE 2. Growth and end product formation in aerobic and anaerobic cultures of *L. viridescens* SMRICC 174, *Lactobacillus* sp. strain SMRICC 173, and *B. thermosphacta* ATCC 11509[T] at different temperatures[a]

Organism	Gas atmosphere	Temp (°C)	Maximum specific growth rate (h^{-1})	Growth yield (g [dry wt] per mol of glucose consumed)	Product yield (mol of product per mol of glucose consumed)[b]								
					D-Lactic acid	L-Lactic acid	Ethanol	Acetic acid	Acetoin	2,3-Butanediol	Isovaleric acid	Isobutyric acid	Formic acid
L. viridescens	Aerobic	25[c]	0.46	23	0.88	0	0.58	0.24	0	0	0	0	0
	Aerobic	15[c]	0.20	22	0.96	0	0.53	0.22	0	0	0	0	0
	Aerobic	8	0.09	23	0.98	0	0.52	0.17	0	0	0	0	0
	Anaerobic	25	0.39	23	1.13	0	0.86	0	0	0	0	0	0
	Anaerobic	8	0.06	18	1.05	0	0.80	0	NA	NA	NA	NA	0
Lactobacillus sp. strain 173	Aerobic	25	Inhibited growth										
	Anaerobic	25	0.37	23	0.88	0.86	0	0	0	0	0	0	0
	Anaerobic	8	Inhibited growth										
B. thermosphacta	Aerobic	25[c]	0.49	27	0	1.42	0.08	0.12	tr	0.04	tr	tr	0.08
	Aerobic	15[c]	0.21	31	0	1.17	0.04	0.07	tr	0.09	tr	tr	0.06
	Aerobic	8[c]	0.08	38	0	0.04	0	0.32	0.39	0.20	0.02	tr	tr
	Anaerobic	25	0.17	22	0	1.52	0.12	0	0	0	tr	tr	0.08
	Anaerobic	8	0.05	20	NA	1.51	0.05	0	0	0	tr	0	tr

[a] Cultures were performed at pH 6.3 in a complex medium with 2% glucose.
[b] 0, Not detected; NA, not analyzed; tr, trace amounts below 0.01 mol of product per mol of glucose consumed.
[c] Mean values from two cultures are reported.

strain 173 was inhibited at 8°C at a low final dry weight.

In aerobic cultures, *B. thermosphacta* grew at μ_{max} values higher than those in the corresponding anaerobic cultures (Table 2). The Y_x varied between 27 and 38 g/mol in aerobic cultures, and an increase was found with decreasing temperature (Table 2). In anaerobic culture, the Y_x was about 21 g/mol and was unaffected by the temperature. In aerobic culture, the product yield of L-lactic acid decreased from 1.42 mol/mol at 25°C to 0.04 mol/mol at 8°C (Table 2). At 8°C in aerobic culture, acetoin dominated the end products together with acetic acid and 2,3-butanediol. In anaerobic culture, L-lactic acid dominated the end products even at 8°C, and no acetoin, acetic acid, or 2,3-butanediol was found.

DISCUSSION

The two *Lactobacillus* strains used in this study were isolated from spoiled smoked pork and spoiled pork. Homofermentative *Lactobacillus* spp. are frequently isolated from meat or meat products (2, 4, 9). *Lactobacillus* sp. strain 173 belonged to the *Lactobacillus* group 1 of Blickstad and Molin (4), representing 2 to 58% of the spoilage flora of cured meat products. *L. viridescens* is found on meat (2) and meat products (4) and is associated with the greening of meat products (12). *Lactobacillus* sp. strain 173 did not meet the description of any approved species of lactobacilli. Phenotypically, this strain can at best be placed somewhere between the type species of lactobacilli, *Lactobacillus delbrueckii* and *Lactobacillus mali*, from which it differs in the following characteristics: ribose, trehalose, and type of lactic acid (*L. delbrueckii*; 5); arginine, ribose, and rhamnose (*L. mali*; 7). Two features of the present strain of *L. viridescens* did not agree with the general description of *L. viridescens* (5), namely, fermentation of cellobiose and production of D-lactic acid instead of DL-lactic acid.

Lactobacillus sp. strain 173 is inhibited in air, due to the production of H_2O_2 (E. Blickstad and G. Molin, submitted for publication). *Lactobacillus* sp. strain 173 was inhibited at 8°C in anaerobic culture (5% CO_2) even though it was isolated from a meat product stored at 4°C in a vacuum package containing a high concentration of CO_2. This implies that neither the amount of CO_2 nor the temperature used should have been inhibitory. Thus, some unknown factor caused or contributed to the inhibition.

B. thermosphacta and *L. viridescens* consume O_2 at a high rate, leading to an O_2-restricted environment when growing at high μ_{max} values (Blickstad and Molin, submitted for publication). In the present study, the growth rates were evaluated during the beginning of the log phase and before the culture might have become O_2 restricted. Thus, the μ_{max} values reported are only affected by pH and temperature and not by O_2 availability.

B. thermosphacta and *L. viridescens* may be competitive in certain environments. Their growth rates were about the same at 8°C in both aerobic and anaerobic gas atmospheres. In an aerobic environment, high pH (6.3) favored the growth of *B. thermosphacta*, whereas *L. viridescens* grew better at a lower pH (5.8).

B. thermosphacta is unable to grow on beef under anaerobic conditions at pH values below 5.8 (6). It was concluded that this inability depends on inhibition by undissociated lactic acid (10). In the present study, *B. thermosphacta* was unable to grow anaerobically at pH 5.3 in broth. The concentration of undissociated lactic acid originating from the inoculum was below 0.05 mM. Thus, at such a low pH as 5.3 the growth of *B. thermosphacta* may be inhibited by the pH per se.

In the present study, it was demonstrated that the aerobic growth of *B. thermosphacta* was also affected by low pH. The growth rate decreased drastically when the pH was decreased from 5.8 to 5.3. Furthermore, not all of the glucose was consumed at pH 5.3. At the point of inhibition, 56 mM of L-lactic acid had been produced. Since *B. thermosphacta* consumes oxygen, the inhibition may have coincided with a depletion of oxygen; that is, the environment turned anaerobic. At a higher pH (5.6), *B. thermosphacta* is able to grow in air in the presence of 100 mM L-lactic acid (10).

A variety of factors such as pH, temperature, gas environment, and substrate availability affect bacterial growth on meat and meat products. The growth and metabolism of *B. thermosphacta* are highly dependent on these factors as shown by the present and by other studies (8; Blickstad and Molin, submitted for publication). It may change from a neutral spoilage organism producing mainly lactic acid to a potent spoiler producing acetoin, acetic acid, 2,3-butanediol, and fatty acids instead of lactic acid. The most critical factor seems to be the gas atmosphere. In anaerobic culture but not in aerobic culture, L-lactic acid dominated the end products even at 8°C. In air, less lactic acid and more acetoin, acetic acid, and 2,3-butanediol were produced as the pH and temperature decreased.

The importance of the gas atmosphere is confirmed by the study done by E. Blickstad and G. Molin (submitted for publication). With an unlimited O_2 supply, *B. thermosphacta* produces mainly acetoin, acetic acid, and isovaleric acid, whereas under anaerobic conditions, L-lactic acid totally dominates the end products. The

variable amounts of L-lactic acid found in the present study are probably caused by variable degrees of O_2 restriction. A very low lactic acid yield was obtained at 8°C, indicating that this culture was not oxygen limited. By comparing product yields from this culture with the ones obtained from a culture not limited by oxygen at 25°C (E. Blickstad and G. Molin, submitted for publication) it can be concluded that the temperature affected the end product formation. When temperature decreases, more 2,3-butanediol and less acetoin are produced.

High pH increases the yield of heterofermentative end products (other than lactate) of lactic acid bacteria (15). In the present study, *L. viridescens* formed the same type of end products irrespective of both pH and temperature but with various yields, and the heterofermentative end products constituted a fairly constant part of the total product yield (Tables 1 and 2).

It can be concluded that *L. viridescens*, *Lactobacillus* sp. strain 173, and *B. thermosphacta* responded in very different ways to the applied environments. *L. viridescens* had the strongest growth capability, whereas *Lactobacillus* sp. strain 173 was the most susceptible. Both *L. viridescens* and *B. thermosphacta* can be considered as inducible, potent spoilage organisms. *B. thermosphacta* may cause off odors and off flavors due to the production of acetoin, 2,3-butanediol, and isovaleric acid. *L. viridescens* may produce H_2O_2, which causes greening of products. The production of the above-mentioned compounds is stimulated by air (O_2). The significance of *Lactobacillus* sp. strain 173 in spoilage is less certain; perhaps this strain favors long shelf life of meat or meat products.

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