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Effect of pH on *Bacillus thermoamylovorans* Growth and Glucose Fermentation†

Y. COMBET-BLANC,[1,2]* K. K. KALAMBA,[2]‡ AND P. Y. KERGOAT[2]

Laboratoire de Microbiologie Institut Français de Recherche Scientifique pour le Développement en Coopération, Université de Provence, 13331 Marseille Cedex 3, France,[1] Laboratoire de Biotechnologie et d'Energétique, ENSUT, Dakar, Sénégal[2]

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The effect of pH on the growth and physiology of *Bacillus thermoamylovorans*, a new moderately thermophilic and non-spore-forming bacterium isolated from palm wine, was studied. Growth occurred from pH 5.4 to 8.5, with optimum growth at 7.0. During the exponential growth phase at optimum pH, glucose was consumed at the maximum rate (qs), 17.87 mmol g^{-1} h^{-1}, and was mainly fermented into acetate, ethanol, and formate (76.5% of metabolites produced). In acidic or alkaline conditions, glucose specific consumption rates were considerably reduced (qs = 8.06 mmol g^{-1} h^{-1} at pH 5.6 and 2.85 mmol g^{-1} h^{-1} at pH 8.4), and a switch in glucose metabolism toward lactate production (62.6% of metabolites produced at pH 5.6 and 41.2% of those produced at pH 8.4) was observed. Moreover, optimum cellular yield ($Y_{x/ATP}$), 14.8 g mol^{-1}, and optimum energy yield ($Y_{ATP/s}$), 2.65 mol mol^{-1}, were observed at neutrality. The results of this study were compared with published data about lactic acid bacteria; this comparison allowed us to complement our previous taxonomic study of *B. thermoamylovorans* and to identify additional phenotypic differences between *B. thermoamylovorans* and lactobacilli.

Previous studies of the microfloras of palm wine, a traditional tropical beverage, have shown that the pH decrease and accumulation of organic acids and ethanol occurring during fermentation greatly reduced the initial diversity of the microflora (23, 25, 27). At the end of the fermentation, microfloras were mostly composed of yeasts and bacteria belonging to the genera *Saccharomyces*, *Lactobacillus*, and *Streptococcus* (2, 18, 23, 25). Otherwise, it has been shown that pH is a key parameter governing the carbohydrate metabolism of lactobacilli and streptococci (9, 15, 19, 20). In these bacteria, a change in pH from acidic to alkaline resulted in a switch from homo- to heterolactic fermentation (9, 20).

We recently isolated from palm wine *Bacillus thermoamylovorans* sp. nov., a bacterium which exhibited phenotypic characteristics similar to those of lactobacilli (i.e., the spectrum of glucose fermentation products and the lack of spores) (5). This study deals with the effects of pH on the growth and end products of glucose fermentation by *B. thermoamylovorans* and compares them with data from the literature dealing with lactobacilli and streptococci.

MATERIALS AND METHODS

Organism. The experiments were conducted with *B. thermoamylovorans* (type strain, DKP; CNCM I-1378) (5). The strain was stored in liquid growth medium as described by Combet-Blanc et al. (5).

Culture methods and medium for fermentors. Batch cultures were performed with a 2-liter fermentor (Labo 2000; Interscience, St.-Nom-La-Bretèche, France) at 50°C, with stirring at 200 rpm. pH was controlled by the addition of 3 N sodium hydroxide with an automatic pH regulator (Interscience). Anaerobiosis was maintained by passing a stream of O_2-free N_2 through the headspace of the culture vessel. The fermentor, containing 1,000 ml of culture medium, was autoclaved for 45 min at 110°C. The medium contained the following (per liter): yeast extract (Difco Laboratories, Detroit, Mich.), 10 g; bio-Trypcase (Biomérieux, Craponne, France), 10 g; NH_4Cl, 3.06 g; KH_2PO_4, 3.15 g; $MgCl_2 \cdot 6H_2O$, 0.47 g; NaCl, 0.3 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $CaCl_2 \cdot 2H_2O$, 0.4 mg; trace element solution (1), 1 ml; and Tween 80, 1 g. Glucose, used as the sole carbon and energy source, was filter sterilized separately and added to a final concentration of 60 mM. The inoculum was grown overnight at 50°C in 90 ml of medium. Batch cultures were duplicated.

Culture methods and medium for Hungate tubes. Tubes for cultures were prepared according to Hungate's anaerobic techniques (11, 16). *B. thermoamylovorans* was cultured on a basal medium containing the following (per liter): yeast extract, 5 g; bio-Trypcase, 5 g; NH_4Cl, 3.06 g; KH_2PO_4, 6 g; $MgCl_2 \cdot 6H_2O$, 0.47 g; NaCl, 0.3 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $CaCl_2 \cdot 2H_2O$, 0.4 mg; trace element solution (1), 1 ml; and Tween 80, 1 g. The medium was adjusted to pH 7.5 with 10 M KOH and boiled and cooled under a stream of O_2-free N_2 at room temperature. It was then distributed under a stream of O_2-free N_2 into Hungate tubes (10 ml of medium). After being autoclaved at 110°C for 45 min, glucose was injected into the Hungate tubes from filter-sterilized solution to a final concentration of 30 mM. Hungate tubes were inoculated with an overnight culture and incubated for 72 h at 50°C. The CO_2 produced was measured directly from the gaseous phase, after the liquid phase had been acidified to pH 2 with 2 N H_2SO_4. The test was carried out in triplicate.

Growth. Growth was monitored by turbidity measurements (660 nm) at 30-min intervals during the exponential phase of growth by a spectrophotometer (Shi-

TABLE 1. Fermentation balance of glucose by *B. thermoamylovorans* at nonregulated pH[a]

Substrate; glucose consumed (mM)	21.4
Products (mM)	
Lactate	22.20
Acetate	8.10
Ethanol	9.90
Formate	12.80
CO_2	5.10
Final pH	5.40
C recovery (%)	93.85
O/R ratio	1.03
(Acetate + ethanol)/(formate + CO_2) ratio	1.01

[a] The fermentation was performed in 10 ml of medium containing 30 mM glucose (final concentration) with a starting pH of 7.5 and under an atmosphere of N_2. The Hungate tubes were incubated for 72 h at 50°C. The results are the averages of triplicate tests.

* Corresponding author. Mailing address: Laboratoire de Microbiologie ORSTOM, Université de Provence, Case 87, 3 Place Victor-Hugo, 13331 Marseille Cedex 03, France. Phone: 33-91 10 64 78. Fax: 33-91 10 64 81.

† This paper is dedicated to the memory of K. K. Kalamba.

‡ Deceased.

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TABLE 2. Effect of pH on the yield of end products of glucose fermentation by *B. thermoamylovorans*

pH	Parameter[a]							
	mol mol of glucose^{-1}					$Y_{x/s}$ (g of cell [dry wt] mol of glucose^{-1})	C recovery (%)	O/R balance
	$Y_{lac/s}$	$Y_{acet/s}$	$Y_{ethan/s}$	$Y_{form/s}$	$Y_{CO_2/s}$			
5.60	1.27	0.19	0.57	0.26	0.50	20.16	101.75	1.02
5.90	0.85	0.46	0.66	0.84	0.29	26.92	98.92	1.01
6.00	0.74	0.50	0.64	0.89	0.26	30.57	94.28	1.02
6.20	0.65	0.56	0.66	1.04	0.17	33.70	93.50	1.01
6.50	0.50	0.70	0.71	1.34	0.07	37.50	95.38	1.01
7.00	0.45	0.73	0.73	1.47	0.00	39.17	95.83	1.00
7.50	0.50	0.71	0.70	1.40	0.01	35.83	95.18	1.00
7.75	0.54	0.67	0.69	1.31	0.04	33.33	94.79	1.00
8.00	0.58	0.60	0.67	1.14	0.13	29.80	92.40	1.01
8.40	0.79	0.42	0.71	0.51	0.62	18.75	95.83	1.06

[a] Parameters were calculated during the exponential growth phase.

madzu UV 160A; Shimadzu Co., Kyoto, Japan) calibrated in grams of cell (dry weight) per liter. To determine the cell dry weight, cells were harvested by centrifugation at 10,000 × *g* for 10 min and washed three times with a solution of NaCl at 0.9%. Washed cells were dried to constant weight at 105°C.

Analyses. Lactic, formic, and acetic acid levels and ethanol and glucose levels were determined by high-performance liquid chromatography with an Analprep 93 pump (Touzart et Matignon, Vitry sur Seine, France), an ORH 801 type column (Interaction Chemicals, Inc., Mountain View, Calif.), and a differential refractometer detector (Shimadzu RID 6 A; Shimadzu Co., Kyoto, Japan). Samples (20 μl) were injected in the column maintained at 35°C. A 25 mM H_2SO_4 solution was used as a solvent with a flow rate of 0.7 ml min^{-1}. The carbon dioxide level was determined with a Girdel series 30 gas chromatograph equipped with a thermal conductivity detector and a stainless steel column (1 m by 3.2 mm) packed with Carbosphere SS (60 to 80 mesh) (column temperature, 150°C; carrier gas, He [10^5 Pa]; injection and detector temperature, 210°C; power of the filament, 90 mA). L-(+)-Lactic and D-(−)-lactic dehydrogenases (Boehringer Mannheim, Mannheim, Germany) were used to assess the stereoisomerism of the lactic acid produced by glucose fermentation.

Fermentation parameters. Fermentation parameters were calculated during the exponential growth phase only. The yields of lactate ($Y_{lac/s}$), acetate ($Y_{acet/s}$), ethanol ($Y_{ethan/s}$), and formate ($Y_{form/s}$), and energy yield on glucose ($Y_{ATP/s}$) were expressed in moles of product per mole of glucose catabolized. The maximum growth rate (μ_{max}), expressed per hour, was estimated by the slope of the linear regression between the Napierian logarithm (natural log) of optical density and growth time. Cellular yield on glucose ($Y_{x/s}$) and cellular yield on ATP ($Y_{x/ATP}$) were expressed in grams of cell (dry weight) per mole. Specific glucose consumption rate (qs), specific consumption rate of glucose fermented into lactate (qs-L), and specific consumption rate of glucose fermented into acetate, ethanol, formate, and CO_2 (qs-AEFC) were expressed in millimoles of glucose per gram of cell (dry weight) per hour and were calculated according to the formulae: $qs = \mu_{max} \times (Y_{x/s})^{-1}$, $qs\text{-}L = 0.5 \times (Y_{lac/s}) \times \mu_{max} \times (Y_{x/s})^{-1}$, and $qs\text{-}AEFC = [0.333 \times (Y_{acet/s} + Y_{ethan/s}) + 0.167 \times (Y_{form/s} + Y_{CO_2/s})] \times \mu_{max} \times (Y_{x/s})^{-1}$ where 0.5, 0.333, and 0.167 are the quantities (moles) of glucose needed for the production of 1 mol of lactate, acetate, or ethanol, formate, and CO_2, respectively.

RESULTS AND DISCUSSION

Estimate of CO_2 produced in batch cultures. During batch cultures in the fermentor, the continuous flow of O_2-free N_2 maintaining the anaerobiosis prevented analysis of the CO_2 produced. In order to measure the amount of CO_2 produced, we used cultures in Hungate tubes at uncontrolled pH. Under these conditions, almost 100% of the carbon from fermented glucose was recovered in the following five metabolites: lactate [96% of L-(+)-isomer], acetate, ethanol, formate, and CO_2 (Table 1). In addition, the ratio between oxidized and reduced carbon products (O/R) was close to 1.0 (Table 1). This suggested that no other metabolite was produced in significant concentrations. Moreover, the ratio of (acetate plus ethanol) to (formate plus CO_2), which was close to 1, showed that CO_2 = acetate + ethanol − formate (products expressed in moles). Consequently, for batch cultures conducted in the fermentor, yields of CO_2 were calculated according to the formula: $Y_{CO_2/s} = Y_{acet/s} + Y_{ethan/s} - Y_{form/s}$.

Effect of pH on *B. thermoamylovorans* growth. The μ_{max} (see Table 3) and $Y_{x/s}$ (Table 2) showed an optimum pH for growth of 7.0. No growth occurred at a pH lower than 5.4 or higher than 8.5 (5). In contrast, the growth of lactobacilli is best in slightly acidic conditions (pH 5 or less), optimal at pH 5.5 to 6.2, and often reduced at neutral or slightly alkaline pH (12, 24).

Effect of pH on end products of glucose fermentation. In experiments conducted in the fermentor, almost all of the carbon of fermented glucose was recovered as lactate, acetate, ethanol, formate, and CO_2, with O/Rs close to 1.0 (Table 2). This indicated that the CO_2 level was correctly estimated and that no other metabolite was produced in these fermentations.

Effect at neutral pH. At neutral pH, glucose was consumed at a qs of 17.87 mmol g^{-1} h^{-1} (pH 7.0) (Table 3) and was mostly fermented into acetate, ethanol, and formate (Table 2). At the end of the growth phase, acetate, ethanol, and formate, for whom the ratio was close to 1:1:2, represented 76.5% of metabolites produced, while lactate represented only 23.5% (Fig. 1a). This balance of fermentation products differed markedly from that of lactobacilli and streptococci. When these bacteria were growing under optimum pH and nutriment supply conditions, glucose was mainly fermented into lactate (12,

TABLE 3. Effect of pH on fermentation parameters during the exponential growth phase of *B. thermoamylovorans*[a]

pH	Parameter					
	μ_{max} (h^{-1})	mmol g of cell^{-1} (dry wt) h^{-1}			$Y_{ATP/s}$ (mol mol of glucose^{-1})	$Y_{x/ATP}$ (g of cell [dry wt] mol^{-1})
		qs	qs-L	qs-AEFC		
5.60	0.16	8.06	5.13	3.07	2.23	9.06
5.90	0.38	14.21	6.04	8.02	2.44	11.02
6.00	0.48	15.57	5.74	8.93	2.39	12.80
6.20	0.56	16.63	5.42	10.13	2.43	13.89
6.50	0.64	17.18	4.25	12.13	2.61	14.39
7.00	0.70	17.87	4.02	13.11	2.65	14.78
7.50	0.62	17.43	4.33	12.25	2.61	13.73
7.75	0.51	15.23	4.12	10.31	2.56	13.01
8.00	0.36	11.99	3.48	7.60	2.45	12.17
8.40	0.05	2.85	1.13	1.60	2.33	8.04

[a] Batch cultures were conducted at 50°C in anaerobiosis with complex medium. Parameters were calculated during the exponential growth phase.

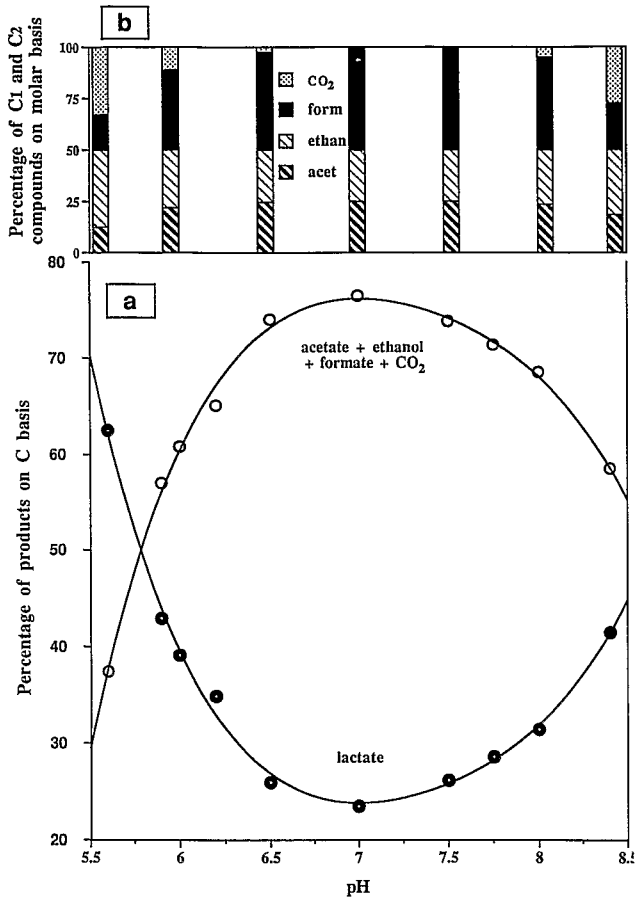

FIG. 1. Effect of pH on the repartition of carbon end products of glucose fermentation. (a) Lactate (●) and acetate plus ethanol plus formate plus CO_2 (○) expressed as a percentage of the total C recovered as product. (b) Relative abundance of C_2 (acetate [acet], ethanol [ethan]) and C_1 (formate [form], CO_2) compounds on a molar basis.

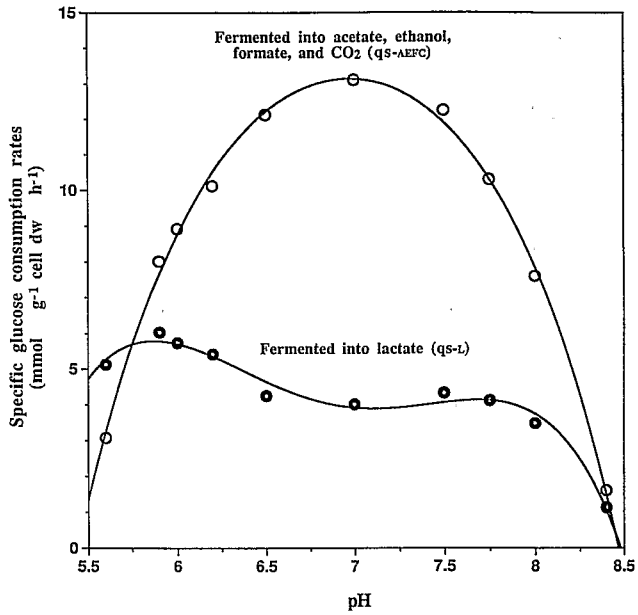

FIG. 2. Effect of pH on the specific glucose consumption rates during fermentation into lactate (qs-L) (○) and acetate, ethanol, formate, and CO_2 (qs-AEFC) (○). dw, dry weight.

24). However, several studies have shown that for the following lactic acid bacteria grown in a chemostat at low growth rates under glucose-limited conditions, acetate, ethanol, and formate were the main products of glucose fermentation (12): *Lactobacillus* sp. (3); *Streptococcus mutans* JC2, *Streptococcus sanguis*, *Streptococcus salivarius*, and *Streptococcus bovis* (4); *Lactobacillus casei* (6); *Streptococcus lactis* (7, 26); and *Lactobacillus delbrueckii* (17).

Effects under acidic and alkaline conditions. Under acidic and alkaline conditions, glucose specific consumption rates were considerably reduced (qs = 8.06 mM g^{-1} h^{-1} at pH 5.6, 17.87 mM g^{-1} h^{-1} at pH 7.0, and 2.85 mM g^{-1} h^{-1} at pH 8.4) (Table 3) and concentrations of lactate increased significantly compared with those of other products (Table 2). At the end of the exponential growth phase, lactate represented 62.6% of the fermentation products at pH 5.6, 23.5% at pH 7, and 41.2% at pH 8.4 (Fig. 1a). Moreover, (i) CO_2 was produced, although it was not formed at pH 7.0 ($Y_{CO_2/s}$ = 0.50 at pH 5.6, 0.0 at pH 7.0, and 0.62 at pH 8.4) (Table 2); (ii) the ratio of $Y_{form/s}$ to ($Y_{acet/s}$ + $Y_{ethan/s}$) decreased (0.34:1 at pH 5.6, 1:1 at pH 7.0, and 0.45:1 at pH 8.4) (Table 2 and Fig. 1b); and (iii) the ratio of $Y_{ethan/s}$ to $Y_{acet/s}$ increased (3:1 at pH 5.6, 1:1 at pH 7.0, and 1.69:1 at pH 8.4) (Table 2 and Fig. 1b).

These results showed that CO_2 production, which occurred to the detriment of formate production, was concomitant with an increase in the ratio of ethanol to acetate. Consequently, at any pH, fermentation was electronically balanced (O/R was close to 1) (Table 2) because formate and ethanol are reduced more than CO_2 and acetate. The flux of glucose that fermented into lactate (qs-L) on one hand and into acetate, ethanol, formate, and CO_2 (qs-AEFC) on the other showed that (i) the activity of the enzymes involved in the lactate metabolic pathway was optimum under acidic conditions (qs-L = 5.5 mmol g^{-1} h^{-1} at pH 5.6 to 6.2, 4.2 mmol g^{-1} h^{-1} at pH 6.5 to 7.75, and 1.13 mmol g^{-1} h^{-1} at pH 8.4), and (ii) the activity of the enzymes involved in the metabolic pathways other than that of lactate was optimum at neutral pH (qs-AEFC = 3.07 mmol g^{-1} h^{-1} at pH 5.6, 13.1 mmol g^{-1} h^{-1} at pH 7.0, and 1.6 mmol g^{-1} h^{-1} at pH 8.4) (Fig. 2 and Table 3). The formate pathway was most sensitive to pH variations (Table 2).

The switch in glucose metabolism to lactate production by *B. thermoamylovorans* that was observed when the culture medium became acidic was also found with streptococci and lactobacilli (*S. liquefaciens* [9]; *L. delbrueckii* [15]; *S. liquefaciens*, *S. faecalis*, *S. durans*, *S. thermophilus*, *S. lactis*, and *S. cremoris* [19]; and *L. bulgaricus* [20]). On the contrary, alkaline conditions decreased lactate production by streptococci and lactobacilli, whereas alkalinity increased that by *B. thermoamylovorans*. Rhee and Pack (20) concluded that the high level of lactate dehydrogenase synthesis under acidic conditions, together with the alkaline preference of the enzymes of the pyruvate split (pyruvate formate lyase [14, 28], aldehyde dehydrogenase [21], acetate kinase [22], and phosphotransacetylase [10]) might explain the shift in the fermentation pattern of *L. bulgaricus* from homofermentative to heterofermentative when the pH changes from acidic to alkaline. This explanation is not suitable for *B. thermoamylovorans* in alkaline conditions. Additional studies are needed to characterize the metabolism of glucose in *B. thermoamylovorans* and its differences from that of lactobacilli. The focus of such studies should be on (i) the identification of the enzymes involved in glucose metabolism and (ii) the synthesis control and properties of enzymes (K_m, V_{max}, pH range, activators, and inhibitors).

Effect of pH on *B. thermoamylovorans* energy and cell yields ($Y_{ATP/s}$ and $Y_{x/ATP}$). The energy yields of glucose breakdown ($Y_{ATP/s}$) were calculated from end product fermentation by considering that 1 mol of ATP was generated for each mol of lactate or ethanol produced and that 2 mol of ATP was generated per mol of acetate produced (Table 3). $Y_{x/ATP}$ and $Y_{ATP/s}$ values obtained at different pHs with *B. thermoamylovorans* showed two phenotypic differences from lactobacilli and streptococci. First, the optimum $Y_{x/ATP}$ for *B. thermoamylovorans* (14.78 g mol^{-1}) (Table 3) was significantly lower than those for lactobacilli and streptococci. Estimates of the $Y_{x/ATP}$ values for lactic acid bacteria, for example, those of *Lactobacillus plantarum* (8, 13), *L. bulgaricus* (20), *L. casei* (6), and *S. lactis* (26), were between 21 and 28 g mol^{-1} under optimum conditions. Second, for *B. thermoamylovorans*, $Y_{x/ATP}$ and $Y_{ATP/s}$ were both optimum at neutral pH (Table 3), whereas for lactobacilli and streptococci, the optimum pHs for $Y_{x/ATP}$ and $Y_{ATP/s}$ were different. For instance, for *L. bulgaricus*, $Y_{x/ATP}$ was optimum at pH 6.5 to 7.0 whereas $Y_{ATP/s}$ was optimum at pH 8.0 (20).

In conclusion, this study has shown important physiological differences between *B. thermoamylovorans* and lactobacilli. These significant phenotypic differences are consistent with the results of the taxonomic study of *B. thermoamylovorans*, which has been classified in the genus *Bacillus* according to 16S rRNA analyses despite the lack of spores (5).

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