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Effect of Organic Complex Compounds on *Bacillus thermoamylovorans* Growth and Glucose Fermentation

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The effect of the concentration of a mixture (1/1 [wt/wt]) of yeast extract and bioTrypcase (YE+bT) on the growth and physiology of a new species, *Bacillus thermoamylovorans*, a moderately thermophilic, non-spore-forming, lactic acid-producing bacterium isolated from palm wine, was studied. At an initial glucose concentration of 100 mM, *B. thermoamylovorans* growth was limited when the concentration of YE+bT was lower than 5.0 g liter^{-1}; under these conditions, cellular yield reached a maximum value of 0.4 g of cells per g of YE+bT. Growth limitation due to deficiency in growth factors led to a significant shift in glucose metabolism towards lactate production. Lactate constituted 27.5 and 76% of the end products of glucose fermentation in media containing YE+bT at 20.0 and 1.0 g liter^{-1}, respectively. This result markedly differed from published data for lactic bacteria, which indicated that fermentative metabolism remained homolactic regardless of the concentration of YE. Our results showed that the ratio between cellular synthesis and energy production increased with the concentration of YE+bT in the culture medium. They indicate that the industrial production of lactic acid through glucose fermentation by *B. thermoamylovorans* can be optimized by using a medium where glucose is present in excess and the organic additives are limiting.

We isolated from palm wine *Bacillus thermoamylovorans* sp. nov., a nonsporulating bacterium which produces lactate, acetate, ethanol, and formate by glucose fermentation. Its phenotypic traits resemble those of lactobacilli (6). In lactic bacteria—lactobacilli and lactococci—specific modifications of culture conditions resulted in a change in the fermentation balance (17, 18, 23). A switch from homo- to heterolactic fermentation was observed upon a change from acidic to alkaline pH (12, 15, 19, 21, 22) or from excessive glucose to glucose-limiting culture conditions (3, 4, 8–10, 20, 26, 28). In contrast, the effects of nutrients such as peptides, amino acids, and vitamins on growth and fermentation balance have been poorly studied. However, several authors have shown that the kinetics of milk acidification by a number of lactic strains was enhanced by the addition of various nutrients, e.g., corn steep (16), yeast extract (YE) (24), cell extracts of lactobacilli (13), and amino acids (14).

In a previous paper, we reported the effect of pH on end products of glucose fermentation and the growth kinetics of *B. thermoamylovorans*. Our results showed that, as with lactobacilli and lactococci, a pH change from neutral to acidic resulted in a switch in glucose metabolism towards lactate production (lactate constituted 62.6 and 23.5% of the products of fermentation at pH 5.6 and 7.0, respectively) (7).

Considering the high biotechnological potential of *B. thermoamylovorans* for lactic acid production (5), we studied the effects of different concentrations of YE and peptides on its growth and the end-product spectrum of glucose fermentation.

MATERIALS AND METHODS

Organism. *B. thermoamylovorans* (type strain DKP [CNCM I-1378]) was revived from cultures stored at −80°C and grown as described by Combet-Blanc et al. (6).

Culture methods and medium. Batch cultures (run in duplicate) were performed in a 2-liter fermentor (Labo 2000 Interscience, St.-Nom-La-Bretèche, France) at 50°C, with stirring at 200 rpm. pH was maintained at 7.0 by using an automatic pH regulator (Interscience) and 3 N sodium hydroxide. Anaerobiotic conditions were maintained by passing a stream of O_2-free N_2 over the headspace of the culture vessel. The fermentor, containing 1,000 ml of culture medium, was autoclaved for 45 min at 110°C. The basic medium contained the following (per liter): 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 an energy source, was filter sterilized separately and added to a final concentration of 100 mM. The inoculum was grown overnight at 50°C in 90 ml of basic medium containing (per liter) 2.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2.0 g of bioTrypcase (bT) (bioMérieux, Craponne, France), and 10.0 g of glucose. Batch cultures were run in duplicate.

Cellular concentration. Growth was monitored by turbidity measurements (660 nm) at 30-min intervals during the fermentation in a spectrophotometer (Shimadzu UV 160A; Shimadzu Co., Kyoto, Japan) calibrated in grams of cells (dry weight) per liter. To determine the cell dry weight, cells were harvested by centrifugation at 10,000 × *g* for 10 min, washed three times with a solution of NaCl at 0.9%, and dried to constant weight at 105°C.

Analyses. Lactic, formic, and acetic acids, ethanol, and glucose were quantified by high-performance liquid chromatography, using 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.). Samples (20 μl) were injected into the column, which was maintained at 35°C. A 25 mM H_2SO_4 solution was used as the eluant, at a flow rate of 0.7 ml min^{-1}.

Fermentation parameters. Fermentation parameters were calculated at the end of the fermentation, when glucose had been fully consumed. The yields of lactate ($Y_{lac/s}$), acetate ($Y_{ace/s}$), ethanol ($Y_{ethan/s}$), and formate ($Y_{form/s}$) and the energy yield derived from glucose ($Y_{ATP/s}$) were expressed in moles of product per mole of glucose catabolized. Cellular yields derived from glucose ($Y_{x/s}$) and ATP ($Y_{x/ATP}$) were expressed in grams of cells (dry weight) per mole. Cellular yield derived from YE+bT ($R_{x/gf}$) was expressed in grams of cells (dry weight) per gram of mixture (1/1 [wt/wt]) of YE and bT. The average hourly growth rate ($\bar{\mu}$) was calculated according to the following equation: $\bar{\mu} = \ln (OD_{final} \times OD_{initial}^{-1}) \times (t_{ferment}^{-1})$, where OD_{final} and $OD_{initial}$ are the optical densities at 660 nm measured at the end and the beginning of the fermentation, respectively, and $t_{ferment}$ is the time needed to completely ferment the glucose (100 mM).

The specific consumption rates of glucose (q_S), glucose fermented into lactate ($q_{S\text{-}L}$), and glucose fermented into acetate, ethanol, and formate ($q_{S\text{-}AEF}$) were expressed in millimoles of glucose per gram of cells (dry weight) per hour and were calculated according to the following equations: $q_S = \bar{\mu} \times (Y_{x/s})^{-1}$, $q_{S\text{-}L} = 0.5 \times (Y_{lac/s}) \times \bar{\mu} \times (Y_{x/s})^{-1}$, and $q_{S\text{-}AEF} = [0.333 \times (Y_{acet/s} + Y_{ethan/s}) + 0.167 \times (Y_{form/s})] \times \bar{\mu} \times (Y_{x/s})^{-1}$, where 0.5, 0.333, and 0.167 are the quantities (moles)

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TABLE 1. Effect of the concentration of YE+bT on the yields of end products of glucose fermentation by *B. thermoamylovorans*[a]

Concn of YE+bT (g liter^{-1})	Parameter[b]							
	Yield (mol mol of glucose^{-1})				$Y_{x/s}$ (g of cells [dry wt] mol of glucose^{-1})	$R_{x/gf}$ (g of cells [dry wt] g of YE+bT^{-1})	C (%)[c]	O/R balance[d]
	$Y_{lac/s}$	$Y_{acet/s}$	$Y_{ethan/s}$	$Y_{form/s}$				
1.00	1.52	0.24	0.23	0.49	5.08	0.41	81.39	1.00
1.33	1.33	0.34	0.34	0.66	6.31	0.39	86.28	1.00
2.00	1.27	0.36	0.37	0.72	7.72	0.39	99.87	1.00
3.50	1.15	0.42	0.44	0.85	13.08	0.39	102.26	1.00
5.00	1.08	0.47	0.44	0.94	16.77	0.32	94.72	1.01
10.00	0.69	0.65	0.66	1.32	27.35	0.26	94.42	1.00
20.00	0.55	0.71	0.72	1.47	33.18	0.17	101.94	1.01

[a] Batch cultures were conducted at 50°C, under anaerobiotic conditions and with pH regulated at 7.0. Data reported are the averages of data obtained from batch cultures run in duplicate.
[b] Parametric values were calculated at the end of the fermentation, when glucose (100 mM) had been fully consumed.
[c] Percent carbon recovery from fermented glucose.
[d] Ratio of oxidized to reduced carbon products.

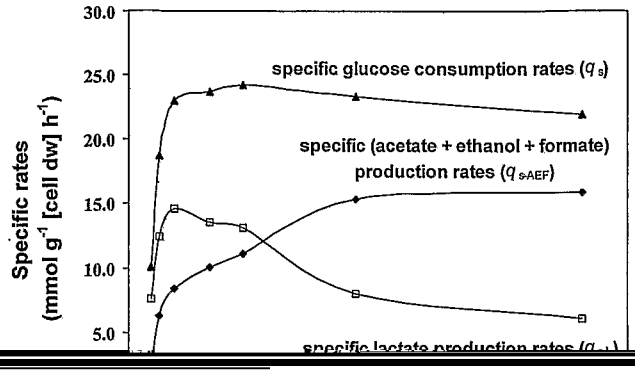
of glucose needed for the production of one mole of lactate, acetate or ethanol, and formate, respectively.

RESULTS

Effect of the concentration of YE+bT on *B. thermoamylovorans* growth. In batch cultures, conducted with an initial glucose concentration of 100 mM, $R_{x/gf}$ decreased from 0.32 to

Effect of the concentration of YE+bT on end products of glucose fermentation. In experiments conducted in the fermentor, almost all carbon from fermented glucose was recovered as lactate, acetate, ethanol, and formate. In addition, the ratio between oxidized and reduced carbon products (O/R balance) was close to 1.0 (Table 1). This suggested that no other metabolite was produced in significant concentration.

With an initial glucose concentration of 100 mM, the de-



such as vitamins and nitrogenous compounds does not modify their fermentative metabolism, which remains homolactic. In contrast, with *B. thermoamylovorans*, a limitation in growth factor(s) resulted in a 3.5-fold increase in lactate yield (Table 1). With lactic bacteria, only the limitation of the growth by glucose or acidic culture conditions is known to significantly shift the product(s) of fermentation from lactate to other end products (3, 4, 8–12, 15, 20, 22, 25, 26, 28).

Furthermore, our results indicated that the coupling between cellular synthesis and energy production ($Y_{x/ATP}$) increased with the concentration of growth factors in the medium relative to that of glucose (Table 2). Such a phenomenon was also observed with *Zymomonas mobilis* by Bélaich et al.

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