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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 0.17 g g^{-1} when the concentration of YE+bT was increased from 5.0 to 20.0 g liter^{-1} (Table 1). At concentrations lower than 5.0 g liter^{-1}, $R_{x/gf}$ was maximum and constant, reaching 0.39 to 0.41 g g^{-1}. These results indicated that, under the experimental conditions used, some unknown growth factor(s) present in YE or bT limited the growth of *B. thermoamylovorans* at YE+bT concentrations lower than 5.0 g liter^{-1}.

When growth was not limited by growth factor(s), i.e., at YE+bT concentrations higher than 5.0 g liter^{-1}, a classical growth response consisting of an exponential phase followed by a stationary phase was observed. Under conditions where growth factors were limiting, growth rates were drastically reduced (Table 2). For the culture conducted with YE+bT at 1 g liter^{-1}, the exponential growth phase rapidly became linear (Fig. 1). Similar effects on *Lactobacillus delbrueckii* growth due to limitation of some unknown nutrients contained in YE were observed by Tsao and Hanson (27), who suggested that this phenomenon was complex and probably implied the existence of several growth factors.

TABLE 2. Effect of the concentration of YE+bT on parameters for fermentation by *B. thermoamylovorans*

Concn of YE+bT (g liter^{-1})	Parameter[a]					
	$\bar{\mu}$ (h^{-1})	Sp consumption rate (mmol g of cell^{-1} [dry wt] h^{-1})			$Y_{ATP/s}$ (mol mol of glucose^{-1})	$Y_{x/ATP}$ (g of cells [dry wt] mol^{-1})
		q_S	$q_{S\text{-}L}$	$q_{S\text{-}AEF}$		
1.00	0.05	10.05	7.63	2.42	2.24	2.27
1.33	0.12	18.72	12.42	6.30	2.34	2.69
2.00	0.18	23.00	14.64	8.36	2.36	3.27
3.50	0.31	23.67	13.57	10.10	2.43	5.39
5.00	0.41	24.21	13.11	11.10	2.45	6.84
10.00	0.64	23.37	8.02	15.35	2.65	10.33
20.00	0.73	21.98	6.08	15.90	2.70	12.28

[a] Data reported are the averages of data obtained from batch cultures run in duplicate.

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 decrease of the YE+bT concentration led to a significant shift in glucose metabolism towards lactate production (Table 1). At the end of glucose fermentation, lactate constituted 27.5 and 76% of the yield in media containing 20.0 and 1.0 g of YE+bT per liter, respectively. On the other hand, kinetic parameters of the fermentations showed that q_S was optimal at YE+bT concentrations higher than 2.0 g liter^{-1} and was drastically reduced at lower concentrations (Table 2 and Fig. 2). The dynamics of the fluxes of $q_{S\text{-}L}$ and $q_{S\text{-}AEF}$ showed that $q_{S\text{-}L}$ occurred optimally at YE+bT concentrations ranging between 2.0 and 5.0 g liter^{-1} and that $q_{S\text{-}AEF}$ increased with YE+bT concentration, reaching an optimum at concentrations higher than 10 g liter^{-1} (Table 2 and Fig. 2). These results indicated that the activities of these metabolic pathways depended

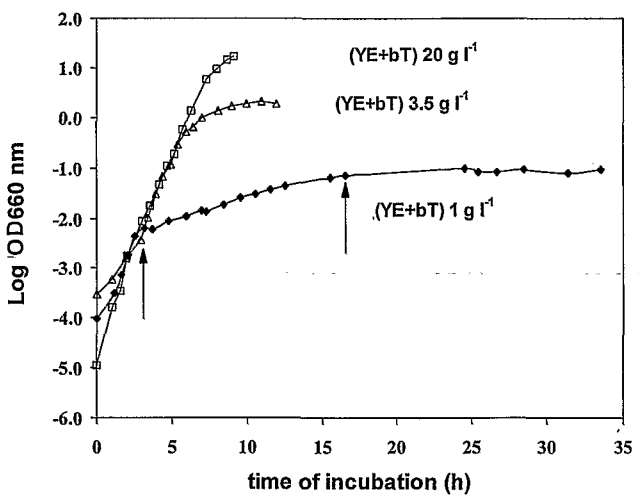

FIG. 1. Effect of the concentration of YE+bT on *B. thermoamylovorans* growth kinetics. Arrows indicate the boundaries of the linear part of the growth phase. Data reported are the averages of data obtained from batch cultures run in duplicate.

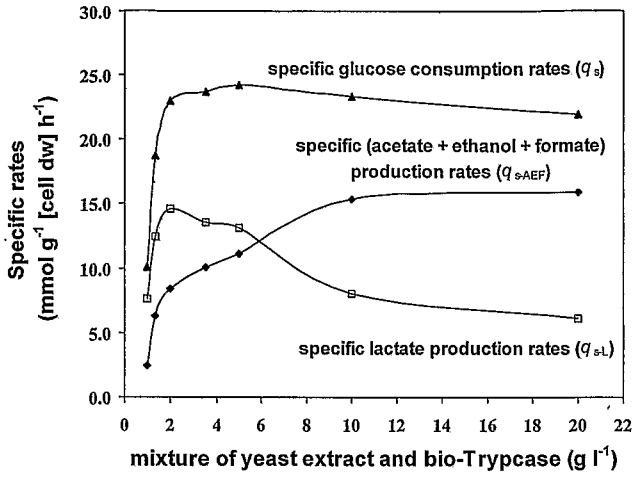

FIG. 2. Effects of the concentration of YE+bT on q_S, $q_{S\text{-}L}$, and $q_{S\text{-}AEF}$. Data reported are the averages of data obtained from batch cultures run in duplicate.

closely on YE+bT concentration. When growth factor(s) was limiting, the lactate-producing pathway was stimulated; when growth factor(s) was not limiting, the lactate-producing pathway had reduced activity and the enzymes involved in other pathways were stimulated.

Effect of the concentration of YE+bT on *B. thermoamylovorans* energy and cellular yields. The value of $Y_{ATP/s}$ was calculated from end-product fermentation by considering that one mole of ATP was generated for each mole of lactate or ethanol produced and that two moles of ATP were generated per mole of acetate produced. When the concentration of YE+bT was increased from 1.0 to 20.0 g liter^{-1}, a switch from homolactic to heterolactic fermentation was observed. This metabolic shift to heterolactic fermentation resulted in an increase of the $Y_{ATP/s}$, from 2.24 mol of ATP per mol of glucose fermented (in a medium limited by growth factor[s]) to 2.70 mol mol^{-1} (in a rich medium) (Table 2).

DISCUSSION

The effect of the concentration of YE+bT on the growth and the metabolic profile of a new species, *B. thermoamylovorans*, was studied in batch cultures. At an initial glucose concentration of 100 mM, $R_{x/gf}$ and the growth kinetics showed that some nutriments present in YE and bT limited the growth of *B. thermoamylovorans* at YE+bT concentrations lower than 5 g liter^{-1} (Table 1 and Fig. 1).

When glucose was present in excess, the growth limitation of the culture by YE+bT led to a marked shift in glucose metabolism from heterolactic to homolactic fermentation. This metabolic shift resulted in a decrease of the $Y_{ATP/s}$ from 2.70 mol of ATP per mol of glucose fermented in a rich medium to 2.24 mol of ATP per mol of glucose in a medium limited by the growth factor(s) present in YE+bT. The comparison of *B. thermoamylovorans* and lactic bacteria, whose products of fermentations are qualitatively the same, showed that fermentation patterns in batch culture differed. With lactic bacteria, grown in a rich medium where growth factors and glucose were not limiting, lactate was the main end product of glucose fermentation (17). In contrast, under the same culture conditions, *B. thermoamylovorans* fermented glucose mainly into acetate, ethanol, and formate. It is generally acknowledged that the limitation of lactic bacterial growth by a deficiency in factors 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. (2): increasing pantothenate concentration from 0.05 to 5,000 μg liter^{-1} in a synthetic medium resulted in an increase of $Y_{x/ATP}$ from 2.5 to 6.5 g of cells mol of glucose^{-1}.

Our results showed that the concentration of some organic compound(s) present in YE and/or bT relative to that of glucose is a key factor to optimize lactic acid production. The industrial production of lactic acid through glucose fermentation by *B. thermoamylovorans* will probably use organic additives other than yeast or bT, because of their high cost. Whatever this source (for example, corn steep), the ratio of glucose to organic compounds will have to be high enough to provide a medium where glucose is present in excess and the organic additives are limiting, in order to optimize lactic acid production.

In conclusion, these results supplement our earlier work on the effect of pH on the growth of *B. thermoamylovorans* during glucose fermentation (7). Physiological differences observed between *B. thermoamylovorans* and lactic bacteria reported here and in the earlier report are consistent with the results of the taxonomic study of *B. thermoamylovorans*, which was classified as a member of the genus *Bacillus* based on the results of 16S rRNA analyses (6). Physiological studies currently being conducted on *B. thermoamylovorans* have already shown close analogies with, but also significant differences from, the fermentative metabolism of lactic bacteria. Explaining these differences will require the characterization of the key enzymes thought to be involved in the glucose fermentation pathways of *B. thermoamylovorans* and the study of their regulation.

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