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Glucose Fermentation by *Propionibacterium microaerophilum*: Effect of pH on Metabolism and Bioenergetic

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Abstract. pH affected significantly the growth and the glucose fermentation pattern of *Propionibacterium microaerophilum.* In neutral conditions (pH 6.5–7.5), growth and glucose fermentation rate (qs) were optimum producing propionate, acetate, CO_2, and formate [which together represented 90% (wt/wt) of the end products], and lactate representing only 10% (wt/wt) of the end products. In acidic conditions, propionate, acetate, and CO_2 represented nearly 100% (wt/wt) of the fermentation end products, whereas in alkaline conditions, a shift of glucose catabolism toward formate and lactate was observed, lactate representing 50% (wt/wt) of the fermentation end products. The energy cellular yields ($Y_{X/ATP}$), calculated (i) by taking into account extra ATP synthesized through the reduction of fumarate into succinate, was 6.1–7.2 g mol^{-1}. When this extra ATP was omitted, it was 11.9–13.1 g mol^{-1}. The comparison of these values with those of $Y_{X/ATP}$ in *P. acidipropionici* and other anaerobic bacteria suggested that *P. microaerophilum* could not synthesize ATP through the reduction of fumarate into succinate and therefore differed metabolically from *P. acidipropionici.*

During the last decades, a number of microbiological studies have been conducted on olive mill wastewater, a very polluting by-product of the agro-industry in Mediterranean countries [6, 22, 23, 30]. Olive mill wastewater, which contains high concentrations of phenolic compounds and carbohydrates, is a favorable environment for lactic and propionic bacteria. A new microaerophilic bacterium species, *Propionibacterium microaerophilum*, was recently isolated from olive mill wastewater [20]. *Propionibacterium* species are known to produce propionic acid, acetic acid, and CO_2 as major end products from lactate or carbohydrates fermentation [10, 32]. Among these fermentation products, propionic acid is of special interest for a number of industrial applications [17, 27]. To improve propionic acid production, (i) screenings of acido-tolerant strains producing a high level of propionic acid were undertaken [1, 28] and (ii) different fermentations processes were tested, including batch fermentations [33], fed-batch fermentations [8, 25], continuous fermentations [7], immobilized cell fermentations [26], and extractive fermentations [16, 18]. Most of these studies emphasized that propionic fermentation was mainly limited by the accumulation of the end products in the fermentation broth and the resulting acidification [5, 14, 24].

Olive mill wastewater is characterized by high acidification resulting from the activity of fermentative bacteria. The aim of this work was to determine the effects of pH on glucose metabolism and bioenergetic of *P. microaerophilum.*

Materials and Methods

Organism. *Propionibacterium microaerophilum* (Type strain = strain $M5^T$, DSM 13435; CNCM I-2360) was stored in liquid medium as described by Koussémon et al. [20].

Culture methods and medium. For cultures at non-regulated pH, fermentation was performed in serum bottles containing 50 ml of medium supplemented with 20 mM of glucose, with a starting pH of 6.04 and under an atmosphere of N_2. The serum bottles were incubated for 5 days at 30°C. Batch cultures were conducted in a 2-liter fermentor (Labo 2000; Interscience, St-Nom-la-Bretèche, France) at 30°C with stirring at 200 rpm. The pH was controlled by the addition of 0.5 M NaOH 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 1500 ml of culture medium, was autoclaved at 110°C for 45 min. The medium contained the following (per liter): 0.6 g of KH_2PO_4, 0.2 g of $MgCl_2$, $6H_2O$, 1 g of NH_4Cl, 1 g of NaCl, 0.1 g of yeast extract (Difco Laboratories, Detroit,

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MI), and 10 ml of a trace element solution [2]. Glucose, used as the sole carbon and energy source, was autoclaved separately, at 110°C for 30 min, and added in the culture medium before the fermentation run. The inoculum was grown at 30°C over 2 days in 100 ml medium. Prior to the study of the effect of pH, limiting glucose conditions were determined by using batch cultures in the fermentor with different glucose concentrations, the temperature and pH being maintained at 30°C and 7.0 respectively. The effect of pH on metabolism and bio-energetic characteristics of *P. microaerophilum* was tested at 15 mM glucose. All batches were run in duplicate.

Culture methods and medium for serum bottles. CO_2 production was estimated from experiments performed, under anaerobiosis (N_2 in the headspace) and at non-regulated pH, in 100-ml serum bottles containing 50 ml of the above medium, supplemented with 20 mM of glucose. It was measured, at the end of growth, directly from the gaseous phase, after acidification of the liquid phase to pH 2 with H_2SO_4 (1 M). The tests were conducted in duplicate.

Analyses. Growth was monitored by turbidity measurements (580 nm) with a spectrophotometer (Shimadzu 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% (wt/wt). Washed cells were dried to a constant weight at 80°C.

Lactic, acetic, propionic, formic, and succinic acids and glucose levels were determined by high-performance liquid chromatography with a pump (Spectra Series P100; Spectra-Physics), an automatic sampler (Spectra Serie AS100), an aminex HPX 87H (300 × 7.8 mm) type column (Bio-Rad), a differential refractometer detector (Shimadzu RID 6 A), and an integrator (Shimadzu C-R 6 A Chromatopac). A 20-μl, cell-free supernatant was injected into the column, which was maintained at 35°C. A 2.5-mM H_2SO_4 solution was used as solvent with a flow rate of 0.6 ml min^{-1}. CO_2 was measured with a gas chromatograph (Chrompack CP 9000) equipped with a thermal conductivity detector and a double column (column temperature, 50°C; carrier gas, He [15 ml min^{-1} at 10^5 Pa]; injection and detector temperature, 50°C and 150°C respectively; injection volume, 0.5 ml). The first column (1.5 m × 2 mm) was packed with silicagel 60–80 mesh. The second column (1.5 m × 2 mm) was packed with a molecular sieve 5 Å 60–80 mesh. 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. When studying the effect of pH on growth and metabolism of *P. microaerophilum*, fermentation parameters were calculated at the end of the growth, when maximum cell concentration had been reached. The yields of lactate ($Y_{lact/s}$), propionate ($Y_{prop/s}$), acetate ($Y_{acet/s}$), formate ($Y_{form/s}$), and CO_2 ($Y_{CO2/s}$) were expressed in moles of product per mole of glucose catabolized. Cellular yields on glucose ($Y_{x/s}$) and on ATP ($Y_{x/ATP}$) were expressed in grams of cell (dry weight) per mole of glucose catabolized. The average hourly growth rate (μ*) was calculated according to the following equation:

$$\mu^* = \ln (OD_{maximum} \times OD_{initial}^{-1}) \times t_{growth}^{-1},$$

where $OD_{maximum}$ and $OD_{initial}$ are the optical densities at (580 nm) measured at the end and the beginning of the growth, respectively, and t_{growth} is the time needed between the beginning and the end of growth.

The specific consumption rates of glucose (qs), glucose fermented into lactate (qs-L), and glucose fermented into propionate, acetate, formate, and CO_2 (qs-PAFC) were expressed in millimoles of glucose catabolized per gram of cells (dry weight) per hour and were calculated according to the following equations:

$$qs = \mu^* \times (Y_{x/s})^{-1},\ qs\text{-}L = \mu^* \times (Y_{x/s})^{-1} \times (Y_{lac/s}) \times 0.5,$$

and

$$qs\text{-}PAFC = qs - qs\text{-}L,$$

where 0.5 is the quantity (moles) of glucose needed to produce one mole of lactate.

Table 1. Fermentation balance of glucose by *P. microaerophilum* at non-regulated pH[a]

Substrate: glucose consumed (mM)	8.42
Products (mM):	
Propionate	4.66
Acetate	2.58
CO_2	2.64
Formate	n.d.[b]
Lactate	n.d.[b]
Final pH	3.87
C-recovery (%)	43.1
O/R ratio	1.03
Acetate/ CO_2 ratio	0.98
Propionate/Acetate ratio	1.81

[a] The fermentation was performed as indicated in Materials and Methods. Results are the average of triplicate tests.
[b] n.d., not detected.

Results and Discussion

CO_2 concentration and end products balance at non-regulated pH. In batch cultures, the flow of O_2-free N_2 in the fermenter prevented the analysis of CO_2 produced during glucose fermentation. CO_2 production was estimated from cultures in serum bottles, in anoxic conditions, with an uncontrolled pH that decreased to a final value of 3.9, stopping fermentation and cellular growth. The sole end products of glucose fermentation detected were propionate, acetate, and CO_2 (Table 1). However, the carbon recovery for these metabolites represented only 43.1% of glucose fermented, with a ratio between oxidized and reduced carbon metabolites (O/R) close to 1 (Table 1). This suggested that about 57% of glucose fermented was (i) converted into one or several product(s) not detectable by our analytical method and (ii) that the carbon oxidation level of the undetected product(s) was close to 0, similar to glucose for instance. Low carbon recoveries were also observed by Crow [9], with *P. acidipropionici* growing on glucose or lactose. In this case, polysaccharide formation was found responsible for such low carbon recoveries.

The ratios of acetate to CO_2 and propionate to acetate close to 1 (0.98) and 2 (1.81) (Table 1) respectively were in accordance with the end-product ratios of glucose fermentation generally calculated in *Propionibacterium* species [32]. Consequently, for the further experiments conducted in the fermenter, the following

Table 2. Effect of pH on the cellular and energetic yields of *P. microaerophilum* fermenting glucose

	Parameters[a]						
				$Y_{ATP/s}$ (mol mol^{-1})		$Y_{x/ATP}$ (g of cell [dry wt] mol^{-1})	
pH	$Y_{x/s}$ (g of cell [dry wt] mol^{-1})	C recovery (%)	O/R balance	−extra ATP[b]	+extra ATP	−extra ATP	+extra ATP
4.8	9.26	46.0	0.84	2.36	5.59	3.92	1.66
5.3	16.63	57.9	0.90	2.43	5.24	6.85	3.17
6.0	31.04	49.3	0.95	2.61	5.07	11.89	6.13
6.5	33.43	51.7	0.93	2.61	5.22	12.83	6.40
7.0	32.10	52.7	0.96	2.68	5.00	11.98	6.42
7.5	34.08	46.4	0.97	2.59	4.72	13.14	7.22
8.0	23.47	52.0	0.94	2.53	4.44	9.28	5.28
8.5	12.64	45.6	1.09	2.35	3.62	5.35	3.49
9.0	10.53	33.5	1.12	2.34	3.05	4.49	3.45

[a] Batch cultures were conducted in anaerobiosis at controlled pH. Parameters were calculated at the end of the growth phase.
[b] The energy yields were calculated for "+extra ATP" column by taking into account extra ATP formed from the reduction of fumarate into succinate.

equations were used to estimated CO_2 production: $Y_{CO_2}/s = Y_{acet/s}$ or $Y_{CO_2}/s = Y_{acet/s} - Y_{form/s}$ when formate was produced.

Effect of pH on *P. microaerophilum* growth. Prior to the study of the effect of pH, the limiting glucose conditions for growth were determined by using batch cultures maintained at pH 7.0 and performed with different glucose concentrations. The results showed that, in our experimental conditions (pH at 7.0 and anaerobic condition), the growth of *P. microaerophilum* was limited by glucose for concentrations lower than 18 mM (data not show). Under these limiting glucose conditions, the cellular yield was 21.6 g of cells (dry weight) per mole of glucose consumed. Subsequently, the results of glucose fermentations, carried out with 15 mM glucose, showed that *P. microaerophilum* cellular yield ($Y_{x/s}$) and growth rate (μ*) (Table 2 and Table 3) were optimum for pH ranging from 6.5 to 7.5, a range close to that of propionibacteria in general [15] and *P. acidipropionici* in particular [16].

Effect of pH on end product of glucose fermentation. In fermentations with controlled pH, glucose was fermented into propionate, acetate, lactate, CO_2, and formate. Lactic acid produced was exclusively the L-(+) form. However, the carbon of fermented glucose was only partly recovered [33.5% to 52.7% (wt/wt)] with O/R ratios close to 1, as already observed in the flask without pH regulation (Table 2). The pH significantly affected (i) glucose-specific fermentation rates (qs) (Table 3) and (ii) the fermentation metabolic pathways (Fig. 1). The glucose-specific fermentation rate (qs), like the growth rate, was optimum at pH between 6.5 and 7.5 and was strongly reduced under more acidic or alkaline conditions (Table 3). Glucose catabolism involved two major pathways, one leading to the production of lactate (qs_{-L}) and one leading to that of propionate, acetate, formate, and CO_2 (qs_{-PAFC}) (Table 3). Figure 2 showed that the activity of the lactate metabolic pathway increased with pH, reaching an optimum under alkaline conditions. Under the latter conditions, half of the fermented glucose flux was converted into lactate, a yield representing up to 50% (wt/wt) of fermented products at pH 9.0 (Fig. 2). In contrast, the activity of the metabolic breakdown of pyruvate and the dicarboxylic acid pathway leading to CO_2, formate, acetate, and propionate was reduced under alkaline conditions and was optimum under both neutral and acidic conditions (Fig. 2). Under these latter conditions, propionate, acetate, and CO_2 were the major end products, representing 92% (wt/wt) of

Table 3. Effect of pH on fermentation kinetic parameters during the growth phase of *P. microaerophilum*

	Parameters[a]			
		mmol g of cell^{-1} (dry wt) h^{-1}		
pH	μ* (h^{-1})	qs	qs_{-L}	qs_{-PAFC}
4.8	0.005	0.54	0.01	0.53
5.3	0.020	1.20	0.10	1.10
6.0	0.040	1.27	0.10	1.17
6.5	0.061	1.82	0.08	1.74
7.0	0.065	2.02	0.16	1.86
7.5	0.063	1.85	0.32	1.53
8.0	0.040	1.70	0.44	1.27
8.5	0.009	0.71	0.22	0.50
9.0	0.005	0.47	0.22	0.25

[a] Parameters were calculated at the end of the growth phase.

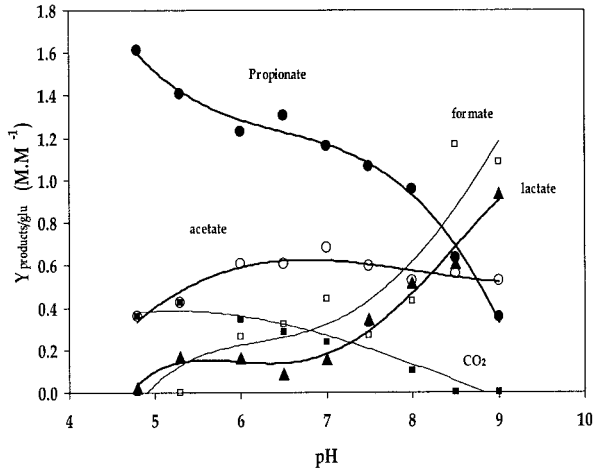

Fig. 1. Effect of pH on the product yields of glucose fermentation: propionate (●), acetate (○), formate (□), lactate (▲), and CO_2 (■).

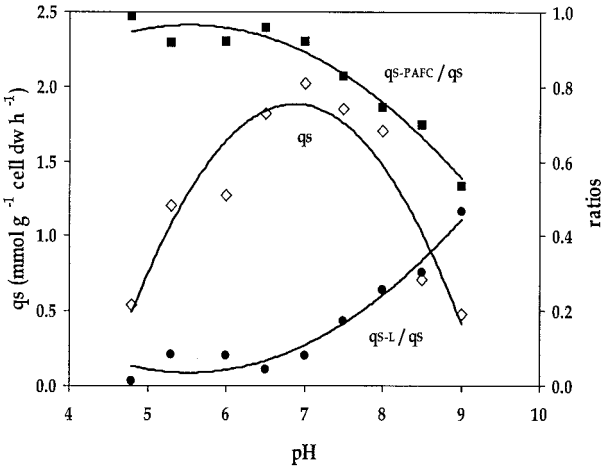

Fig. 2. Effect of pH on the specific glucose consumption rate (qs) (◇), the ratio of the rate of glucose fermented into lactate to qs (qs-L/qs) (■), and the ratio of the rate of glucose fermented into propionate, acetate, formate, and CO_2 to qs (qs-PAFC/qs) (●).

fermented products at pH 7.0 and 99% (wt/wt) at pH 4.8 (Fig. 2). According to the theoretical yields of propionic and acetic acids from glucose or lactate fermentation, based on the balanced equation for dicarboxylic and the acid metabolic pathway, Playne [27] found a yield of 1.33 mol mol^{-1} for propionate and 0.67 mol mol^{-1} for acetate. With *P. microaerophilum*, the values for propionate and acetate yields were close to the theoretical values only for pH 6.0 to 7.0 (Fig.1). Formate production increased with the pH, replacing CO_2 production (Fig. 1). The increase of formate and lactate yields, occurring concomitantly with the inhibition of the reductive pathway leading toward propionate, might be explained as electron sink alternative reactions preventing the accumulation of reducers, such as $NADH_2$, generated during glucose catabolism, thus allowing control of the cellular electronic balance.

A similar change in glucose metabolism was observed with *P. acidipropionici* growing in alkaline conditions [16]. However, contrary to *P. microaerophilum*, for which glucose catabolism switched markedly from propionate, acetate, and CO_2 toward lactate and formate production, in *P. acidipropionici* glucose catabolism was diverted toward pyruvate and succinate production, without lactate or formate production [16]. Finally, in *P. microaerophilum*, in contrast to *P. acidipropionici* [16, 21], the acetate yields were significantly affected by pH (Fig. 1).

Cellular and energy yields of *P. microaerophilum* ($Y_{X/ATP}$ and $Y_{ATP/S}$). In *Propionibacterium* species, the energy yields of glucose breakdown ($Y_{ATP/S}$) are calculated from end product fermentation by considering generally that 1 mol of ATP was generated for each mole of lactate or propionate produced and that 2 mol of ATP were generated per mole of acetate produced. Applying this calculation to *P. microaerophilum*, the energy yields ($Y_{ATP/S}$) were 2.6 mol mol^{-1} and $Y_{X/ATP}$ 11.9–13.1 g mol^{-1} at optimum pH for growth (Table 2).

On the other hand, De Vries et al. [12] and Bauchop and Eldsen [3], dealing with ATP generation in *P. freudenreichii* and *P. pentosaceum* (renamed as *P. acidipropionici*), postulated for the calculation of $Y_{ATP/S}$ values that 2 extra moles of ATP were formed from the reduction of 1 mole of fumarate into succinate. Taking into account this oxidative phosphorylation during anaerobic electron transport, the $Y_{ATP/S}$ values in *P. acidipropionici* growing on glucose varied consequently from 4.6 mol mol^{-1} on synthetic medium to 4.9 mol mol^{-1} on complex medium, while $Y_{X/ATP}$ varied from 14 g mol^{-1} on synthetic to 16.7 g mol^{-1} on complex medium. Applying the latter method to *P. microaerophilum*, the energy yield $Y_{ATP/S}$ was 4.7–5.1 mol mol^{-1} and $Y_{X/ATP}$ 6.1–7.2 g mol^{-1} at the optimum pH for growth (Table 2).

Finally, from comparison of cellular yields to ATP ($Y_{X/ATP}$) (calculated according to the two methods mentioned above), it appeared that, when the extra ATP formed from the reduction of fumarate into succinate was taken into account, the cellular yield of *P. microaerophilum* (6.1–7.2 g mol^{-1}) was significantly lower than that of *P. acidipropionici* (14–16.7 g mol^{-1}) and several other anaerobes (10–20 g mol^{-1}, depending on the composition of the culture medium and the bacterial species) [3, 4, 11, 13, 19, 29, 31]. In contrast, when the extra ATP was omitted from the calculation, the range of value of $Y_{X/ATP}$ (11.9–13.1 g mol^{-1}) in *P. microaerophi-*

lum was close to that of *P. acidipropionici* and other anaerobic bacteria.

In conclusion, these results would tend to show significantly that *P. microaerophilum* could not synthesize ATP through the reduction of fumarate to succinate. Therefore, the energetic metabolism of *P. acidipropionici* and *P. microaerophilum*, both species very close, would be different. To clarify this important question, further studies should characterize the key enzymes involved in the glucose catabolism pathways of *P. microaerophilum* and *P. acidipropionici.*

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