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Batch Fermentation Model of Propionic Acid Production by *Propionibacterium acidipropionici* in Different Carbon Sources

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Abstract Propionic acid (PA) is widely used as additive in animal feed and also in the manufacturing of cellulose-based plastics, herbicides, and perfumes. Salts of propionic acid are used as preservative in food. PA is mainly produced by chemical synthesis. Nowadays, PA production by fermentation of low-cost industrial wastes or renewable sources has been an interesting alternative. In the present investigation, PA production by *Propionibacterium acidipropionici* ATCC 4965 was studied using a basal medium with sugarcane molasses (BMSM), glycerol or lactate (BML) in small batch fermentation at 30 and 36 °C. Bacterial growth was carried out under low dissolved oxygen concentration and without pH control. Results indicated that *P. acidipropionici* produced more biomass in BMSM than in other media at 30 °C (7.55 g l^{-1}) as well as at 36 °C (3.71 g l^{-1}). PA and biomass production were higher at 30 °C than at 36 °C in all cases studied. The best productivity was obtained by using BML (0.113 g l^{-1} h^{-1}), although the yielding of this metabolite was higher when using glycerol as carbon source (0.724 g g^{-1}) because there was no detection of acetic acid. By the way, when using the other two carbon sources, acetic acid emerged as an undesirable by-product for further PA purification.

Keywords *Propionibacterium acidipropionici* · Propionic acid · Batch fermentation · Carbon sources · Sugarcane molasses · Glycerol · Lactate

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Introduction

Propionibacteria are, in general, Gram-positive, nonmotile, catalase-positive, nonspore-forming, rod-shaped, anaerobic to aerotolerant bacteria, able to ferment different carbohydrates and certain polyalcohols to produce mainly propionic acid, acetic acid, and carbon dioxide. Propionic acid is the major product from the dicarboxylic acid pathway, also known as succinate pathway. This organic acid and its salts are able to inhibit microorganisms [1–5]. The prevailing view is that propionic acid disturbs the pH gradient across the cell membrane, an essential motive force for facultative anaerobes to transport nutrients and metabolites. Due to the hydrophobic nature of both propionic acid and cell membrane, the undissociated acid can diffuse through the bacterial membrane into the cytoplasm and then dissociate into a proton and propionate anion inside the cell. Thus, an inward 'leak' of protons is created. In order to maintain the functional proton gradient across the membrane, extra adenosine triphosphate (ATP) must be consumed by H^+-ATPase to extrude the proton out, diminishing the available ATP for cell metabolism [6, 7]. Therefore, the growth of fungus, yeasts, and some bacteria is inhibited.

Propionic acid is used in chicken and other animal feed to prevent infection from mould [8]. It is also used for the manufacturing of cellulose-based plastic, herbicides, and perfumes. Calcium, sodium, and potassium salts of propionic acid are used as food preservatives, generally recognized as safe food additives. Presently, there has been an increasing interest in the production of propionic acid by fermentative processes [9–11]. In recent years, concerns about the uncertain supply and the eventual depletion of world petroleum reserves have fostered investigations into alternative ways to produce petrochemical derivatives. One example is the propionic acid, which is currently produced commercially by the oxidation of liquid-phase propane or propionaldehyde [4, 12, 13]. If good yields of propionic acid could be obtained by fermentation of low-cost industrial wastes or renewable sources, biological production could become economically competitive, besides reducing the contaminant industrial wastes [14].

There are few reports and data about propionic acid production by glycerol fermentation, although glycerol has become a cheap carbon source because the new processes of fuel production from vegetable oils provide a large amount of glycerol as a by-product, which needs new applications [15].

The objective of the present investigation was to study the use of glycerol by *Propionibacterium acidipropionici* by comparing it with other carbon sources in batch fermentation model and, in addition, evaluate the capacity of propionic acid production at two different temperatures.

Materials and Methods

Microorganism and Media

The *P. acidipropionici* ATCC 4965 used in this study was grown in a synthetic basal medium making use of lactate, glycerol, or sugarcane molasses as carbon sources. The culture was grown in deep agar at 30 °C and stored at 4 °C. The conservation medium contained per liter of deionized water: 1 g KH_2PO_4, 2 g $(NH_4)_2HPO_4$, 5 mg $FeSO_4{\cdot}7H_2O$, 10 mg $MgSO_4{\cdot}7H_2O$, 2.5 mg $MnSO_4{\cdot}H_2O$, 10 mg $CaCl_2{\cdot}6H_2O$, 10 mg $CoCl_2{\cdot}6H_2O$, 5.0 g yeast extract, 5.0 g sodium lactate, and 7.0 g agar, and pH was adjusted to 6.8 before autoclaving.

The preculture and the inoculum media had the same composition as the conservation medium without agar. Moreover, sodium lactate concentration was increased to 20 g l^{-1}, whereas yeast extract concentration was increased to 10 g l^{-1}.

The fermentation medium for batch fermentation had 20 g l^{-1} glycerol [basal medium with glycerol (BMG)], 30 g l^{-1} sodium lactate [basal medium with lactate (BML)], or 40 g l^{-1} sugarcane molasses [basal medium with sugarcane molasses (BMSM)] as carbon source. The basal medium and the carbon sources were prepared independently. The pH of these two solutions was adjusted to 6.8–7.0 before autoclaving. After heat sterilization at 121 °C and 1 bar for 15 min, they were mixed aseptically in the fermentation flasks.

Inoculum Preparation

One isolated colony from deep agar plate was transferred to 2 ml of preculture medium and incubated at 30 °C for 48 h. A portion of this culture (0.4 ml) was transferred to 40 ml screw-cap flask containing 40 ml of inoculum medium broth. *P. acidipropionici* was grown without agitation for 24–36 h at 30 °C in inoculum medium broth (final $A_{660}\approx 0.8$) and was inoculated at 1% (*v*/*v*), into 1 l of fermentation broth in the 1-l customized flasks.

Batch Fermentations

The batch fermentation model was performed in three different media, BML, BMG, and BMSM, and was conducted in 1-l customized flasks with a final volume of 1 l in order to reduce the presence of oxygen. The fermentations were carried out without pH control in static incubation at two different temperatures, 30 and 36 °C. The experiments were performed in duplicate, and the obtained results are the average of those independent trials. Samples of 40 ml were removed at the beginning of the fermentation and at periodic intervals during 6 days.

Carbon Source and Organic Acid Determination

Lactate, glycerol, sucrose, propionic acid, and acetic acid concentrations were determined by high-performance liquid chromatography (Shimadzu Liquid Chromatograph, model RID-10A refractive index detector) by using the Aminex® HPX-87H ion exclusion column (Bio-Rad, Hercules, CA, USA) operated at 60 °C, with 5 mM H_2SO_4 as the mobile phase at 0.6 ml/min flow rate. Samples for analysis were diluted with purified water (milliQ) according to their concentration and centrifuged to remove cells, then were filtered through 0.22 μm-pore-size filters (Millipore), and stored at −20 °C before analysis. A Shimadzu Chromatopac (model C-R6A) was used to analyze the data and plot the chromatograms. The product concentration was calculated by comparing the peak areas with those of external standards. The propionic acid concentration was twofold plotted (×2), for clear visualization.

Biomass and pH Determination

The pH of the 40 ml portion sample was measured in a HANNA HI9321 microprocessor pHmeter, which was calibrated with two standard points before the measures.

Optical density (OD) of the cells was measured at 660 nm in a Shimadzu UV-1601 spectrophotometer. A standard curve was plotted between cell dry weight and absorbance and was used to determine the cell weight in the samples from their absorbance. Samples were diluted with distilled water in order for OD to be between 0.2–0.8.

Results

Batch Fermentation by Using Glycerol as Carbon Source

In order to determine yield and productivity parameters to evaluate propionic acid fermentation, the experiments were carried out with different carbon sources at two different temperatures by using a fermentative batch model.

Figure 1a and b shows a typical fermentation pattern using BMG at 30 and 36 °C, respectively. Fermentation at 30 °C has just consumed half of the substrate, and 6.77 g l^{-1} of propionic acid was produced without any acetic acid formation. Biomass production only reached 2.42 g l^{-1}. Cell growth and glycerol consumption achieved the plateau after 70 h, and it was observed that propionic acid production continued. The pH variation was very high and quick, that is, the pH changed 2.5 points in less than 40 h, and it reached a plateau phase around 4.4.

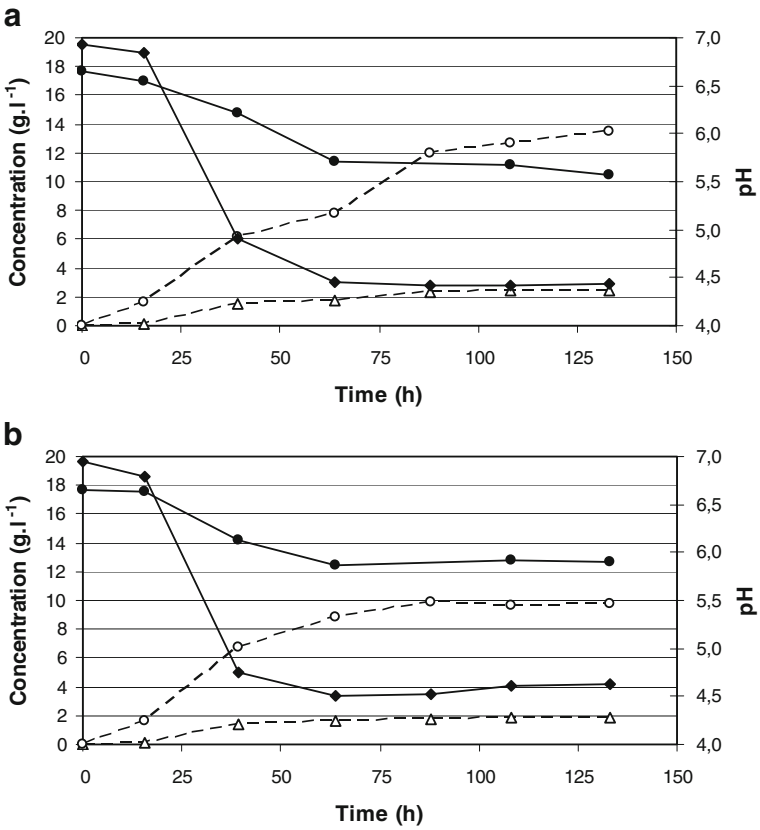

Fig. 1 Propionic acid fermentation in BMG. **a** Fermentation at 30 °C. **b** Fermentation at 36 °C. Concentration in gram per liter of: biomass (dry weight; *open triangle*), glycerol (*closed circle*), propionic acid (×2; *open circle*), and pH (*closed diamond*). The propionic acid concentration was twofold plotted (×2) for clear visualization

Propionibacteria fermentation in BMG at 36 °C was less productive, and it showed almost the same profiles as the one at 30 °C; with the difference at this temperature, propionic acid production was higher.

Batch Fermentation by Using Lactate as Carbon Source

Typical propionibacteria fermentations, by using lactate at 30 and 36 °C, are shown in Fig. 2a and b, respectively. In relation to batch fermentation at 30 °C, higher amounts of propionic acid (15.06 g l^{-1}) and acetic acid (5.59 g l^{-1}) were produced when using the other carbon sources. Cell growth and production of organic acids entered the stationary phase when lactate was depleted. The fermentation time was around 130 h with small pH variation, and biomass production was similar to BMG and achieving 3.31 g l^{-1}.

In relation to batch fermentation at 36 °C, a considerable amount of propionic acid (13.32 g l^{-1}) was produced from the lactate and acetic acid production achieved (3.58 g l^{-1}), but both organic acid concentrations were lower than the fermentation at 30 °C. The same occurred with the biomass production, which reached just 1.29 g l^{-1}. The sodium lactate

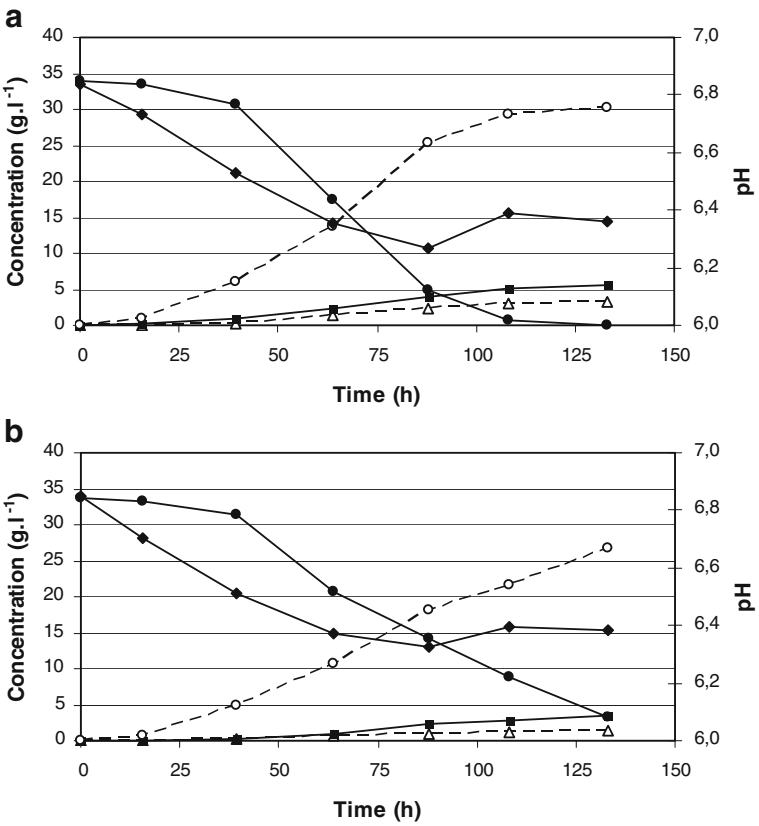

Fig. 2 Propionic acid fermentation in BML. **a** Fermentation at 30 °C. **b** Fermentation at 36 °C. Concentration in gram per liter of: biomass (dry weight; *open triangle*), sodium lactate (*closed circle*), acetic acid (*closed square*), propionic acid (×2; *open circle*), and pH (*closed diamond*). The propionic acid concentration was twofold plotted (×2) for clear visualization

was not totally depleted, and the cell continued growing in a slow way, according to the lactate consumption. The pH variation was also very short, that is, around 0.5.

Batch Fermentation by Using Sugarcane Molasses as Carbon Source

Propionic acid fermentations in BMSM at 30 and 36 °C are shown in Fig. 3a and b, respectively. Considering all experiments, fermentation at 30 °C produced the higher concentration of biomass (7.55 g l^{-1}) of all experiments, although the propionic acid concentration was similar with glycerol; but it was not so significant when using lactate as carbon source. Cell growth entered the stationary phase around 65 h, and the propionic acid production continued as the sucrose concentration decreased. Acidification was rapid, and the pH decreased 2.6 points in less than 40 h, thus reaching a plateau phase around pH 4.1.

At 36 °C, a small amount of biomass was produced from sugarcane molasses (3.71 g l^{-1}), but it was observed that both organic acids, propionic (6.92 g l^{-1}) and acetic (1.65 g l^{-1}), were lower than in the fermentation at 30 °C.

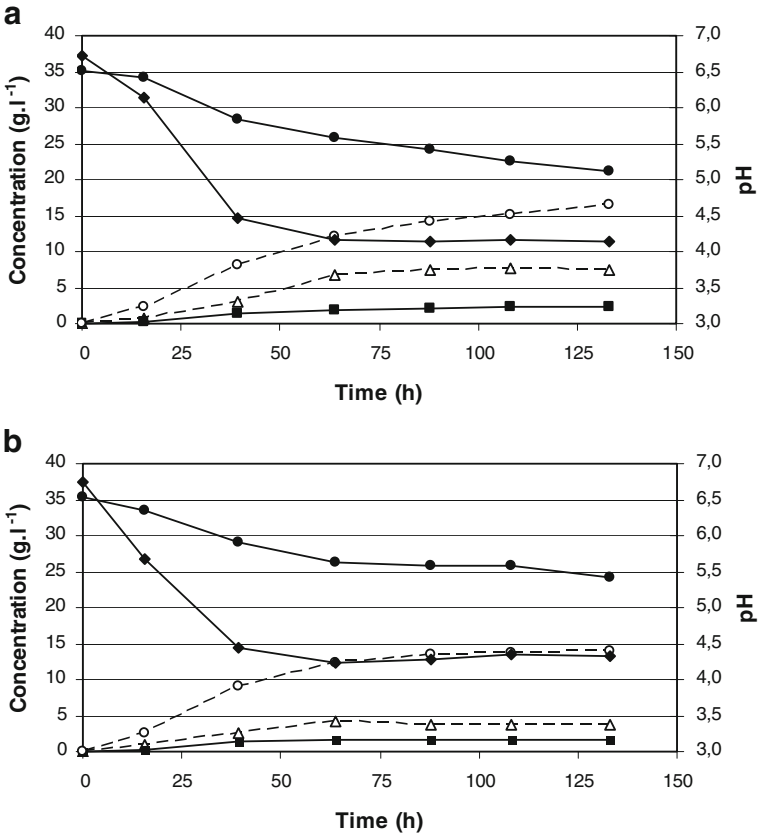

Fig. 3 Propionic acid fermentation in BMSM. **a** Fermentation at 30 °C. **b** Fermentation at 36 °C. Concentration in gram per liter of: biomass (dry weight; *open triangle*), sugarcane molasses (*closed circle*), acetic acid (*closed square*), propionic acid (×2; *open circle*), and pH (*closed diamond*). The propionic acid concentration was twofold plotted (×2) for clear visualization

Comparing Parameters: Use of Different Carbon Sources at Two Different Temperatures

As already shown in Figs. 1, 2, and 3, propionic acid fermentations in BMG, BML, and BMSM at different temperature levels display different kinetic parameters. The differences were also evident in comparing the cell growth rate (μ), acidification (ΔpH), propionic-to-acetic acid ratio (P/A), and cell (X) and product yields (Tables 1 and 2).

As shown in Tables 1 and 2, cell yield depends on both temperature and the carbon source used. The cell yield from sugarcane molasses was about four times higher than that from lactate; nevertheless, propionic acid was similar. Cell growth rate as well as biomass final concentration, biomass productivity, and biomass yield was better when using BMSM than with BML or BMG, mainly at 30 °C.

The propionic acid production had small differences according to the temperature level, but in all of the carbon sources tested, it could be noticed that 30 °C temperature presented better condition to produce propionic acid and dry propionibacteria biomass than 36 °C. The best carbon source for *P. acidipropionici* ATCC 4965, comparing the propionic acid final concentration and the propionic acid productivity, was BML. However, with glycerol, there was no acetic acid production, and the propionic acid yield was maximum, as an experimental estimative ratio P/A was 100:1.

Discussion

Considering the experimental batch fermentations without pH control, a maximum concentration of propionic acid was obtained with lactate at 30 °C; that was higher than when using molasses, even with a lower cell concentration. In other words, lactate was converted into propionic acid faster than from molasses because it does not need to be degraded via glycolytic pathway, and its biosynthesis is easier. With lactate, the average substrate consumption rate, at 30 °C, was approximately 0.26 g l^{-1} h^{-1} per gram of cells, whereas with molasses, at 30 °C, it was approximately 0.14 g l^{-1} h^{-1} per gram of cells. Thereby, sugarcane molasses is seen as a good carbon source to produce biomass once it is cheaper than lactate and it is a renewable source. A process developed in two steps could be interesting, first growing cells in BMSM and then feeding lactate.

It is relevant to point out that a small amount of biomass using lactate at 30 °C is able to produce the highest propionic acid concentration, thus reducing the downstream

Table 1 Effects of carbon source and temperature on propionic acid fermentation after 133 h.

Carbon source	Temperature (°C)	$pH_{initial}$	ΔpH	μ (h^{-1})	Final concentration (g l^{-1})			Average ratio P/A
					X	P	A	
Lactate	30±0.1	6.87	0.48±0.03	0.0395	3.31±0.11	15.06±0.56	5.59±0.22	2.7:1
	36±0.1	6.87	0.46±0.02	0.0156	1.29±0.12	13.32±0.89	3.58±0.29	3.7:1
Glycerol	30±0.1	6.98	2.50±0.10	0.0311	2.42±0.13	6.77±0.11	ND	100.0:1[a]
	36±0.1	6.98	2.32±0.08	0.0303	1.81±0.08	4.87±0.36	ND	100.0:1[a]
Sugarcane molasses	30±0.1	6.77	2.58±0.03	0.1269	7.55±0.07	8.23±0.12	2.25±0.02	3.7:1
	36±0.1	6.77	2.40±0.09	0.0689	3.71±0.01	6.92±1.17	1.65±0.09	4.2:1

ND None detected

[a] Estimating 1% of acetic acid in the glycerol fermentative broth

Table 2 Effects of carbon source and temperature on propionic acid fermentation related to productivities and yields after 133 h.

Carbon source	Temperature (°C)	Productivity (g l^{-1} h^{-1})			Yield coefficients (g g^{-1})		
		P_X	P_P	P_A	$Y_{X/S}$	$Y_{P/S}$	$Y_{A/S}$
Lactate	30±0.1	0.025±0.001	0.113±0.004	0.042±0.002	0.097±0.004	0.442±0.020	0.164±0.008
	36±0.1	0.010±0.001	0.100±0.007	0.027±0.002	0.042±0.001	0.436±0.002	0.117±0.002
Glycerol	30±0.1	0.018±0.001	0.051±0.001	ND	0.259±0.004	0.724±0.015	ND*
	36±0.1	0.014±0.001	0.037±0.003	ND	0.261±0.002	0.703±0.024	ND*
Sugarcane molasses	30±0.1	0.057±0.001	0.062±0.001	0.017±0.001	0.417±0.001	0.455±0.002	0.124±0.001
	36±0.1	0.028±0.001	0.052±0.009	0.012±0.001	0.297±0.003	0.555±0.089	0.132±0.006

ND None detected

processing costs in large-scale production. Besides that, it reduces the time and energy required for removing biomass, as well as minimizes the waste-disposal problem [14]. On the other hand, if propionic acid fermentation was carried out by using molasses in a two-step-process, a high propionic acid production should be accompanied with a high yield of biomass, which would be useful for an eventual extraction of vitamin B_{12} [17, 18].

Temperature [16] and pH control [18] are two factors that determine high yields. Seshadri and Mukhopadhyay [16] reported that propionic acid production decreases from 30 to 37 °C with temperature increments, whereas the biomass production increases. In our experiments with the strain ATCC 4965, the opposite was observed, that is, both propionic acid production and biomass growth rate were better at 30 °C than at 36 °C in all assays.

The propionibacteria are very sensitive to pH and usually require pH control because little growth occurs below pH 5.0 [3, 7]. Lactate as carbon source has a clear advantage over glycerol and over sugarcane molasses when tight pH control cannot be implemented, as previously described [11], once it was shown that pH variation was faster when using BMG and BMSM in comparison to BML.

Boyaval et al. [15] showed similar profiles of the propionic acid production, that is, biomass growth rate and pH variation, in batch fermentation of glycerol at 30 °C with *Propionibacterium thoenii* NCDO 1082. However, in the present investigation, a higher glycerol-to-propionic acid yield was obtained; it means that almost all the carbon source was transformed into propionic acid by *P. acidipropionici* at 30 °C as well as at 36 °C.

The production of organic acids using propionic acid bacteria is inhibited by fermentation final products, acetic and propionic acids [4, 9, 12]. As acetic acid is an inhibitor considered stronger than the propionic acid itself, glycerol fermentation was tested, and it was observed that it did not produce any acetic acid [15]. Obviously, glycerol has a clear advantage over lactate and molasses as carbon sources for undergoing that process. Furthermore, acetic acid is a by-product in the propionic acid fermentation, and it may increase the downstream steps, reducing the final quality of the product. Anyway, propionic acid fermentation making use of glycerol has a lot of advantages and a big potential over other carbon sources such as lactate, glucose, molasses, or whey [19]. Since in the near future glycerol will be an industrial waste derived from biodiesel production, it is worthwhile to carry out more researches in order to use this novel technology.

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