Organic Acid Production by *Propionibacterium shermanii*: Effect of pH, Temperature and Vitamin-Nitrogen Source

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Production of propionic acid by *Propionibacterium shermanii* CDB 10014 was enhanced by a pH value of 6.5 and by temperatures in the range 35–37 °C. Depending on the type of yeast extract, succinic acid can be produced in higher proportions, with decreasing propionic acid yields. With respect to propionic acid production, Difco yeast extract has shown the best results when yeast extract preparations from other different suppliers were compared. To replace yeast extract by a cheaper vitamin-nitrogen source, corn-steep liquor was tested. A complete depletion of glucose was achieved, yielding a final propionic acid concentration of over 35 g/l. These results are even better than those obtained with Difco yeast extract and suggest the possibility of an economical process based on corn-steep liquor.

Introduction

Propionic acid is used as a preservative in poultry feed and in bakery products. It is also used in the manufacture of cellulose-based plastics, perfumes, herbicides, and medicaments (Playne, 1985). Propionic acid is normally produced from petrochemical materials, but it is well known that some bacteria, especially those belonging to the genus *Propionibacterium*, produce this acid in high concentrations. Bacterial propionic acid formation has the advantage that the biomass can be used as a source of vitamin B_{12} (Florent, 1986), as a cheese-starter (Cummins and Johnson, 1992), in silage processing (Flores-Galagarza *et al.*, 1985; Tomes, 1991), and as a probiotic (Mantere-Alhonen and Mäkinen, 1987).

Propionibacteria, similar to other acid producing bacteria, are strongly dependent on pH (Hsu and Yang, 1991). In the literature, a pH-range between 6.0 and 7.0 is reported as optimum for both *Propionibacterium* growth and propionic acid production (Playne, 1985; Crespo *et al.*, 1990).

Temperature is another important factor affecting this process. Depending on the strain, different optimum temperatures, for growth and for propionic acid formation, have been reported, 24 °C (Hettinga and Reinhold, 1972), 30 °C (Crespo *et al.*, 1990), 37 °C (Champagne *et al.*, 1989; Quesada-Chanto *et al.*, 1994).

Propionibacteria growth is dependent on the vitamins normally present in yeast extract (Hettinga and Reinhold, 1972). Although propionibacteria can grow with inorganic nitrogen salts as a nitrogen source, it is well known that they grow much better on media containing organic-nitrogen compounds such as casamino acids or yeast extract (Cummins and Johnson, 1992). Considering the high price of that kind of media component, interest in using cheaper raw materials is increasing (Playne, 1985; Lewis and Yang, 1992).

Since the information in the literature about these aspects is insufficient, the objective of this study was to find the optimal values of pH and temperature for growth and acid production by *Propionibacterium shermanii* and also to test some inexpensive substitutes for yeast extract. Furthermore, some experiments comparing different type of yeast extract were performed.

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Material and Methods

Microorganism

Propionibacterium shermanii CDB-10014 (formerly *P. shermanii* PZ-3) was obtained from the Centro de Desenvolvimento Biotecnológico, Joinville, S. C., Brazil.

Media

The maintenance medium contained per litre deionized water: 20 g glucose, 2 g KH_2PO_4, 4 g $(NH_4)_2$ HPO_4, 5 mg $FeSO_4$ x $7H_2O$, 10 mg $MgSO_4$ x $7H_2O$, 2.5 mg $MnSO_4$ x H_2O, 10 mg $CaCl_2$ x $6H_2O$, 10 mg NaCl, 10 mg $CoCl_2$ x $6H_2O$, 10 g yeast extract (Oxoid, Brangstoke, England), and 15 g agar, with a pH before autoclaving of 6.8–7.2. Except for the agar, the preculture medium had the same composition.

The basic fermentation medium contained per litre of deionized water: 80 g glucose, 1 g KH_2PO_4, 2 g $(NH_4)_2$ HPO_4, 5 mg $FeSO_4$ x $7H_2O$, 10 mg $MgSO_4$ x $7H_2O$, 2.5 mg $MnSO_4$ x H_2O, 10 mg $CaCl_2$ x $6H_2O$, 10 mg NaCl, 10 mg $CoCl_2$ x $6H_2O$, and 10 g yeast extract (Oxoid). The pH before autoclaving was 6.8–7.2.

In some experiments, Oxoid yeast extract was replaced by Fluka (Buchs, Switzerland) or Difco (Detroit, USA) yeast extract (10 g/l); Prodex (Prodesa, São Paulo, Brazil), an unpurified yeast extract, (12 g/l); soy brand (Vila Nova, Joinville, Brazil) (10 g/l); or corn-steep liquor -C. S. L. (Refinações de Milho Brasil, Mogi Guaçu, Brazil) (100 g/l).

Inoculum preparation

The culture was transferred from a deep agar into an Erlenmeyer flask with 150 ml of the preculture medium and allowed to stand at 35 °C. After 72 h, 20 ml of this culture were inoculated in 150 ml of the preculture medium and allowed to stand at 35 °C. After 24 h, the bioreactor was inoculated with 15% (v/v) of this culture.

Fermentation process

The runs were carried out in batch mode in a Biostat Q system (B. Braun Biotech, Melsungen, Germany). This system consists of four 400 ml bioreactors. The effect of the pH was studied in the range 5.5–7.0, at 35 °C. Temperatures varying from 25 to 37 °C, at pH 6.5, were tested. The influence of the different vitamin-nitrogen sources was evaluated at 35 °C and pH 6.5. In all cases, the pH was automatically controlled by adding 7 N NaOH.

Analysis

Cell growth was estimated by measuring the optical density of cell suspensions at 560 nm wavelength in a Shimadzu UV 160A spectrophotometer (Tokyo, Japan). The cell mass was estimated by a correlation between optical density and cell dry weight. Glucose and organic acid concentrations were measured by HPLC equipped with an ORH-801 Column (Interaction Chromatography, San José, USA) with a RI detector (Knauer, Bad Homburg, Germany). H_2SO_4 0.005 M was used as mobile phase.

Results and Discussion

Effect of pH

The pH values studied were: 5.5, 6.0, 6.5 and 7.0. After 75 h of fermentation, only a slight variation of both the final cell and by-products (acetic and succinic acids) concentrations was observed in the evaluated pH-range. With respect to the final propionic acid concentration, however, a higher value was obtained with pH 6.5.

On the other hand, the best cell yield ($Y_{X/S}$) and the best propionic acid yield ($Y_{P/S}$) were obtained at pH 5.5 (Table I). At that pH, however, the overall process rate decreased and, consequently, the volumetric productivity (Q_v) was lower than that

Table I. Effect of pH and temperature on yield, volumetric productivity and glucose consumption, in a batch fermentation of *Propionibacterium* shermanii CDB 10014 after 75 h ($Y_{P/S}$, propionic acid produced/glucose consumed; $Y_{X/S}$, biomass produced/glucose consumed; Q_v, volumetric productivity; ΔS, glucose consumption).

Conditions		$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	Q_v (g/l×h)	ΔS (%)
pH	5.5	0.45	0.31	0.16	30.1
	6.0	0.37	0.24	0.18	44.4
	6.5	0.40	0.26	0.21	38.6
	7.0	0.40	0.27	0.17	36.8
Temperature (°C)	25	0.42	0.22	0.13	33.8
	30	0.44	0.29	0.16	40.3
	35	0.43	0.27	0.19	48.0
	37	0.44	0.23	0.19	46.3

achieved at other pH values. Such behaviour has also been reported by Hsu and Yang (1991). Thus, due to the higher Q_v, pH 6.5 was chosen for the next experiments. These results agree with most of the results reported in the literature, where a pH-range between 6.0 and 7.0 were reported as the optimum for growth and propionic acid production (Playne, 1985; Crespo *et al.*, 1990).

Effect of temperature

The studied temperatures were: 25, 30, 35 and 37 °C. After a 75-hour cultivation, the highest concentrations of cells and products were obtained in the range 35–37 °C (Fig. 1). At 25 and 30 °C, lower concentrations were achieved. Nevertheless, as demonstrated in Table I, similar propionic acid yields ($Y_{P/S}$) were calculated for any condition. From these results, one can conclude that temperature affects propionic acid production rate but not the stoichiometry of its formation from glucose. In contrast, cell growth ($Y_{X/S}$) was favoured by temperatures between 30 and 35 °C. The highest propionic acid volumetric productivities (Q_v), as expected, were found at 35–37 °C. The $Y_{P/S}$ is higher at 35 °C than at 37 °C, and therefore, this temperature was selected for the next experiments.

Effect of the vitamin-nitrogen source

There are reports about the influence of the yeast extract concentration on the growth of propionibacteria (Quesada-Chanto, *et al.* 1994). However, no information about the influence of the type of yeast extract is available. For many microorganisms, it probably makes no difference. For propionibacteria, our results have demonstrated that not only the growth and the final concentration of the organic acids were affected, but also the yield of each acid (Table II, Fig. 2). The best fermentation yields and the highest final cell and propionic acid concentration were achieved with Difco yeast extract, while the worst results were found utilising Prodex. It should be noticed that with Prodex, and also with Fluka yeast extract, an abnormally high succinic acid concentration was produced, resulting in decreasing propionic acid yields. With Prodex, for instance, succinic acid was more than 37% of the total acid concentration while with Difco yeast extract this proportion was less than 7%. This finding has not been described

Table II. Effect of the vitamin-nitrogen source on the yield, volumetric productivity and glucose consumption, in a batch fermentation of *Propionibacterium* shermanii CDB 10014 after 75 h.

Vitamin-nitrogen source		$Y_{P/S}$ (g/g)	$Y_{X/S}$ (g/g)	Q_v (g/l×h)	ΔS (%)
Yeast extract	Oxoid	0.50	0.31	0.24	47.5
	Fluka	0.42	0.33	0.29	69.1
	Difco	0.49	0.34	0.31	61.5
	Prodex	0.32	0.17	0.23	60.6
Corn-steep liquor		0.42	0.26	0.47	100
Soy brand		0.43	0.10	0.05	12.4

For abbreviation see legend of Table I.

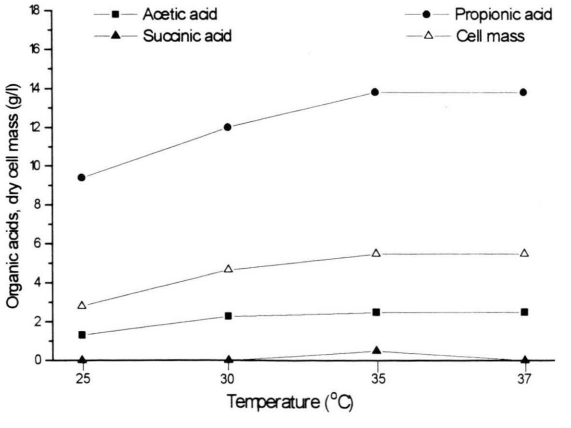

Fig. 1. Growth and organic acid production by *P. shermanii* at different temperatures.

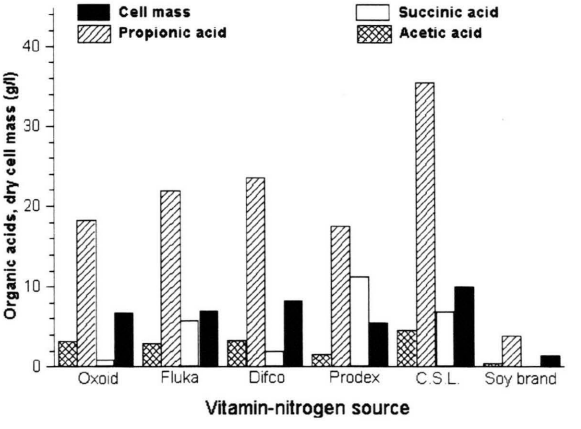

Fig. 2. Effect of different vitamin-nitrogen sources on growth and organic acids production by *P. shermanii*.

before, possibly due to the fact that in most of propionic acid fermentation studies gas chromatography has been used to measure the final products (propionic and acetic acid). Succinic acid is not detected by this procedure.

Although there is no experimental evidence, such behaviour could be explained by lack or presence of any substance interfering with the conversion of succinic acid to methylmalonyl-CoA in the metabolic pathway to produce propionic acid (methylmalonyl-CoA pathway (Schlegel, 1985)).

As to the other two vitamin-nitrogen sources tested, our results have shown that soy brand does not provide the growth requirements for *P. shermanii.* On the other hand, the presence of corn-steep liquor (C. S. L.) in the fermentation medium led to final cell and product concentrations higher than under any other condition (Table II, Figure 2). In this case, a complete depletion of glucose was reached and a final propionic acid concentration of over 35 g/l was achieved, with a normal conversion yield. The results with corn-steep liquor suggest that the relatively low propionic acid concentrations obtained with the other vitamin/nitrogen sources could be improved by increasing their concentrations in the fermentation media. Considering the high price of yeast extract, however, an economic process based on this material would be impossible. On the contrary, corn-steep liquor is inexpensive and, therefore, it could be used at any concentration, making this biotechnological process economically attractive.

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