Research Article

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Complex whey utilization: the propionic acid alternative

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Abstract: Whey is the complex waste of the dairy industry. Despite the fact, that it has numerous applications (like different form of food supplements), its major amount is still handled as waste. The carbohydrate, protein and lactic acid content, as well as the COD and BOD, are sufficiently high warranting disposal as waste resulting in high costs; however, their levels are insufficient for the cost-effective isolation and purification. Most of the numerous reports on whey utilisation focus on lactose utilization, while lactic acid removal is complex, but necessary, particularly in case of sour whey decontamination. According to our best knowledge among the microbial fermentation, the only lactic acid (as carbon source) utilization process is propionic acid fermentation. Propionic acid is an attractive product with a wide application range. In this study, two propionic acid producing microorganisms were investigated in terms of industrial applicability. The propionic acid producing bacteria are generally characterized by anaerobic metabolism (except the pathogenic P. acne); but, for application in a biorefinery, facultative anaerobe behavior is the most appropriate and cost-effective. In this study, the aero-tolerances of Propionibacterium freudenreichii subsp. shermanii and Propionibacterium acidipropionici were examined; their propionic acid-producing properties (yield, concentration, substrate preference, productivity) were compared.

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1 Introduction

Whey is a major by-product of the dairy industry with its volume increasing at about the same rate as that of milk production [1]. Whey exerts a considerable oxygen demand and its cost-effective disposal or utilization has become increasingly important to the modern dairy industry [2,3]. Whey is a solution mainly consisting of protein, carbohydrates and lactic acid, and many of the difficulties encountered in whey handling stem from this fact [4]. Although several possibilities of whey utilization have been considered over the last 50 years, half of the world whey production is not treated [5].

Propionibacteria are Gram-positive, non-motile, anaerobic to aero-tolerant bacteria, able to ferment different carbohydrates and certain polyalcohols to produce mainly propionic acid, acetic acid, and carbon-dioxide [6]. Strains of the genus *Propionibacterium* are used in several industrial processes. Propionibacteria are used by the dairy industry as a starter culture for the production of Swiss-type cheese [7]. The products from the metabolism of lactic acid are responsible for the characteristic eyes and contribute to the flavor, texture, and shelf life of Swiss cheeses [8]. Although they are used mainly in cheese production, Propionibacteria are also used industrially as silage inoculum, as a probiotic agent, and for the production of vitamin B_{12} and propionic acid [9].

Propionic acid also has many varied uses as an antifungal agent in foods and feeds [10]. As a preservative, propionic acid extends the shelf-life of food products by inhibiting molds and some bacteria [7]. It is also used for manufacturing of cellulose-based plastics, herbicides, and perfumes [11]. Calcium, sodium, and potassium salts of propionic acid are used as food preservatives, and are generally recognized as safe food additives [12].

Presently, there has been an increasing interest in the production of propionic acid by fermentative processes [13]. Although preservatives deriving from *Propionibacterium* fermentations are also available, most propionic acid used by the food industry is produced by chemical synthesis. If good yields of propionic acid could be obtained by fermentation of low-cost industrial wastes

or renewable sources, biological production of propionic acid could become economically competitive, besides reducing the contaminating industrial wastes [14]. At present, the maximum reported yield of propionic acid obtained by fermentation is still too low to be economically competitive with chemical synthesis. Therefore, our aim is to study propionic acid fermentation in different integrated processes, like biorefineries, to obtain roboust and cost-efficient technology. Thus, the first results reported here focused on comparison of different available strains in terms of aero-tolerance and propionic acid productivity.

2 Materials and methods

2.1 Microorganism

Propionibacterium freudenreichii subsp. *shermanii* (DSM 4902 equivalent to ATCC 9614), and *Propionibacterium acidipropionici* (DSM 20273 equivalent to ATCC 4965) were used in this study.

2.2 Media

The PYG medium for cell growth and inoculum preparation contained the following compositions per liter: 5.00 g trypticase peptone, 5.00 g peptone, 10.00 g yeast extract, 5.00 g beef extract, 5.00 g glucose, 2.00 g K_2HPO_4, 1.00 ml Tween-80, 40.00 ml salt solution (see below), 15 g agar (Difco, USA). While glucose was sterilized separately, every media component and equipment were sterilized in autoclave (Tuttnauer ELV3800 ELV) and added aseptically to the broth. The salt solution contained per liter: 0.25 g $CaCl_2$ x 2 H_2O, 0.50 g $MgSO_4$ x 7 H_2O, 1.00 g K_2HPO_4, 1.00 g KH_2PO_4, 10.00 g $NaHCO_3$ and 2.00 g NaCl.

Whey was obtained from a Hungarian dairy. It was stored in 1000 ml flasks at -18°C. The whey components were measured using high performance liquid chromatography (HPLC) as detailed under *Analytical Methods*. It contained 15.00 g/l lactic acid and 5.50 g/l lactose as usable carbon source.

2.3 Investigation of difference in the aero-tolerance between two *Propionibacterium*

To compare the aero-tolerances of the two strains, the first investigation was carried out on PYGA media (media PYG supplemented with 15 g/L agar) in two Petri-dishes per strain. The pH of media was adjusted to 6.9 using 25% aqueous ammonia solution, and sterilized at 121°C for 20 min in an autoclave. For inoculation each strain was grown in 150 ml PYGB (PYG without agar i.e. broth) containing Erlenmeyer flask and was shaken at 200 rpm at 30°C for 72 h under anaerobic conditions. One Petri-dish per strain was also incubated at the same time under aerobic conditions and under anaerobic conditions (Anaerobic jar, Merck) at 30°C (Memmert VO200) for 72 h.

To verify the above obtained results, another investigation was carried out in two 250 ml Erlenmeyer flasks containing 150 ml PYGB. One flask was inoculated with 20 ml of *P. acidipropionici* grown for 72 h at 30°C under anaerobic conditions (OD_{600nm}: 5.12). The other flasks was inoculated with 20 ml of *P. freudenreichii* grown for 72 h under anaerobic conditions (OD_{600nm}: 4.81) Both flask were incubated under aerobic conditions at 30°C in a shaking incubator (New Brunswick Scientific Excella E24) at 200 rpm.

2.4 Fermentation conditions

Propionic acid fermentation was carried out in 1000 ml bioreactors (B. Braun Biostat Q) containing 630 ml whey. The experimental culture was inoculated with 10% (v/v) propionic acid bacteria inoculum which was grown for 72 h at 30°C in a shaking incubator at 200 rpm. The culture pH was automatically adjusted to pH 6.5 by addition of 20 % NaOH. The culture temperature was controlled at 37°C, and the magnetic stirrer was set to 300 rpm. The samples were removed at regular intervals for further analysis.

2.5 Analytical methods

Cell growth was monitored by measuring the optical density (OD) at 600 nm in a 1 mL cuvette using a spectrophotometer (Pharmacia LKB Ultrospec Plus, 80-2092-26). Broth samples with suspended cells were diluted a priori five-fold with distilled water. Lactose and organic acids were quantified by using HPLC (Waters Breeze) with an organic acid analysis column (Aminex HPX- 87H, Bio-Rad) operated at 65°C with 0.5 mM H_2SO_4 as the mobile phase at 0.5 ml/min.

3 Results and discussion

3.1 Aero-tolerance

Both strains formed colonies under anaerobic conditions but only *Propionibacterium acidipropionici* formed

colonies under aerobic conditions. *Propionibacterium freudenreichii* did not grow under aerobic conditions. The two aerobically cultivated Erlenmeyer flasks were sampled after three days. The optical density of and pH of samples were determined. The OD_{600nm} for *Propionibacterium freudenreichii* was 0.825±0.08 and pH 6.5. The *Propionibacterium acidipropionici* had OD_{600nm} of 3.16±0.37 and pH 5.5.

As seen from these results, *Propionibacterium acidipropionici* have a higher tolerance against oxygen compared to *Propionibacterium freudenreichii*. Under aerobic conditions, the *Propionibacterium acidipropionici* also produced more acid than *Propionibacterium freudenreichii*.

3.2 Fermentation results

The results of whey based propionic acid bacteria fermentation in 1-L bioreactors (grown at 37°C, pH 6.5,) are shown in Figure 1.

As shown in Fig 1 *Propionibacterium freudenreichii subsp. shermanii* first consumed the lactic acid content and then the lactose. All of the lactic acid was consumed in the first 45 hours. The initial lactic acid and lactose concentrations were 15 g/L and 4.5 g/L, respectively. The fermentation time was 75 hours. At the end of the fermentation, 8.1 g/L propionic acid was produced with a 0.40 g/g yield.

Fig 2 shows whey fermentation by *Propionibacterium acidipropionici*. Similarly, this strain also preferably consumed lactic acid before metabolizing lactose. Most of the propionic acid was produced in parallel with lactic acid consumption. Lactose was only utilized when its concentration dropped under a critical level of lactic acid. The biomass production in Fig. 2 shows typical diauxic cell growth according to catabolit repression. The lactose consumption was also much slower than lactic acid consumption. The lactose content was not depleted until the end of the 100-hour fermentation.

As shown on Table 1, *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* produced 6.5 g/L and 5.5 g/L propionic acid from lactic acid, respectively. The productivity (from lactic acid) was 0.14 and 0.10 g/L*h for *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici*, respectively. The reason of this is that the *Propionibacterium acidipropionici* needed more time to convert the lactic acid into propionic acid. The final combined (lactose and lactic acid based) propionic acid concentrations from were 8.2 g/L for *Propionibacterium acidipropionici* and 6.1 g/L for *Propionibacterium freudenreichii*. The productivity of propionic acid was 0.11 g/l*h for *Propionibacterium freudenreichii* and 0.06 g/l*h for *Propionibacterium acidipropionici*, with propionic acid yields of 0.40 g/g and 0.35 g/g for these two respective strains.

4 Conclusions

Results of conducted experiments indicate that *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici* are able to utilize whey under microaerofil conditions, with average final propionic acid concentrations of 8.2 g/l and 6.1 g/l, respectively. Therefore, *Propionibacterium acidipropionici* has a better aero–tolerance compared to *Propionibacterium freudenreichii*. *However, Propionibacterium freudenreichii* can utilise lactose content in a shorter time and reach higher yield than *Propionibacterium acidipropionici*.

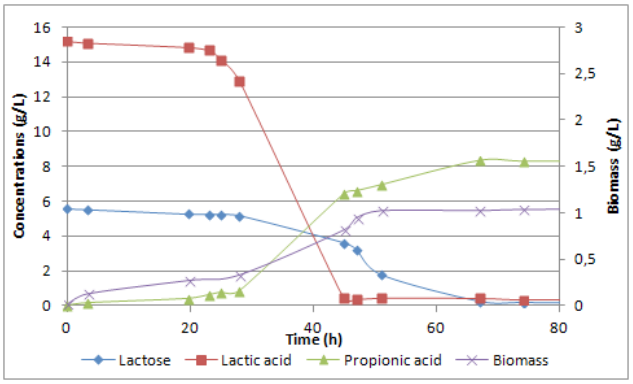

Figure 1. *Propionibacterium freudenreichii subsp. shermanii* fermentation on whey

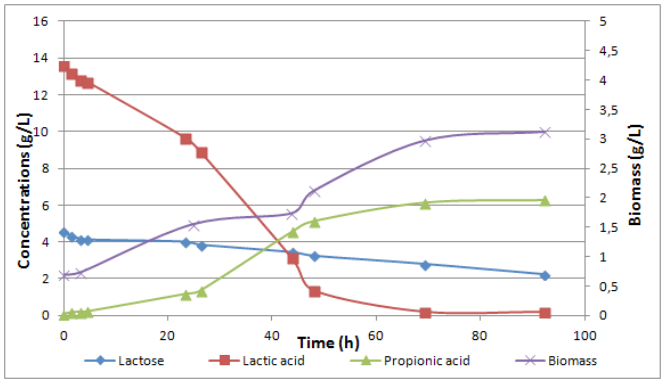

Figure 2. *Propionibacterium acidipropionici* fermentation on whey

Table 1. Whey fermentation with Propionibacterium species

Strains	Fermentation time (h) of lactic acid	Propionic acid production from lactic acid (g/L)	Productivity (g/L*h) from lactic acid	Yield (g/g) from lactic acid
Propionibacterium freudenreichii	45	6.5	0.14	0.43
Propionibacterium acidipropionici	55	5.5	0.10	0.41
	Fermentation time (h) of whey	**Final concentration (g/L) of propionic acid**	**Productivity (g/L*h)**	**Yield (g/g)**
Propionibacterium freudenreichii	75	8.2	0.11	0.40
Propionibacterium acidipropionici	100	6.1	0.06	0.35

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