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Simultaneous production of lactobionic and gluconic acid in cheese whey/glucose co-fermentation by *Pseudomonas taetrolens*

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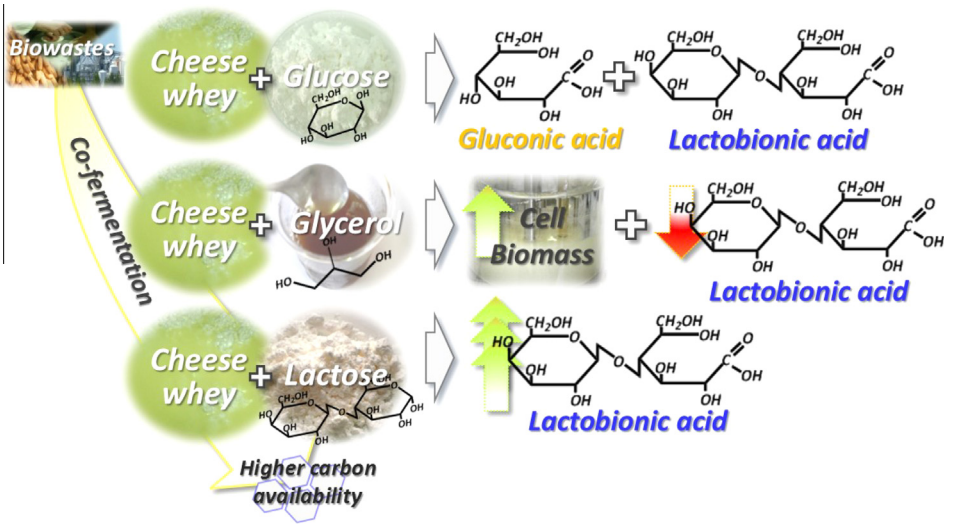
HIGHLIGHTS

- Novel bioprocess to co-produce simultaneously lactobionic and gluconic acid.
- Substrate versatility of *P. taetrolens* was evaluated in a co-fermentation system.
- Glycerol supply promoted cell growth but gave lower lactobionic acid yields.
- Overproduction of lactobionic acid was achieved by lactose feeding.
- Cellular functionality was strongly reliant on the type of co-substrate supplied.

GRAPHICAL ABSTRACT



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ABSTRACT

Substrate versatility of *Pseudomonas taetrolens* was evaluated for the first time in a co-fermentation system combining cheese whey and glucose, glycerol or lactose as co-substrates. Results showed that *P. taetrolens* displayed different production patterns depending on the co-substrate supplied. Whereas the presence of glucose led to a simultaneous co-production of lactobionic (78 g/L) and gluconic acid (8.8 g/L), lactose feeding stimulated the overproduction of lactobionic acid from whey with a high specific productivity (1.4 g/g h) and yield (100%). Co-substrate supply of glycerol conversely led to reduced lactobionic acid yield (82%) but higher cell densities (1.8 g/L), channelling the carbon source towards cell growth and maintenance. Higher carbon availability impaired the metabolic activity as well as membrane integrity, whereas lactose feeding improved the cellular functionality of *P. taetrolens*. Insights into these mixed carbon source strategies open up the possibility of co-producing lactobionic and gluconic acid into an integrated single-cell biorefinery.

1. Introduction

The rapidly expanding commercial applications for lactobionic acid have converted this organic acid into an emerging chemical

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that can be used either as biofunctionalization, protective, antioxidant, or gelling agent (Alonso et al., 2013a). Lactobionic acid has indeed become a key chemical platform in the development of biocompatible nanoparticles, biomaterials, and targeted drug delivery systems with promising biomedical uses (Feng et al., 2009; Zhang et al., 2011). As a result, the market niche for lactobionic acid is forecasted to increase significantly in the coming years, growing in both food and pharmaceutical applications (Affertsholt, 2007).

Currently, lactobionic acid is exclusively produced via chemical synthesis from refined lactose in an energy-intensive and costly process (Alonso et al., 2013a). However, biotechnological production of lactobionic acid from an inexpensive source like cheese whey has recently appeared as a cost-effective and sustainable alternative for its industrial production (Alonso et al., 2011, 2012a, 2013b). Lactobionic acid constitutes a non-growth associated sugar organic acid produced by *Pseudomonas taetrolens* in a fermentation system that can be impaired either by over-supply of dissolved oxygen (Alonso et al., 2012a) or inappropriate pH-control strategies (Alonso et al., 2013c). Both metabolic overflow and stress-induced physiological responses may likewise arise due to imbalance bioprocessing conditions, leading thereby to a reduced biological performance of *P. taetrolens* cells (Alonso et al., 2012b, 2013c). Addressing such physiological information has been proven effective in the selection of feeding strategies that sustain enhanced lactobionic acid production outputs (Alonso et al., 2013b). Though the physiological robustness was preserved by implementing novel feeding approaches, the substrate versatility of *P. taetrolens* remains to be elucidated. The supply of a second carbon source may lead to higher cell densities, and therefore enhanced overall productivities. The integration of such co-substrate strategies may additionally open-up the application of biorefinery approaches for high-yield lactobionic acid production.

Impelled by the depletion in carbon sources and the increase in the fermentation costs, the search for alternative feedstocks has become the driving force behind the development of innovative biorefinery approaches targeting the microbial production of organic acids (Dishisha et al., 2013; Lin et al., 2011). Such approaches have the potential to attain a cost-competitive organic acid biomanufacturing while upgrading high-strength polluting biowastes (Alonso et al., 2014). Co-fermentation strategies combining several unrelated-carbon sources have additionally received considerable interest as promising cultivation modes for channelling both carbon and energy fluxes towards improved metabolite production patterns (Kang et al., 2011; Wang and Yang, 2013). Likewise, synergies between mixed substrates and co-production systems can be drawn to design integrated biorefinery cell platforms in which metabolite formation may be modulated depending on the carbon source supplied (Liang and Qi, 2014). In this sense, improved value-added product portfolios with complementary commercial applications can be targeted via innovative co-production systems (Kang et al., 2011; Lazar et al., 2011).

Maintaining non-limiting nutrient conditions by supplying extra carbon sources may undoubtedly be a potential strategy for prolonging the cellular growth phase, and therefore leading to higher cell densities during lactobionic acid production. This study aimed at implementing a novel co-fermentation system with glucose, lactose and glycerol as co-substrates for high-yielding bio-production of lactobionic acid, emphasizing the use of cheese whey as feasible and inexpensive feedstock. Assessment of *P. taetrolens*' substrate versatility resulted in a novel system to co-produce two sugar organic acids into an integrated single-cell biorefinery using a co-fermentation strategy. However, both higher carbon availability and carbon-limited conditions should be carefully balanced against a reduced cellular functionality. To this end, the present study additionally evaluated how C-source shifts impact on the adaptive physiological responses at the single-cell level, providing new insights into process robustness for effective carbon uptake during a mixed carbon source process.

2. Methods

2.1. Microorganism

P. taetrolens LMG 2336, obtained from the Belgian Coordinated Collection of Microorganisms (Gent, Belgium), was maintained frozen (in 40% [v/v] glycerol at −20 °C). The strain was subsequently subcultured on Nutrient Broth (NB, containing 1 g/L meat extract, 2 g/L yeast extract, 5 g/L peptone and 5 g/L NaCl) agar plates, incubated for 48 h at 30 °C, and then preserved at 4 °C.

2.2. Inoculum preparation

A loopful of *P. taetrolens* from a fresh Nutrient Broth agar plate was used to inoculate a 500 mL Erlenmeyer flask containing 100 mL of Nutrient Broth medium. This flask was incubated on an orbital shaker (New Brunswick Sci., NJ, USA) at 250 rpm and 30 °C for 10 h. Actively growing cells from this culture were subsequently employed as inoculum for bioreactor seed cultures or shake-flask cultivations on whey.

2.3. Sweet whey preparation

Diluted sweet cheese whey was prepared by diluting sweet cheese whey (provided by ILAS S.A., Asturias, Spain) 1-fold with distilled water (1:1) and adjusted to pH 6.5 (by adding 6 M NaOH) prior to sterilization using a tangential microfiltration device equipped with a 0.22 μm pore size PVDF membrane-cassette (Millipore, Massachusetts, USA). Concentrated cheese whey for fed-batch bioreactor cultures was prepared as above via microfiltration of undiluted sweet cheese whey.

2.4. Shake-flask cultivations

Shake-flask cultivations were conducted in 500 mL Erlenmeyer flasks containing 100 mL of sweet whey inoculated with 0.14 g/L of wet biomass from NB inoculum cultures harvested by centrifugation at 11,000*g* for 10 min. Cultivations on cheese whey were carried out with and without carbon source supplementation in order to study the substrate versatility of *P. taetrolens*. To this end, 500 mL Erlenmeyer flasks containing 100 mL of whey were supplemented with glucose (Panreac, Spain) or glycerol (Panreac, Spain) at concentrations of 5, 10, 20, and 40 g/L. These cultures were subsequently incubated on an orbital shaker (Excella E24, New Brunswick Sci., NJ, USA) at 250 rpm and 30 °C. Samples were aseptically withdrawn periodically to determine bacterial growth and pH. Biomass was removed by centrifugation at 16,000*g* for 5 min, the cell-free supernatants being stored frozen (−20 °C) until further analysis.

2.5. Fed-batch bioreactor cultivations

Bioreactor cultivations were performed in a 2-L bioreactor (BioFlo 110, New Brunswick Sci., NJ, USA) with an initial working volume of 1 L at 30 °C and an aeration rate of 1 Lpm. Agitation was automatically adjusted to keep dissolved oxygen tension (DOT) above 10% via an agitation cascade (from 350 to 500 rpm). Excessive foam formation was prevented by automatic addition of diluted (1:10) Y-30 emulsion (Sigma–Aldrich, Germany). The bioreactor was equipped with a pH meter (Mettler Toledo, Switzerland) and a polarographic dissolved oxygen electrode