RESEARCH
ARTICLE

Lactobionic acid production by *Pseudomonas taetrolens* in a fed-batch bioreactor using acid whey as substrate

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Acid whey causes a major disposal problem for the dairy industry due to its low pH and high salt concentration. Lactobionic acid (LBA) production by whey fermentation is an inexpensive process. The goal of this work was to employ acid whey for LBA production. Acid whey was fermented in a bioreactor, adding Pseudomonas taetrolens *LMG 2336 at 30°C and 6.5 pH, with 1.5-Lpm oxygen aeration and 350-rpm agitation. Three fermentation tests were carried out with a different amount of inoculum (10%, 30% and 10% three times within 24 h). Results indicated that repeatedly adding* P. taetrolens *inoculum to the acid whey substrate allows a complete lactose conversion into LBA, while the lactose oxidation process was insufficient in the sample where the inoculum was only added at the beginning of the fermentation process (only 29.7% LBA production with 10% inoculum). The physiological heterogeneity of* P. taetrolens *was determined by multiparametric flow cytometry, and results showed that there was a phenotypic adaptation of the microorganism due to the changes observed in its heterogeneity and physiological state. The results achieved will help to recycle acid whey for value-added product production such as LBA production.*

Keywords Lactobionic acid, Acid whey, Lactose oxidation, *Pseudomonas taetrolens*.

INTRODUCTION

Whey is widely used as a raw material in different fields in the food industry due to the high nutritional value of its main components, but nonetheless, far more is produced than is recycled as a raw material. Acid whey is a major by-product for which the dairy industry has, for a considerable time, struggled to find a sustainable use, despite the fact that whey disposal without proper treatment causes serious environmental pollution problems due to its high oxygen demand, both chemically (~70 000 mg/L) and biologically (~40 000 mg/L) (Macwan *et al.* 2016). It is estimated that acid whey production in the European Union is 40 million tonnes per year, while the Greek yoghurt and cottage cheese market annually generates billions of kilograms of this by-product. These large amounts of acid whey generated by the industry

have led to increasingly widespread research into methods for making use of it (Rocha-Mendoza *et al.* 2021). Acid and sweet whey contain comparable quantities of carbohydrates, but acid whey commonly has a higher concentration of salts and a slightly lower amount of protein. Unfortunately, some of the chemical properties of acid whey make it difficult to process and frequently limit its use in the food industry (Mano *et al.* 2020). Certainly, acid whey's low pH must be fully or partially neutralised before its addition to food products as a supplement designed to produce changes in their physicochemical and sensory properties (Sahar *et al.* 2020).

Nonetheless, the worldwide reality of the low cost and surplus of lactose, the principal component of acid whey, has inspired scientists to seek innovative technologies for producing valuable derivatives from lactose and extend their usage

in the food, chemical and pharmaceutical industries (Gänzle *et al*. 2008). The goal in the dairy field is to obtain an income flow from dairy production waste so as to generate products with high added value that have potential profitability (Mano *et al*. 2020).

Whey is a cheap substrate for the biobased production of lactobionic acid (LBA) by microorganisms on an industrial scale (Sarenkova and Ciprovica 2018). An effective and sustainable bioproduction system has been optimised to produce LBA from sweet whey, using *Pseudomonas taetrolens* (Alonso *et al*. 2011, 2012a, 2012b, 2013). Lactose can also be transformed by the enzymatic complex of glucose–fructose oxidoreductase and glucose lactonase of *Zymomonas mobilis*, which leads to a high LBA yield in the fermentation substrate. *P. taetrolens* possesses an efficient agent for the biosynthesis of LBA in the form of its lactonase and lactose oxidase enzymes (Alonso *et al*. 2013, 2017; Delagustin *et al*. 2019). The economic feasibility of the synthesis of organic acids by bioproduction demands a yield of more than 1 g/L/h, and the final product concentration needs to reach more than 50 g/L (Yang *et al*. 2007).

Lactobionic acid is an aldonic acid and an oxidation product of lactose, containing gluconic acid and galactose, which has potential applications in the food, medicine, cosmetic, pharmaceutical and chemical industries (Alonso *et al*. 2013). In recent years, LBA has received attention as a food additive due to its functions, such as flavour enhancement, as well as being a texturiser, acidulant, solubilising agent, gelling agent, low-calorie sweetener and humectant; furthermore, it is recognised as a prebiotic (Alonso *et al*. 2013; Goderska 2019; Cardoso *et al*. 2019). Currently, the price of LBA varies from one supplier to another and it costs between USD 40 and 649 per 100 g. Lactobionic acid as a laboratory reagent with higher purity is more expensive. The Food and Drug Administration (FDA) has approved the use of calcium lactobionate (obtained from LBA) as a food additive, and it is cheaper than LBA, costing between USD 22 and 142 per 100 g (Sarenkova and Ciprovica 2018). With current worldwide regulations, food applications with LBA are rare (Cardoso *et al*. 2019). Nonetheless, LBA has rapidly grown in importance as a strategic functionalisation molecule in the development of nanoparticle-based platforms and biomaterials with promising therapeutic applications (Alonso *et al*. 2013).

Already, several research studies aiming to produce LBA by biosynthesis from cheese whey have been carried out. Nearly all the studies deal with the use of cheese whey as a fermentation broth (Alonso *et al*. 2011, 2012a, 2012b, 2017; Goderska *et al*. 2014; Giorgi *et al*. 2018). Treatment of acid whey (pH ~ 4.5) to obtain LBA by biosynthesis has not yet been announced as effective, however, because bacterial reproduction is low in this substrate. Thus, the objective of this study was to achieve the valorisation of acid whey by developing a fermentation process to obtain LBA with high lactose conversion and LBA production employing different strategies, especially adding fresh *P. taetrolens* LMG 2336 inoculum to the acid whey substrate periodically during fermentation.

MATERIALS AND METHODS

Microorganisms
P. taetrolens LMG 2336, obtained from the BCCM (Belgian Coordinated Collection of Microorganisms, Gent, Belgium), was maintained frozen (in 40% (v/v) glycerol at −20°C). The bacterial strain was cultured on NB (Nutrient Broth, with 20 g/L agar, 5 g/L NaCl, 5 g/L peptone, 2 g/L yeast extract and 1 g/L meat extract, all from Sigma-Aldrich, Steinheim, Germany) agar plates (20 g/L agar; VWR Chemicals, Radnor, PA, USA). The agar plates were incubated at 30°C for 48 h and used directly for the preparation of inoculum.

Inoculum preparation
Erlenmeyer shaker flasks containing 100 mL of NB were inoculated with a 10 μL loopful of *P. taetrolens* from the NB agar plates. These samples were incubated for 10 h at 30°C in an orbital shaker (New Brunswick Sci., Edison, NJ, USA) with an agitation rate of 350 rpm. The biomass of *P. taetrolens* was separated by centrifugation at centrifugation speed of 20 720 x g for 10 min. and then used as a bulk starter.

Acid whey preparation
Acid whey (artisan cheesemaker Ca Sanchu, Asturias, Spain) was pasteurised at 90°C for 45 min. and filtered through cheesecloth. Then, it was purified with a tangential microfiltration device equipped with a 0.22-μm pore size polyvinylidene difluoride (PVDF) membrane cassette (Millipore, Bedford, MA, USA). Acid whey used for fermentation experiments had the following parameters: pH, 4.57 ± 0.06; lactose, 4.2 ± 0.14%; proteins, 0.14 ± 0.04%; and fats, 0.01 ± 0.01%. For fat determination, a MilkoScan™ Mars (Foss, Hillerød, Denmark) analyser was used. Protein content was measured using the Kjeldahl method (ISO 8968-1:2014) and the lactose concentration by the high-performance liquid chromatography (HPLC) (detailed in the 'Analytical Methods' section).

Culture conditions and fermentation experiments
The fermentation process was performed in a 2-L bioreactor (BioFlo 110; New Brunswick Scientific, Edison, NJ, USA) with a final working volume of 1 L. Three different types of inocula with the fresh biomass of *P. taetrolens* were prepared. The amount of inoculum was selected according to the results of previous studies with the same microorganism in sweet whey (Alonso *et al*. 2011). In sample AW1, acid whey was inoculated with 10% (v/v) inoculum at the

beginning of the fermentation process. For sample AW2, acid whey was inoculated with 10% (v/v) inoculum at the beginning of the experiment, with 10% (v/v) inoculum at 12 h and with 10% (v/v) inoculum at 24 h after fermentation started, amounting to 30% total inoculum, separated in time. In sample AW3, the acid whey was inoculated with 30% (v/v) inoculum at the beginning of the fermentation process. All experiments were performed at 30°C for 72 h with 1.5-Lpm aeration rate and 350-rpm agitation. The pH was detected using an InLab® Expert Pro-ISM pH electrode (Mettler Toledo, Greifensee, Switzerland) and maintained at the value 6.5 by computer-controlled peristaltic pumps, which added 6 M NaOH (Sigma-Aldrich, Hamburg, Germany) automatically to avoid excessive acidification of the fermentative medium. Dissolved oxygen tension (DOT) was detected with a polarographic dissolved oxygen electrode (InPro 6830; Mettler Toledo, Greifensee, Switzerland). To remove the excess of foam, Y-30 emulsion (1:10; Sigma-Aldrich, Saint Louis, MO, USA) was added automatically. All fermentation experiments were performed in triplicate. Samples were taken during the fermentation process at different times to determine the following parameters: dry cell weight, optical cell density (OD), bacterial growth on NB agar plates, lactose and LBA concentration.

Staining procedures and multiparametric flow cytometry

Multiparametric flow cytometry was performed (FCM) to check the physiological status of *P. taetrolens* in order to differentiate between viable, damaged and dead subpopulations during fermentation experiments. Samples were taken at times 0, 12, 24, 36, 48, 60 and 72 h. They were centrifuged at centrifugation speed of 20 720 x g for 10 min, and the pellet was washed twice with phosphate-buffered saline (PBS, pH 7.4, sterile and 0.22-μm filtered; Sigma-Aldrich) and adjusted to a cellular density of between 10^6 and 10^7 cells/mL in the same PBS buffer. Before staining, and in order to prevent bacterial aggregation, samples were sonicated for 2 s at a temperature of 20°C with ultrasound at 37 kHz and 7 W/cm^2.

Cells were stained with a mixture of three fluorescent dyes, SYBR Green (Life Technologies Corporations, Eugene, OR, USA), ChemChrome V6 (CV6, Combourg, Chemunex, France) and propidium iodide (PI, Molecular Probes). SYBR Green stained all bacteria (live and dead) and was used to establish the bacterial concentration for FCM. Metabolic activity and membrane integrity were checked by staining with CV6 and PI, respectively. Staining solutions were prepared according to García *et al.* (2017) with some modifications. SYBR Green was diluted 1:10 000 in TE buffer (10 mM Tris-HCl and 1 mM EDTA (both from Sigma-Aldrich), pH 8.0, 0.22-μm filtered); CV6 was diluted 1:10 in sterile distilled water (0.22-μm filtered), and PI was prepared at a concentration of 1 mg/mL in

sterile distilled water (0.22-μm filtered). 6 μL, 8 μL and 7.5 μL of SYBR Green, CV6 and PI staining solutions, respectively, were added to 200 μL of cellular suspension. Samples were incubated for 15 min in the dark at room temperature before testing.

To set up gates in the FCM density dot plots, different control samples of *P. taetrolens* were prepared. Dead bacteria were obtained by heating samples at 90°C for 45 min and immediately frozen after the incubation time. Live bacteria were tested in the early exponential growth phase, and a mixture containing both dead and live bacteria (1:1) was also prepared. Unstained cells were used as the control.

Flow cytometry measurements were performed employing a CytoFLEX S flow cytometer (Beckman Coulter, Life Sciences, Indianapolis, IN, USA). Green fluorescence, corresponding to SYBR Green and CV6-stained cells, was collected on the FL1 channel (561 nm), while PI fluorescence was measured on the FL4 channel (638 nm). Analyses were carried out at a low flow rate setting (between 3000 and 4000 events/s). Data acquisition and analysis were performed using CytExpert 2.1 (Beckman Coulter) software.

Analytical methods

Bacterial growth was determined spectrophotometrically from the optical density at 600 nm (Thermo Scientific Helios Gamma UVG, Loughborough UK) after sample centrifugation at centrifugation speed of 20 720 x g for 10 min. Dry cell weight was determined using 1 mL of fermented substrate and was expressed in g/L. After two cycles of washing with phosphate-buffered saline (PBS, pH 7.4, sterile and 0.22-μm filtered; Sigma-Aldrich) followed by sample centrifugation at centrifugation speed of 20 720 x g for 10 min, the pellet was dehydrated by drying at 45°C until reaching constant weight. The viable cell count was performed by the spread plate method in NB agar. Colony forming units (cfu) were counted after incubating NB agar plates at 30°C for 48 h. Results were expressed as cfu/mL.

Lactose and LBA concentrations in cell-free culture samples were detected by HPLC (Agilent 1200; Agilent Technologies, Inc., Santa Clara, CA, USA), employing a column (Coregel ION 300; Teknokroma, Barcelona, Spain) coupled to a refractive index detector at 40°C. Sulphuric acid (0.450 mM, pH 3.1) was used as the mobile phase, with a column temperature of 75°C and a flow rate of 0.3 mL/min. Data acquisition and analysis were performed with ChemStation software (Agilent).

Fermentation parameters

Different parameters have been calculated for the fermentation processes. Growth rate μ (h^{-1}) was calculated as the slope of the regression line between ln (x/x_0) versus time during a specific time interval using Eqn (1) (Shuler and Kargi 2002; Alonso *et al.* 2017).

$$\mu = \frac{In\left(\frac{x}{x_0}\right)}{t - t_0}, \qquad (1)$$

where x is the corresponding biomass concentration at t (g/L); x_0 is the corresponding biomass concentration at t_0 (g/L); t is the time at the end of the exponential phase (h); and t_0 is the initial time of the exponential phase (h). Volumetric productivity is defined as the total amount of LBA formed per unit volume per time. The LBA productivity in the fermentation process WP (g/L/h) was calculated according to Eqn (2) (Prasirtsak *et al.* 2019).

$$WP = \frac{P}{t_{dead} + t}, \qquad (2)$$

where P is the amount of LBA produced during the process (g/L); t_{dead} is the time of the lag phase (h); and t is the process time (h). Biomass productivity of the fermentation process WX (g/L/h) was evaluated according to Eqn (3) (Hempel *et al.* 2002).

$$WX = \frac{X}{t_{dead} + t}, \qquad (3)$$

where X is the amount of biomass produced during the process (g/L).

The specific LBA production rate qLBA (g/g/h) was calculated (Alonso *et al.* 2017) by dividing the volumetric LBA productivity WP (g/L/h) by the average biomass ΔX (g/L) during the fermentation process using Eqn 4.

$$qLBA = \frac{WP}{\Delta X}, \qquad (4)$$

The ratio of biomass produced with respect to the amount of lactose consumed during fermentation $Y^X/_S$ (g biomass/g substrate) was calculated using the following Eqn (5) (Kargi 2009).

$$Y\frac{X}{S} = \frac{X_t - X_{t0}}{S_{t0} - S_t}, \qquad (5)$$

where X_{t0} is the initial concentration of fresh biomass (g/L); X_t is the final concentration of the biomass at time t (g/L);
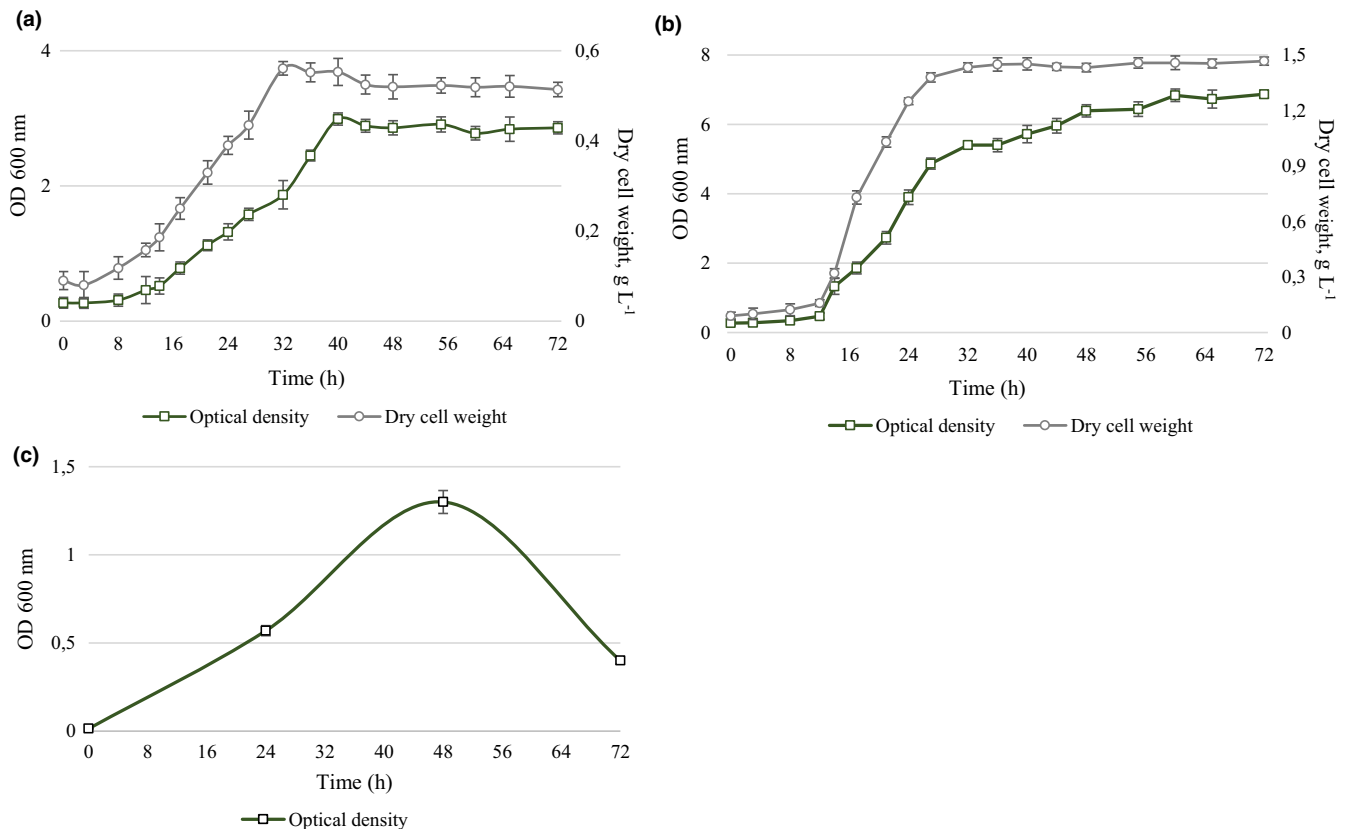
Figure 1 Time course graph of optical density (600 nm) and dry cell weight (g/L) of *Pseudomonas taetrolens* during the fermentation of acid whey in bioreactor for 72 h. (a) AW1 fermentation with 10% (v/v) inoculum at the beginning of the fermentation process; (b) AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started; and (c) AW3 fermentation with 30% inoculum at the beginning of the experiment.
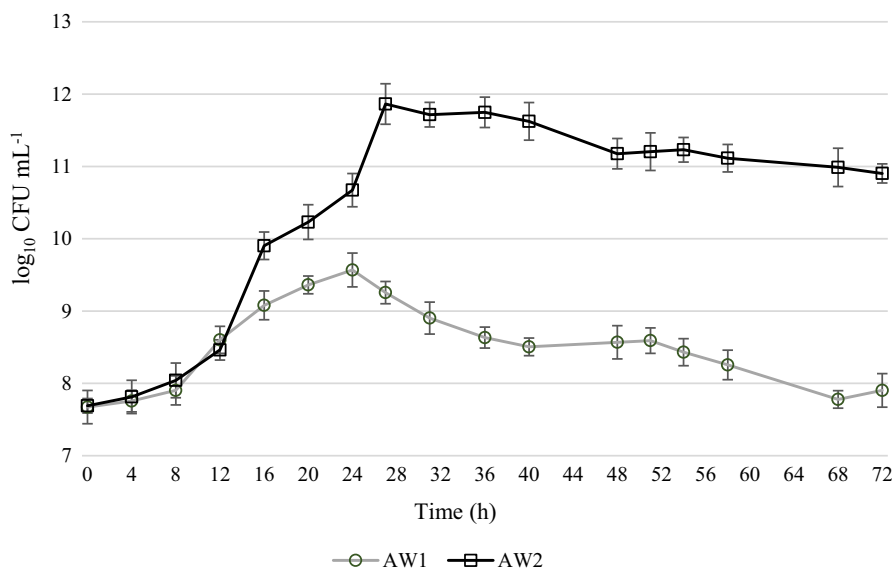
Figure 2 Time course graph of *Pseudomonas taetrolens* viable cell count during 72 h of fermentation. The number of microorganisms is expressed as $\log_{10}$ cfu/mL. AW1 fermentation process with 10% (v/v) inoculum at the beginning of the fermentation process; AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started.

S_{t0} is the initial concentration of substrate (g/L); and S_t is the concentration of substrate at time t (g/L).

Statistical analysis

Three independent experiments were carried out, and the results were expressed as the mean value. Data acquisition and analysis were performed with the variance analysis method ANOVA, and *t*-tests were performed. A level of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Pseudomonas taetrolens growth in acid whey

An analysis was performed to determine whether *P. taetrolens* can grow in acid whey and how the inoculum concentration affects the fermentation process, dry cell weight and OD (Figure 1), as well as viable cell count (Figure 2).

Figure 1 shows the OD and dry cell weight for the AW1 (Figure 1a) and AW2 (Figure 1b) samples and the OD for AW3 (Figure 1c) fermentation. As can be seen in Figure 1, the addition of 30% inoculum at the beginning of the experiment presented a significantly lower optical density in the AW3 sample, with a maximum of 1.3 OD after 48 h, showing that in acid whey, this high initial inoculum volume (30%) is inhibited by the low availability of substrate for the high quantity of biomass, and is therefore inappropriate. Due to these results, the dry cell weight of the AW3 sample was not analysed. In the AW1 sample, optical density and dry cell weight from 16 h until the end of fermentation were lower than in the AW2 sample, with a significant difference
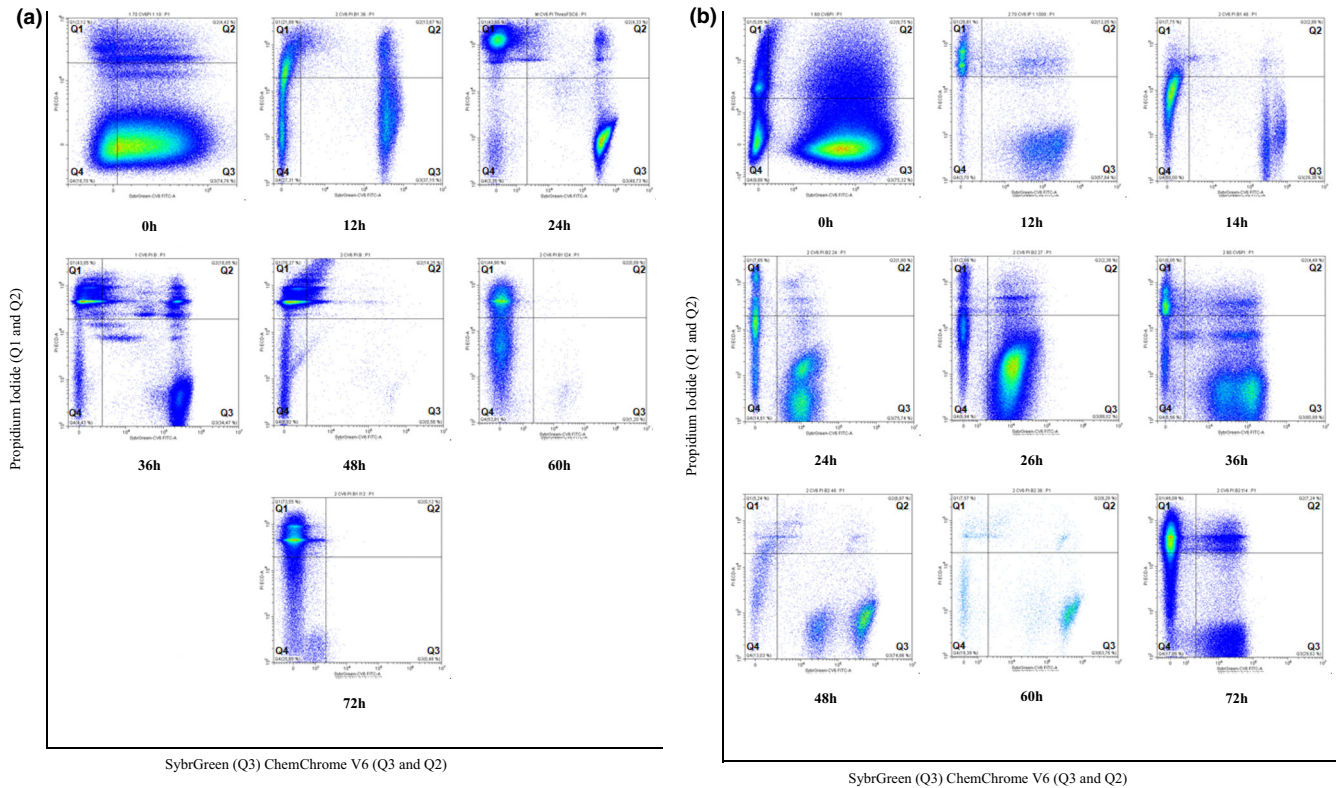
Table 1 Summary of values acquired in AW1 and AW2 acid whey fermentation processes. AW1 fermentation process with 10% (v/v) inoculum at the beginning of the fermentation process; AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started

	AW1	AW2
μ (h^{-1})	0.06 ± 0.01^a	0.15 ± 0.01^b
μ_{max} (h^{-1})	0.08 ± 0.01^a	0.27 ± 0.01^b
Duration of the lag phase (h)	16^a	14^b
WP (g/L/h)	0.22 ± 0.01^a	0.74 ± 0.02^b
qLBA (g/g/h)	0.60 ± 0.02^a	0.73 ± 0.03^b
Maximum optical density, 600 nm	2.99 ± 0.02^a	6.87 ± 0.09^b
Maximum bacterial count ($\log_{10}$ cfu/mL)	9.57 ± 0.17^a	11.82 ± 0.23^b
$Y^{X}/_{S}$ (g/g)	0.035 ± 0.002^a	0.034 ± 0.004^a
WX (g/L/h)	0.010 ± 0.002^a	0.027 ± 0.001^b
Maximum dry cell weight (g/L)	0.56 ± 0.12^a	1.47 ± 0.18^b
Fermentation time (h)	72^a	48^b
Conversion yield (%)*	29.7 ± 1.6^a	99.85 ± 0.01^b

*Yield was shown as the percentage of lactose converted into LBA at the end of the fermentation process.

[a,b]Means in the same row followed by different letters differ at $P < 0.05$. μ, specific growth rate; μ_{max}, maximum specific growth rate; qLBA, the specific LBA production rate; $Y^{X}/_{S}$, the amount of biomass produced in relation to the amount of lactose consumed during fermentation; WX, biomass productivity of the fermentation process; WP, LBA productivity of the fermentation process.

Figure 3 Density dot plots representing *Pseudomonas taetrolens* heterogeneity and physiological state during the fermentation process. Density plots display parameters as a frequency distribution, each cell is represented as a dot: red, yellow, green, light blue and dark blue dot spots represent the density gradient, from the highest to the lowest bacterial concentration, in (a) AW1 fermentation process with 10% (v/v) inoculum at the beginning of the fermentation process and in (b) AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started.

($P > 0.05$). During the first 14 h of fermentation, there were no significant differences ($P < 0.05$) between the AW1 and AW2 samples. After 14 h in AW2, the second inoculum became adapted to the medium and differences between the two fermentations are noticed. The maximum optical density and dry cell weight achieved in samples are represented in Table 1. All results showed that the bacterial population grew moderately in acid whey, but repeatedly adding inoculum of *P. taetrolens* helped to increase the growth and the bacteria adapted successfully, even reaching an amount of biomass similar to that noticed by other authors who employed the same bacteria but used sweet whey as substrate (Alonso *et al*. 2011, 2012a, 2012b, 2017).

As unsatisfying results were obtained with the AW3 fermentation, the growth curve of viable bacteria was only measured for the AW1 and AW2 samples (Figure 2). As Figure 2 shows, significantly different results were obtained with AW2, reaching a maximum of 11.82 $\log_{10}$ cfu/mL, while in AW1, the maximum was 9.56 $\log_{10}$ cfu/mL. The slope of the bacterial growth curve for the AW2 fermentation was steeper between 12 and 14 h and also between 24 and 26 h. These were the points when the fresh bacterial inoculum was added. The viable cell count

fits with the results obtained for OD and dry cell weight data (Figure 1).

During the fermentation processes, the pH was adjusted to a value of 6.5 using 6 M NaOH by adding between 10 and 30 mL of 6 M NaOH to 1 L of acid whey. It is assumed that the growth of *P. taetrolens* bacteria in acid whey is influenced negatively by Na^+ ions, and as a result of the pH adjustment, the acid whey substrates contain more Na^+ than the sweet whey substrates. Studies have shown that Gram-negative bacterial growth decreases in substrates with a high presence of Na^+ and/or Cl^- ions. As a result, under such conditions the bacterial cell membrane easily transports these ions into the cell and changes its osmotic pressure, which can affect the viability of the bacteria. Researchers have noticed that the activity of *P. aeruginosa JCM5962* (T) enzymes and other species of *Pseudomonas* decreases when the medium has a high Na^+ ion concentration (Gautam and Azmi 2017; Sachan *et al*. 2017).

Physiological status of *Pseudomonas taetrolens* monitoring by multiparametric flow cytometry

Multiparametric flow cytometry was used to determine the cellular state of the microorganisms and to verify whether the

strategy of adding inoculum progressively was effective in improving the fermentation process. As the AW3 fermentation showed the worst results, these analyses were done exclusively with samples AW1 and AW2. Results are shown in Figure 3.

In Figure 3density plots display parameters as a frequency distribution, each cell is represented as a dot: red, yellow, green, light blue and dark blue dot spots represent the density gradient, from the highest to the lowest bacterial concentration. Density plots depicts the distribution of cells within a population characterized by a very high density of events. The dual staining made it possible not only to distinguish dead cells (Q1-PI-stained) from living ones (Q3-CV6-stained) but also to detect damaged cells (Q2-CV6/PI-stained), which may subsequently recover their activity.

Figure 3 (a) shows the results for the AW1 fermentation. At the beginning of the fermentation process, the percentage of live *P. taetrolens* was 74.76%. However, it was observed that after 48 h, most of the microorganisms were dead (76.27%) and the rest were damaged (14.25%). Similar results were obtained for 60 and 72 h.

For AW2 fermentation (Figure 3b), the number of live bacteria was similar to AW1 fermentation at the beginning of the experiment (75.32%). However, the addition of new inoculum at 12 and 24 h resulted in a higher population of live bacteria during 72 h of fermentation, with 29.62% live organisms at the endpoint and 7.24% damaged. The positive effect of the addition of new biomass was mainly observed after the addition of inoculum at 24 h. At 26 and 36 h of fermentation, the percentages of live bacteria were 88.02 and 80.89%, respectively.

In both cases, it was observed that the number of damaged but living cells was low compared with the numbers of live or dead cells, showing that the fermentation conditions led to a brief damaged period before the cells died, perhaps due to nutritional limitation causes.

Figure 4 shows the percentages of *P. taetrolens* subpopulations (dead, live and damaged) in both fermentations. In Figure 4 (a), it can be noticed how rapidly dead cells appeared in the AW1 fermentation, since after 36 h the bacteria were mostly dead. Thus, *P. taetrolens* can adapt properly to acid whey substrate. On the contrary, in the AW2 fermentation metabolically live bacteria dominated throughout the 72 h of fermentation. The AW2 fermentation shows similar physiological heterogeneity of *P. taetrolens* as in the sweet whey medium (Alonso *et al*. 2012b), where the smallest part also consisted of dead cells, while viable cells dominate, especially during the growth phase.

The flow cytometry results obtained could be explained by a repeated adaptation of *P. taetrolens* as inoculum is added. Although there are no scientific data in the literature on *P. taetrolens*, information on *P. aeruginosa* strains is available. It has been shown that the cells of these bacteria are inhibited by high concentrations of sea salt in the substrate, but over time, they can adapt to the substrate and even can survive for several years. Such adaptation is considered phenotypic rather than genetic (Elabed *et al*. 2019). This adaptability could be compared with the growth of *P. taetrolens* in acid whey, resulting in the inhibition of the bacteria in the studied substrate. However, a small proportion of bacterial cells slowly begin to adapt and survive, indicating the potential of acid whey for LBA production. Adding additional inoculum of bacteria to the acid whey, in which some bacteria have already adapted, could promote the bioconversion of lactose into LBA.

pH and DOT change during fermentation

The pH and DOT were monitored in the AW1 and AW2 fermentations (Figure 5), since these parameters can indicate the behaviour of the bacteria in substrates. In both cases, the initial pH was 4.57 and was adjusted automatically to 6.5 by adding 6 M NaOH. The pH increased at the end of the fermentation process (after 40 h in AW1 and after 58 h in AW2). After 40 h of fermentation, there were significant differences ($P < 0.05$) in pH between the samples. A pH of 6.5 is the optimum for *P. taetrolens* to convert lactose into LBA at the highest rate, and decreasing or increasing the pH from 6.5 can affect the LBA production rate (Alonso *et al*. 2013). The pH reached 8.76 in the AW1 and 7.14 in the AW2 samples at the end of the fermentation. Usually, pH decreases during the lactose oxidation process, because of the conversion of lactose into LBA. However, the decomposition of dead bacterial cells provides more nitrogen compounds to the substrates and the nitrogen is converted into ammonium ions, which promote an increase in pH (Strock 2008).

In Figure 5, it is also observed that *P. taetrolens* cells were barely consuming oxygen in the AW1 sample when compared to the AW2 sample, since in AW1, the DOT values were very close to the saturation level. The dissolved oxygen profile was related to the bacterial growth phase since dissolved oxygen in the substrate was expended while the bacteria were growing. In sample AW2, the inoculum was added periodically and this could be the reason why the dissolved oxygen rate decreased more than in the AW1 sample. Thus, there were a greater number of live bacteria, as multiparametric flow cytometry results showed (Figures 3 and 4). Therefore, this bacterial growth required a supply of oxygen, so the DOT in AW2 decreased. Oxygen supply plays a key role in metabolite production and cellular growth in many bioprocesses (Lozano *et al*. 2011). In this case, the DOT level only dropped to 78.2% in AW1, while in AW2, it dropped to 24.1%. These results suggested that *P. taetrolens* grows much better in acid whey if inoculum is added periodically every 12 h.

Lactose consumption and LBA production during fermentation

Results of lactose consumption and LBA production during fermentation are shown in Figure 6. In AW3, no LBA was

(a)

(b)
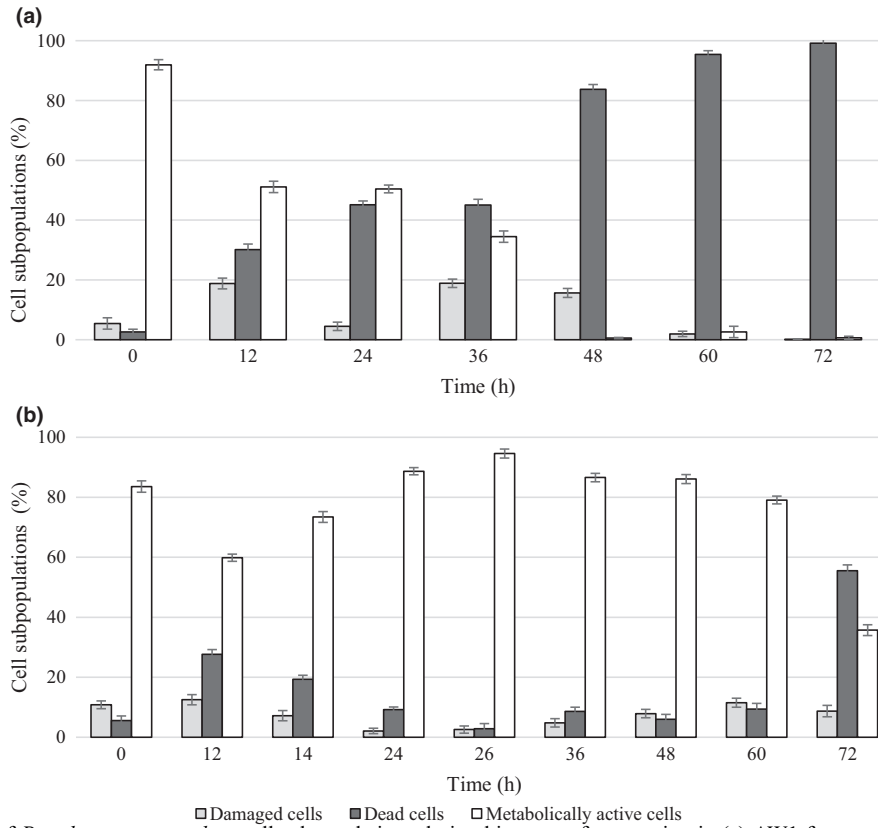
Figure 4 Percentages of *Pseudomonas taetrolens* cell subpopulations during bioreactor fermentation in (a) AW1 fermentation process with 10% (v/v) inoculum at the beginning of the fermentation process and in (b) AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started.
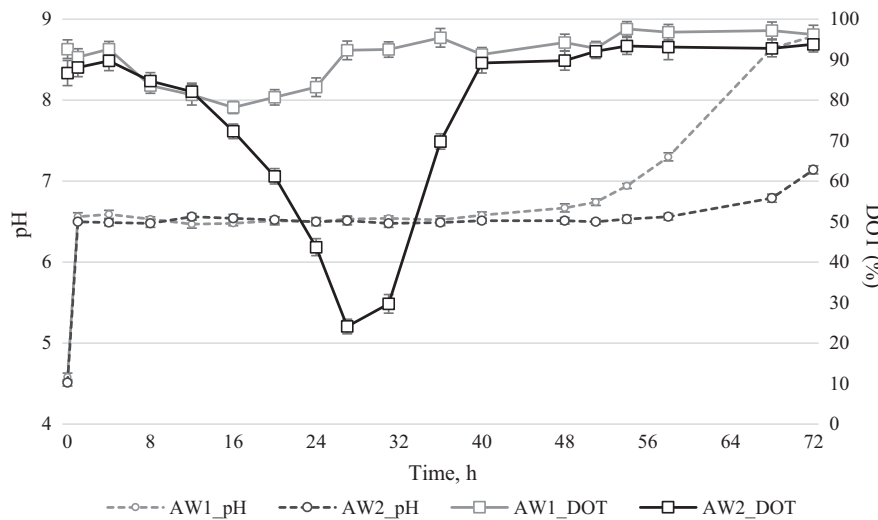
Figure 5 Time-course graph of pH and dissolved oxygen tension (DOT) during bioreactor fermentation in AW1 fermentation process with 10% (v/v) inoculum at the beginning of the fermentation process and in AW2 fermentation process with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started.
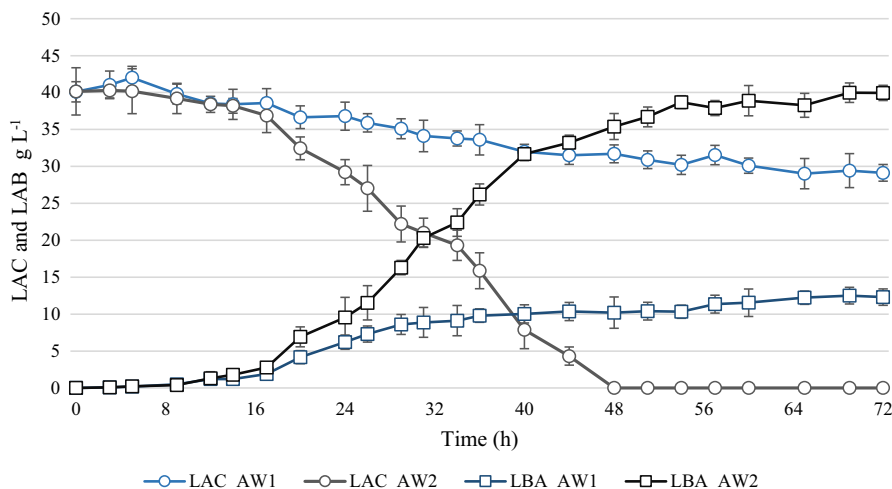
Figure 6 Time-course graph of lactose (LAC) and lactobionic acid (LBA) concentrations during fermentation processes in AW1 fermentation with 10% (v/v) inoculum at the beginning of the fermentation and in AW2 fermentation with 10% (v/v) inoculum at the beginning of the experiment and at 12 and 24 h after fermentation started.

produced, probably due to an excess of the initial inoculum. After 24 h, there was a concentration of 39.32 ± 0.41 and 1.90 ± 0.21 g/L of lactose and LBA, respectively. At the endpoint of the fermentation at 72 h, there was hardly any lactose consumption, with a final value of 39.42 ± 0.34 and an LBA concentration of 3.27 ± 0.52 (detailed data in the Figure S1 and Table S1).

Significant differences were established between the two samples ($P < 0.05$) after 16 h of fermentation. The production process was effective in the AW2 sample, since it reached 100% lactose conversion after 48 h, while in AW1, only 29.7% of the initial lactose was converted after 72 h of fermentation. The results clearly show the importance of adding fresh biomass to the substrate to achieve the main goal of a complete conversion of lactose into LBA. Also, a correlation between DOT values (Figure 5) and LBA production (Figure 6) can be observed.

In previous research using acid whey as substrate (Sarenkova *et al*. 2019a, 2019b, 2021), a lactose conversion yield of <32% was achieved. This study proves that bacteria have only a limited reproduction in acid whey (Figure 1), even if the acid whey is supplied with oxygen and the pH is optimal for bacterial growth. However, repeatedly adding fresh *P. taetrolens* biomass improves bacterial growth (Figure 1b) and lactose conversion (Figure 6).

Bioprocess parameters during fermentation of acid whey samples

To better establish the efficiency of the fermentation, bioprocess parameters were calculated and are presented in Table 1. All parameters were more favourable in the AW2 sample than in the AW1 sample, except for $Y^{X}/_{S}$, which was the same in both samples – there was no significant difference ($P > 0.05$). It showed that in both samples, the same weight of biomass grew per unit of lactose weight.

The specific growth rate was higher in the AW2 sample, so bacterial growth was faster than in AW1, reaching a maximum specific growth rate of 0.27 (h^{-1}) in AW2, while in the AW1 sample, it was only 0.08 (h^{-1}). The duration of the lag phase was similar, but in AW2, the inoculum was added at 12 h of the fermentation process and the exponential phase started 2 h earlier than in the AW1 sample. Lactobionic acid productivity and biomass productivity were significantly higher in AW2. The specific LBA production rate showed how much LBA can be produced by 1 g of biomass per hour, and the results were close, but the higher value was in AW2, since there were more metabolically active bacteria in AW2 than in AW1. When there are more metabolically active bacteria in a medium, there will be a higher yield of dry cell weight, OD and viable bacterial count, which is confirmed by the results obtained for AW2 in comparison with sample AW1.

P. taetrolens growth is limited in acid whey, and the lactose conversion process is slow, which is why adding inoculum during the fermentation process helps obtain higher results, quite similar to the results obtained by other authors for LBA production employing the same bacteria using sweet whey as substrate (Alonso *et al*. 2011, 2012a, 2012b, 2017; Goderska *et al*. 2014; Giorgi *et al*. 2018). Alonso *et al*. (2011) achieved a 100% LBA yield in sweet whey substrate maintaining the pH at 6.5 and adding 30% (v/v) *P. taetrolens* inoculum. In this research with sweet whey, a bioconversion of 100% was obtained in 32 h, while in the present study with acid whey, the same result was obtained in 48 h. Maximum volumetric LBA productivity of 1.12 (g/L/h) was thus achieved with the 30% inoculation level in

sweet whey substrate. These data show that sweet whey is still more suitable for LBA production, the fermentation time is shorter and productivity of LBA is higher with sweet whey than with acid whey substrate. With sweet whey, it is easier to convert lactose into LBA than with acid whey.

CONCLUSIONS

The results of the study showed that acid whey can be a suitable substrate for LBA bioproduction employing *P. taetrolens*. The initial amount of inoculum is one of the key parameters for obtaining an optimal productivity rate. An inoculum of 30% turned out to be excessive, and it barely achieved growth or LBA production. As for the 10% inoculum (the optimum in sweet whey), it gave a much lower yield than the addition of 10% inoculum three times in 24 h. Furthermore, the physiological status of *P. taetrolens* in the samples differed; in the AW1 sample, dead cells appeared rapidly, while in the AW2 sample, metabolically active bacteria lived and dominated throughout the fermentation process. These results clearly indicate the importance of repeatedly adding inoculum to the acid whey substrate in order to obtain a complete conversion of lactose into LBA. This approach constitutes a step forward towards achieving scalable LBA bioproduction systems with high bioconversion rates.

Although the experimentation developed in this article is at the laboratory level, it would not be complex to scale it up to the industrial level, as adding fresh biomass in sterile conditions in this type of process does not present great difficulty. Therefore, LBA production is practicable by this research methodology, but still not fully suitable for industry, for which reason the authors recommend further research into downstream processes to recover and purify LBA in order to permit the implementation of the process on an industrial level.

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AUTHOR CONTRIBUTIONS

Inga Sarenkova: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Visualization; Writing – original draft. **Sara Sáez-Orviz:** Conceptualization; Investigation; Methodology; Software; Visualization; Writing – review & editing. **Inga Ciprovica:** Project administration; Supervision; Validation; Visualization; Writing – review & editing. **Manuel Rendueles:** Project administration; Supervision; Validation; Visualization; Writing – review & editing. **Mario Díaz:** Resources; Validation; Writing – review & editing.

DATA AVAILABILITY STATEMENT

Research data are not shared.

REFERENCES

Alonso S, Rendueles M and Díaz M (2011) Efficient lactobionic acid production from whey by *Pseudomonas taetrolens* under pH-shift conditions. *Bioresource Technology* **102** 9730–9736.

Alonso S, Rendueles M and Díaz M (2012a) Role of dissolved oxygen availability on lactobionic acid production from whey by *Pseudomonas taetrolens*. *Bioresource Technology* **109** 140–147.

Alonso S, Rendueles M and Díaz M (2012b) Physiological heterogeneity of *Pseudomonas taetrolens* during lactobionic acid production. *Applied Microbiology and Biotechnology* **96** 1465–1477.

Alonso S, Rendueles M and Díaz M (2013) Bio-production of lactobionic acid: Current status, applications and future prospects. *Biotechnology Advances* **31** 1275–1291.

Alonso S, Rendueles M and Díaz M (2017) Tunable decoupled overproduction of lactobionic acid in *Pseudomonas taetrolens* through temperature-control strategies. *Process Biochemistry* **58** 9–16.

Cardoso T, Marques C, Dagostin J L A and Masson M L (2019) Lactobionic acid as a potential food ingredient: recent studies and applications. *Journal of Food Science* **84** 1672–1681.

Delagustin M G, Goncalves E, Carra S, Barcellos T, Bassani V L, da Silveira M and Malvessi E (2019) Sodium, potassium, calcium lactobionates, and lactobionic acid from *Zymomonas mobilis*: a novel approach about stability and stress tests. *Journal of Pharmaceutical and Biomedical Analysis* **174** 104–114.

Elabed H, Gonzalez-Tortuero E, Ibacache-Quiroga C, Bakhrouf A, Johnston P, Gaddour K, Blazquez J and Rodriguez-Rojas A (2019) Seawater salt-trapped Pseudomonas aeruginosa survives for years and gets primed for salinity tolerance. *BMC Microbiology* **19** 1–13.

Gänzle M G, Haase G and Jelen P (2008) Lactose: Crystallization, hydrolysis and value-added derivatives. *International Dairy Journal* **18** 685–694.

García C, Rendueles M and Díaz M (2017) Microbial amensalism in *Lactobacillus casei* and *Pseudomonas taetrolens* mixed culture. *Bioprocess and Biosystems Engineering* **40** 1111–1122.

Gautam M and Azmi W (2017) Purification of extracellular collagenase from *Pseudomonas* sp.: remarkable collagenolytic activity. *Advances in Biotechnology & Microbiology* **4** 33–40.

Giorgi S D, Raddadi N, Fabbri A, Toschi T G and Fava F (2018) Potential use of ricotta cheese whey for the production of lactobionic acid by *Pseudomonas taetrolens* strains. *New Biotechnology Journal* **42** 71–76.

Goderska K (2019) The antioxidant and prebiotic properties of lactobionic acid. *Applied Microbiology and Biotechnology* **103** 3737–3751.

Goderska K, Szwengiel A and Czarnecki Z (2014) The utilization of *Pseudomonas taetrolens* to produce lactobionic acid. *Biotechnology and Applied Biochemistry* **173** 2189–2197.

Hempel N, Petrick I and Behrendt F (2002) Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. *Journal of Applied Phycology* **24** 1407–1418.

Kargi F (2009) Re-interpretation of the logistic equation for batch microbial growth in relation to Monod kinetics. *Letters in Applied Microbiology* **48** 398–401.

Lozano E, Galindo E and Pena C F (2011) Oxygen transfer rate during the production of alginate by *Azotobacter vinelandii* under oxygen-limited and nonoxygen-limited conditions. *Microbial Cell Factories* **10** 1–13.

Macwan S R, Dabhi B K, Parmar S C and Aparnathi K D (2016) Whey and its utilization. *International Journal of Current Microbiology and Applied Science* **5** 134–155.

Mano J, Liu N, Hammond J H, Currie D H and Stephanopoulos G (2020) Engineering *Yarrowia lipolytica* for the utilization of acid whey. *Metabolic Engineering* **57** 43–50.

Prasirtsak B, Thitiprasert S, Tolieng V, Assabumrungrat S, Tanasupawat S and Thongchul N (2019) D-Lactic acid fermentation performance and the enzyme activity of a novel bacterium *Terrilactibacillus laevilacticus* SK5–6. *Annals of Microbiology* **69** 1537–1546.

Rocha-Mendoza D, Kosmerl E, Krentz A, Zhang L, Badiger S, Miyagusuku-Cruzado G, Mayta-Apaza A, Giusti M, Jimenez-Flores R and Garcia-Cano I (2021) Invited review: acid whey trends and health benefits. *Journal of Dairy Science* **104** 1262–1275.

Sachan S, Chandra V Y, Yadu A and Singh A (2017) Cobalt has enhancing effect on extracellular lipases isolated from Pseudomonas aeruginosa JCM5962(T). *International Journal of PharmTech Research* **10** 45–49.

Sahar T, Francisco S, Chen G Q, Chen X, Bathurst K and Kentish S E (2020) Pilot study on the removal of lactic acid and minerals from acid whey using membrane technology. *ACS Sustainable Chemical Engineering* **8** 2742–2752.

Sarenkova I and Ciprovica I (2018) The current status and future perspectives of lactobionic acid production: a review. *Research for Rural Development* **1** 233–239.

Sarenkova I, Ciprovica I and Cinkmanis I (2019a) Effect of different salts on *Pseudomonas taetrolens*' ability to lactobionic acid production. *World Academy of Science, Engineering and Technology. International Journal of Nutrition and Food Engineering* **13** 208–213.

Sarenkova I, Ciprovica I and Cinkmanis I (2019b) The effect of concentrated whey solids on Lactobionic acid production by *Pseudomonas taetrolens*. In Baltic Conference on Food Science and Technology: Conference Proceedings. pp. 250–253.

Sarenkova I, Saez Orviz S, Ciprovica I, Rendueles M and Diaz M (2021) Lactobionic acid production from acid whey under different fermentative conditions. *Journal of Advanced Agricultural Technologies* **8** 35–40.

Strock J S (2008) *Encyclopedia of Ecology: Reference Module in Earth Systems and Environmental Sciences*, 1st edn, pp 162–165. Amsterdam, Netherlands: Elsevier Science, Academic Press.

Shuler M L and Kargi F (2002). *Bioprocess Engineering: Basic Concepts*, 2nd edn, p. 576. New York, USA: Pearson.

Yang S T, Huang H, Tay A, Qin W, De Guzman L and San Nicolas E C (2007) Extractive fermentation for the fermentation of carboxylic acids. In Yang S T (Ed.), *Bioprocessing for Value-Added Products from Renewable Resources*, pp. 421–446. Oxford: Elsevier Inc.

SUPPORTING INFORMATION

The following supporting information is available for this article:

Figure S1. Lactobionic acid bioproduction using acid whey.

Table S1. Lactose consumption and LBA production in AW3.