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Acid Whey Valorization for Biotechnological Lactobionic Acid Bio-production

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Abstract

The dairy industry is facing a problem associated with 1.6 billion tons of acid whey per year as a waste stream. The extended amount of acid whey has encouraged studies for novel approaches of acid whey utilization. The production of lactobionic acid (LBA) using dairy waste has been in rapid demand as an economically feasible and environmentally friendly approach. The composition of acid whey makes lactose conversion into LBA by *Pseudomonas taetrolens* complicated. Therefore, the aim of the current research was to evaluate factors (quality of whey (salts, protein concentration, pH), volume of inoculum, and cultivation time) with the purpose of increasing the suitability of acid whey for biotechnological LBA production. LBA production was performed in a 4L bioreactor, which was equipped with a pH electrode and a dissolved oxygen electrode. The whole experiment was performed at a temperature of 30 °C under 350 rpm agitation. The continuous aeration was set at 0.5 L/min. The current study presents the study of acid and sweet whey combinations in different ratios (100:0; 50:50; 60:40, 70:30, 80:20, respectively) inoculated with 10% or 30% v/v of fresh *P. taetrolens* inoculum reaching up to 59.9 ± 1% LBA yield during cultivation. Increasing protein and pH in a substrate slows down the lactose converting ability of *P. taetrolens*. Results demonstrated that increasing the acid whey amount in a substrate can affect the LBA yield, and a combination of sweet and acid whey could be a good solution for biotechnological LBA production using dairy waste.

Keywords: Aacid Whey; Bioreactor; *Pseudomonas Taetrolens*; Lactose Oxidation; Lactobionic Acid.

1. Introduction

In recent years, acid whey production has considerably increased due to extended amounts of cottage cheese, Greek yogurt, and acid-coagulated cheeses [1, 2]. The dairy industry is facing a problem associated with 1.6 billion tons of acid whey per year as a waste stream [3]. The extended amount of acid whey has encouraged studies for novel approaches of acid whey utilisation [4]. The high biological oxygen demand for acid whey (52,400 to 62,400 mg/L) and cottage cheese whey (31,900 to 40,000 mg/L) causes a problem in utilizing the waste without placing a negative impact on the ecosystem [5].

The application of acid whey is limited due to its low pH and short shelf-life [5, 6], as well as its slightly salty taste [7]. However, acid whey is a valuable product containing lactose, whey proteins, peptides, lipids, minerals, and vitamins

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[8]. One of the perspective whey utilisation methods is fermentation, still it does not only decrease the biological oxygen demand (by 90–95%) of acid whey but also offers value-added bio-compound production [4].

Whey is a suitable biomaterial for lactobionic acid (LBA) production [9]. LBA (4-0-β-D-galactopyranosyl-D-gluconic acid) is composed of galactose linked to gluconic acid by an either-like glycosidic bound [10]. LBA molecular weight is 358.3 Da and acid dissociation constant (pKa) is 3.6, represents white solid powder, freely soluble in water with a melting point in the range of 120 to 130 °C. In terms of nutrition, LBA has a low energy value – 2 kcal [11]. Nowadays LBA is used in the pharmaceutical, food, medicine, cosmetic and chemical industries [10], for example, in anti-aging and skincare products formulations [9, 10, 12], in the food industry as a stabilizer, antioxidant, gelling agent in ice cream production, acid regulator in fermented dairy products and an aging inhibitor in bread production [9]. Due to its widespread usage, the production of LBA from recycling food by-products has increased [12]. The first results of LBA bio-production were 75% of LBA yield during 165 h of lactose oxidation by *Pseudomonas* spp. [13]. Alonso and co-authors (2011, 2012) [14, 15] got a 42.4 g/L of LBA yield during 32 h of fermentation using sterilized sweet whey, which substantiates the potential of whey as an inexpensive medium for LBA production [16]. Similar studies have been performed with acid whey using *Pseudomonas taetrolens*, which are able to convert up to 25% of initial lactose into LBA [17, 18]. The composition of acid whey makes lactose conversion into LBA by *P. taetrolens* complicated, therefore, the aim of the current research was to evaluate factors (chemical composition of whey (metal ions, protein concentration, pH), volume of inoculum, and cultivation time) with the purpose to increase suitability of acid whey for biotechnological LBA production.

2. Materials and Methods

2.1. Culture

Freeze-dried *P. taetrolens* DSM 21104 pure culture was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). Microorganisms were activated in nutrient agar (Scharlab S.L., Spain) (1 L contains: 10 g agar, 1 g meat extract, 5 g peptone, 2 g yeast extract and 5 g NaCl) and incubated at 30 °C for 48 h for further inoculum preparation.

2.2. Inoculum Preparation

A loopful with 10 µL of *P. taetrolens* strain from previously incubated Petri-dishes was inoculated in 100 ml of a nutrient broth liquid medium (1 L contains: 5 g peptone, 1 g meat extract, 2 g yeast extract and 5 g NaCl) in a 200 ml flask. The flask was incubated at 30 °C in an environmental shaker incubator ES-20 (Biosan Ltd., EU) at 230 rpm for 18 h. Broth cultures were centrifuged (Hermle Labortechnik GmbH, Germany) at 6000 rpm for 10 min to get inoculum of *P. taetrolens*, which is further used as an inoculation seed culture in the batch fed bioreactor [17, 18].

2.3. Whey Preparation

Acid whey and sweet whey, high protein sweet whey concentrate obtained from local producer (LTD Jaunpils) were used for further studies with the following average composition: acid whey: lactose 5.6 ± 0.11%; fats 0.01 ± 0.01%, total solids 7.07 ± 0.15%; and pH 4.58 ± 0.01, sweet whey concentrate: lactose 13.9 ± 0.12%; fats 0.01 ± 0.01%, total solids 15.9 ± 0.19% and pH 5.78 ± 0.01. Samples were prepared by mixing acid whey and sweet whey concentrate in a ratio of 50:50, 60:40, 70:30, and 80:20, respectively. The pH of all samples was raised to 6.5 by adding 6 M NaOH [9] and further pasteurised at 72 °C for 15 s. All samples were marked with letters and numbers, as stated in Table 1.

Table 1. Composition of whey samples used in the study

Sample*	Total solids, g/L	Lactose, g/L	Salts, g/L
B100	$67.4^{b} \pm 1.0$	$54.1^{c} \pm 0.92$	$5.3^{f} \pm 1.0$
B100^{p}	$74.8^{b} \pm 5.0$	$57.0^{c} \pm 1.35$	$4.7^{g} \pm 1.0$
B50:50$_{a}$	$143.6^{a} \pm 2.3$	$116.4^{a} \pm 0.47$	$20.6^{a} \pm 1.5$
B50:50$_{b}$	$138.2^{a} \pm 9.6$	$109.0^{b} \pm 0.1$	$22.2^{b} \pm 1.0$
B60:40	$127.8^{a} \pm 7.2$	$103.5^{b} \pm 0.73$	$11.9^{e} \pm 1.5$
B70:30	$123.2^{a} \pm 10.2$	$100.9^{b} \pm 0.84$	$16.5^{c} \pm 1.5$
B80:20	$119.0^{a} \pm 8.3$	$98.9^{b} \pm 0.76$	$14.6^{d} \pm 1.5$

Different superscripts within a column (a, b, c) are significantly different (p<0.05);

*Where; B - bioreactor; a - 10% v/v inoculated; b - 30% v/v inoculated; p - high protein acid whey; 100-100% acid whey substrate.

2.4. Fed-Batch Bioreactor Method

LBA production was performed in a 4 L bioreactor (BIOSTAT® B plus, Germany) with a working volume of 3 L. The bioreactor was equipped with a sampler, pH electrode (Mettler Toledo, Switzerland) and dissolved oxygen electrode (Mettler Toledo, Switzerland) to monitor the pH and dissolved oxygen values. The experiment was performed at a 30 °C temperature under 350 rpm agitation, the continuous aeration was set at 0.5 L/min, which contains 0.1 L/min of oxygen supply via ring sparger located at the bottom of the vessel. The experiment was operated by using the pH-shift strategy where pH was set at 6.5. 1 M NaOH was used to regulate the medium pH with peristaltic pump. Excessive foam formation was controlled by adding a silicone polymer based anti-foaming agent (1:10 v/v) (Sigma-Aldrich, Steinheim, Germany). These prior conditions were applied for all cultivations.

The experiment was conducted by adding 3 L of sample to the bioreactor and then inoculated with 10% v/v inoculum, except sample B50:50$_b$ which was inoculated with 30% v/v. The 48 h cultivation time was adopted from Alonso *et al*. (2011, 2013a, 2017) [9, 14, 19] studies. All samples were cultivated for 48 h, but for sample B100, B100^p – 72 h.

2.5. Analytical Methods

The *lactose* concentration in whey was determined prior to the study using MilcoScan™ Mars (Foss, Denmark).

The *lactose and LBA concentration* were measured using high-performance liquid chromatography (Prominance HPLC system, Shimadzu LC-20, USA). All samples before HPLC analyses were centrifuged for 10 min at 15 000 rpm to remove cell debris and other water-insoluble substances. The LBA was determined using a refractive index detector RID-10A (YMC-C18, 4.6 mm × 250 mm, 5 µm column). Mobile phase isocratic elution (2 L elution contained 1.15 mL H_3PO_4, 14.36 g KH_2PO_4 and 20 mL acetonitrile) was used. Lactose content was determined using the refractive index detector DAD SPD-M20 A (Alltech NH_2, 4.6 nm × 250, 5 µm column). Mobile phase isocratic elution was 84% of acetonitrile, 16% of deionized water. The volume of the sample injected was 10 µL, temperature 35 °C and flow rate 1 mL/min. Samples were quantified according to HPLC-grade external analytical standards with higher purity, lactose (Sigma Aldrich, Germany) and LBA (Acros Organics, India).

Protein concentration was measured by the Kjeldal method (ISO 8968-1:2014), which was followed by sampling, digestion (FOSS Tecator™ Digestor, Denmark), distillation (FOSS Kjeltic™ 2100, Denmark) and titration.

Determination of *non-protein nitrogen* (ISO 8968-4:2001) was followed by sampling, digestion (FOSS Tecator™ Digestor, Denmark), distillation (FOSS Kjeltic™ 2100, Denmark) and titration.

Salts concentration was calculated as the difference between total solids and the sum of lactose, fats and proteins.

pH value was measured with an InLab® Expert Pro-ISM pH electrode (Mettler Toledo, Switzerland).

2.6. Pseudomonas Taetrolens Colony Forming Units

P. taetrolens colony forming units (CFU) were measured using nutrient agar (Scharlau, Europe) with the incubation temperature at 30 °C for 48 h and counted with the Acolyte colony counter (SYNBIOSIS, UK). *P. taetrolens* growth curves were obtained as a function of time by plotting log CFU/mL.

2.7. Calculation of Lactobionic Acid Yield

LBA yield (bioconversion yield) was calculated as a percentage based on the amount of initial lactose oxidized into LBA at the end of cultivation [14]:

$$\text{LBA yield } \% = \frac{m_{LBA}}{m_{LAC}} \times 100 \quad (1)$$

where m $_{LBA}$ is LBA content obtained at the end of cultivation, g/L, and m $_{LAC}$ is initial lactose concentration, g/L.

2.8. Data Processing

Data analysis and acquisition were completed using Microsoft Office Excel v16.0 and SPSS 19.0 software. Analysis of variance (ANOVA) and Tukey's test were used to compare means, differences were considered significant at a level of $p<0.05$. All results were displayed as the average data from three independent experiments unless specified.

3. Results and Discussion

3.1. Factors affecting LBA yield

The oxidation process of lactose depends on *P. taetrolens* dehydrogenase and lactonase activity. Many investigations were done by Alonso *et al*. (2011, 2013a, b, 2015, 2017) [9, 14, 19, 20] analysing the growth of *P. taetrolens* in sweet

whey. According to the above-mentioned studies, *P. taetrolens* growth ability depends on bacteria strains [10], cultivation temperature, pH and temperature [14, 16], composition of substrate [10, 18], initial amount of inoculum [14, 21], seed culture age [14] and others. In addition, oxygen supply and proper pH maintenance in the cultivation process are key factors influencing the outcome of the LBA yield [9]. Alonso *et al*. (2012)] [15] have concluded that LBA production is adversely affected by the presence of excessive oxygen and high (more than 350 rpm) agitation speed. The effect of whey solids has also been studied, revealing that LBA production increases with increasing of whey solids [22].

3.2. Pseudomonas Taetrolens Growth Monitoring

The growth of *P. taetrolens* was observed during cultivation (Figure 1). *P. taetrolens* viable cell count was achieved in substrate from 10^{10} to 10^{12} CFU/mL after 16 h of cultivation and remained unchanged till the end of cultivation. No significant differences ($p>0.05$) of *P. taetrolens* CFU were observed among analysed samples during the cultivation process.

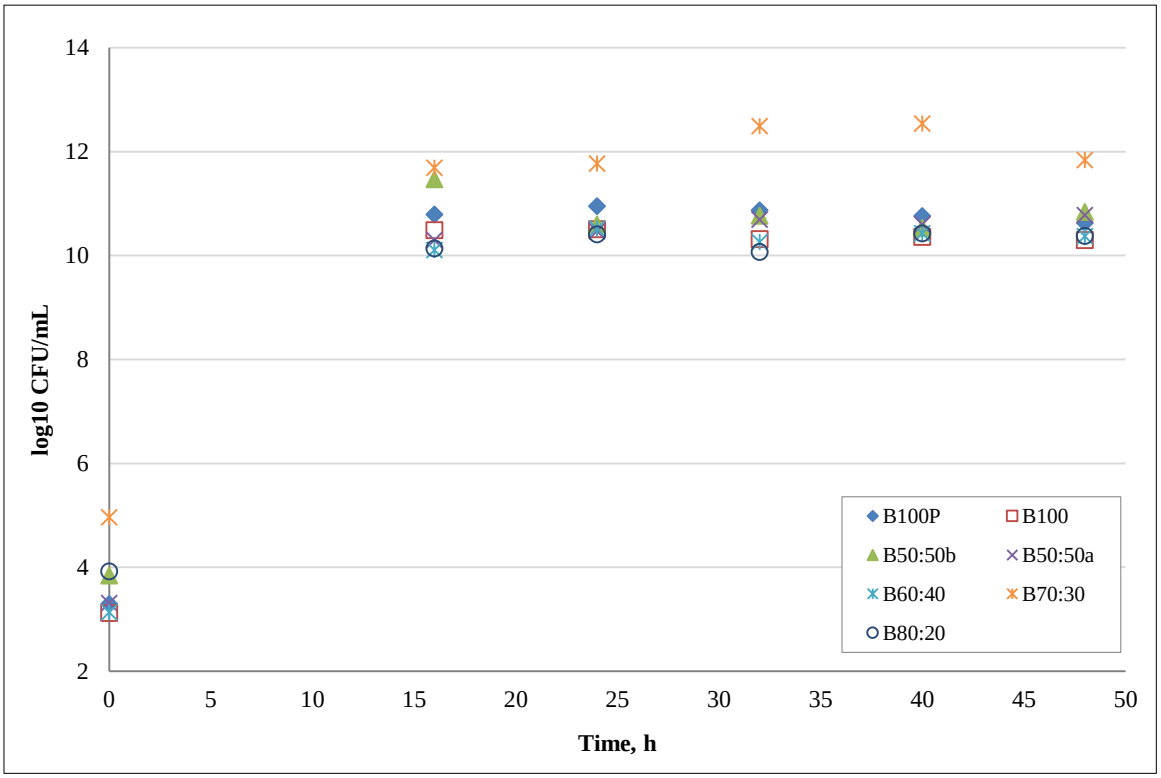

Figure 1. Growth of *Pseudomonas taetrolens* in whey samples during the cultivation in batch-fed bioreactor *Where, a - 10% v/v inoculated, b - 30% v/v inoculated; p - indicates high protein acid whey

Sarenkova's *et al*. (2019a) [17] established higher *P. taetrolens* (10^{10} CFU/mL) concentration after 30 h of acid whey cultivation using the shake-flask method. In the current study, we have determined around of 10^{11} CFU/mL in all samples during 30 h of cultivation. The higher CFU was achieved by providing optimal cultivation conditions (pH 6.5, aeration 0.5 L/min) in the bioreactor. According to Sarenkova *et al*. (2019a) [17], the pH of media after 48 h of cultivation was around 4.25 ± 0.50 in acid whey samples using the shake–flask method. In the current study, pH in the acid whey sample B100 after 48 h of cultivation had reached pH 6.5 ± 0.7. The pH 6.5 to 6.7 is necessary for *P. taetrolens* lactonase production to oxidase lactose into LBA [9, 23]. In samples B60:40, B70:30, B80:20 and B100 the final pH was around 6.5. Obtained results are in agreement with Alonso *et al*. (2011, 2013a, 2017) [9, 14, 19] and De Giorgi *et al*. (2018) [10] using sweet whey as a substrate, the pH after 24 h of the lactose oxidation process reduced from 8.0 to 6.5 due to LBA production. All studies prove that the bioreactor is more suitable for LBA production, compared to the shake-flask method.

3.3. Ratio of Sweet and Acid Whey

The LBA yield after 48 h of cultivation is given in the plotted graph (Figure 2) in percentages. In the samples, $B50:50_a$ and B60:40 have a higher LBA yield (47.5 ± 1.3% and 44.56 ± 1.98% respectively) compared to samples with a higher acid whey proportion: B70:30 (42.47 ± 0.97%), B80:20 (41.02 ± 1.63%) and B100 (40.68 ± 1.56%). Eventually, the study results proved that as the amount of acid whey in substrate decreases, controversially, LBA yield increases.

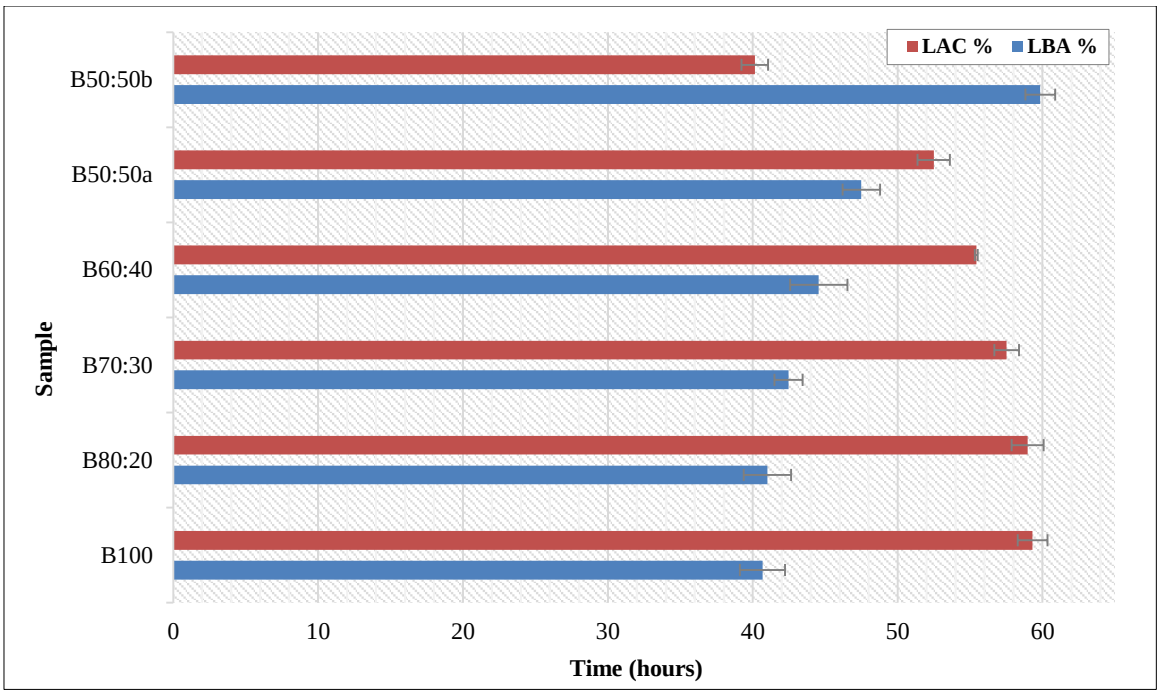

Figure 2. Lactobionic acid (LBA) yield and remained lactose (LAC) in different whey samples after 48 h of cultivation
***Where, a - 10% v/v inoculated, b - 30% v/v inoculated**

3.4. Composition of Whey

Sarenkova *et al*. ([2019b) [18] analysing acid whey using shake flask–method determined 20% of LBA yield. Published data on the production of LBA using a combination of acid and sweet whey in a bioreactor is not available for comparison of the study results.

The lower amount of LBA yield in samples with a higher acid whey proportion could be explained by composition, as acid whey has a higher concentration of ***lactic acid and salts*** [3, 6]. The strong positive linear correlation (r = 0.72) between LBA yield and salts concentration was established (Figure 3). Some elements can inhibit *P. taetrolens* enzyme activity, correspondingly lactose oxidation into LBA. In previous studies, Sarenkova and co-authors proved the impact of metal ions on the cultivation process, concluding that Mn^{2+} and Mg^{2+} ions enhanced cultivation compared to K^{+} ions in acid whey substrate. Results proved that Mn^{2+} and Mg^{2+} ions are necessary for *P. taetrolens* metabolism [17].

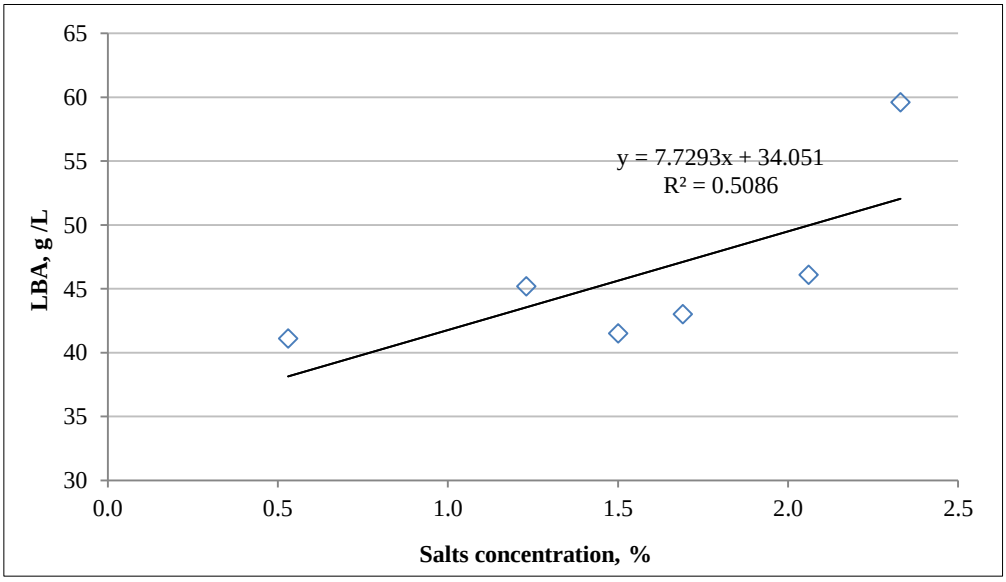

Figure 3. Correlation between LBA and salts concentration in substrate

Knowing, that Ca^{2+} ion concentration is significantly higher in acid whey substrate and they could negatively affect *P. taetrolens* lactonase activity. Majore *et al*. (2017) [24], analysing β galactosidase activity, made the conclusion that Ca^{2+} ions can inhibit this enzyme activity.

Enzyme activity could affect the amount of Na^+ ions as the osmotic pressure in bacteria cells is growing. The internal osmotic pressure must be higher than in the external environment in order to maintain bacterial growth and viability. If osmotic pressure is too low/high, it could also affect enzyme activity [25, 26]. To maintain an acid whey substrates pH, a significant amount of 1 M NaOH is used in samples with a higher proportion of acid whey (Figure 4), influencing lactonase activity and the lactose conversation rate into LBA.

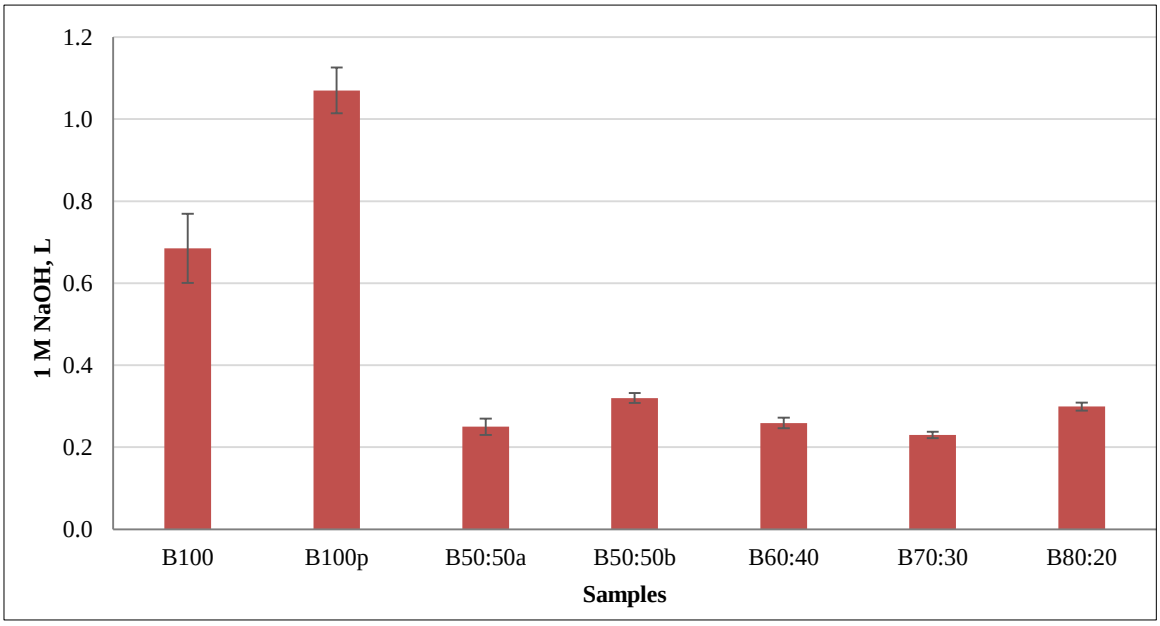

Figure 4. Amount of 1 M NaOH used during cultivation

Another explanation of limited LBA production in acid whey, is a significantly higher concentration of lactic acid (sweet whey – 0.13 g/L [27], acid whey – 0.58 g/L [3] and bacteriocins [28, 29].

The initial concentration of lactose in sample B100 was 54 ± 0.92 g/L, after 48 h of cultivation 21.97 ± 1.56 g/L of LBA was produced, but after 72 h of cultivation – 23.74 ± 1.4 g/L. There was no significant difference ($p>0.05$) between LBA yield obtained after 48 and 72 h in the bioreactor (Figure 5). The highest conversion rate of lactose into LBA was observed between 28 to 48 h of cultivation, during the same period a slow reduction of the protein concentration in the substrate was also observed (Figure 6). Results are in agreement with De Giorgi *et al*. (2018) [10], where no significant difference in LBA yield was found after 48 and 72 h of incubation (34.25 g/L and 36.32 g/L respectively) using Ricotta cheese whey as a substrate and *P. taetrolens* as inoculum.

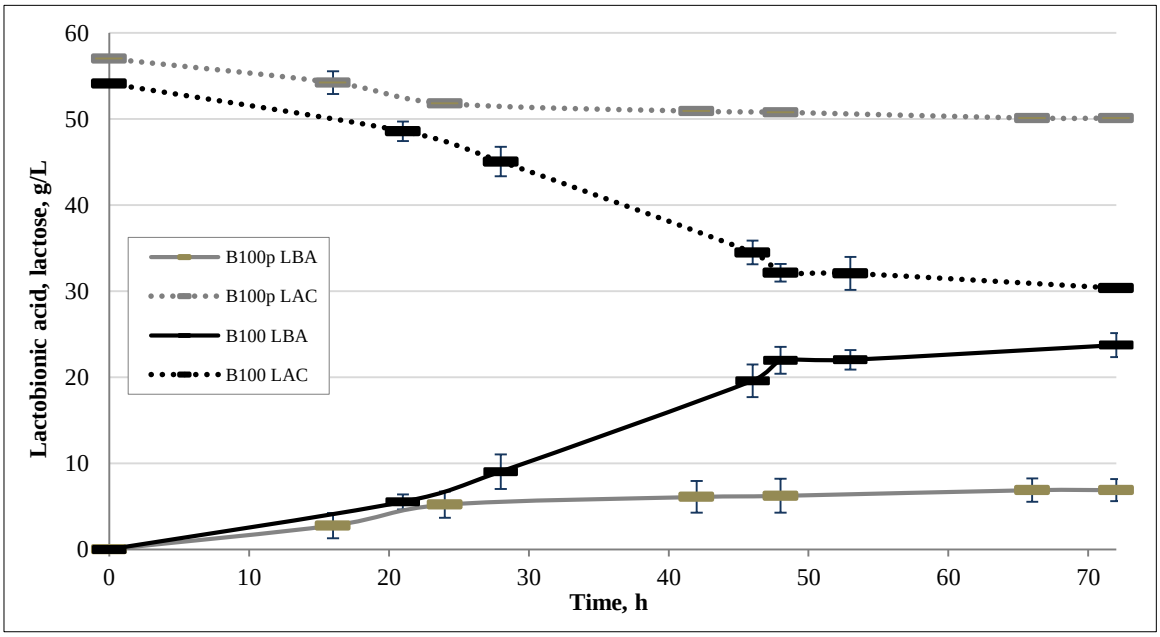

Figure 5. Lactobionic acid (LBA) yield and remained lactose (LAC) in B100p and B100 whey samples during 72 h of cultivation, *p - indicates high protein acid whey

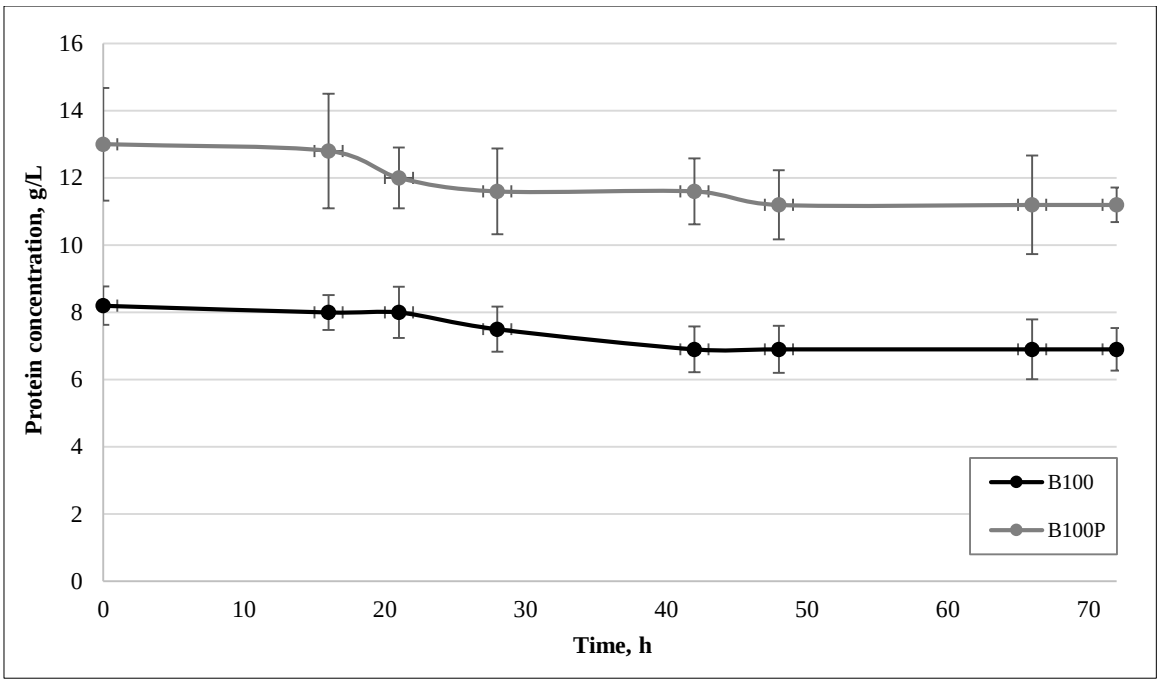

Figure 6. Changes in protein concentration during B100 and B100p samples cultivation *p - indicates high protein acid whey

Table 2. Changes in protein and non-protein nitrogen concentration after 48 h of whey samples cultivation

Sample	Before cultivation, g/L		After 48 h cultivation, g/L	
	Protein	Non-protein nitrogen compounds (total)	Protein	Non-protein nitrogen compounds (total)
$B50:50_a$	$5.80^A \pm 0.78$	$0.061^A \pm 0.001$	$2.50^B \pm 0.32$	$0.063^A \pm 0.001$
$B50:50_b$	$6.90^A \pm 1.30$	$0.064^A \pm 0.001$	$3.20^B \pm 0.25$	$0.066^A \pm 0.001$
B60:40	$6.40^A \pm 0.79$	$0.067^A \pm 0.001$	$3.10^B \pm 0.15$	$0.069^A \pm 0.001$
B70:30	$6.20^A \pm 0.97$	$0.070^A \pm 0.001$	$2.80^B \pm 0.51$	$0.070^A \pm 0.001$
B80:20	$5.90^A \pm 1.02$	$0.069^A \pm 0.001$	$2.60^B \pm 0.92$	$0.069^A \pm 0.001$

Different superscripts within a line for protein or non-protein nitrogen compounds (A, B, C) are significantly different ($p<0.05$).

Significantly ($p<0.05$) lower LBA yield was observed in the sample $B100_p$ with a higher amount of protein, respectively 6.23 ± 1.03 g/L and 6.9 ± 1.29 g/L of LBA after 48 h and 72 h of cultivation. Other researchers [15, 21-23] achieved a higher LBA conversion rate using protein free permeate as a substrate.

The protein concentration of whey samples B100 and $B100_p$ was ($p<0.05$) reduced during 48 h of cultivation from 8.2 to 6.9 g/L and from 13.0 to 11.2 g/L, respectively, and remained unchangeable till the end of cultivation (72 h) (Figure 6). Similar reduction of protein values was happened during 48 h of cultivation in remaining samples as well (Table 2). Other authors analysed substrates with a lower protein concentration 2.6 to 5.8 g/L [10] and 0.38 to 0.88 g/L [17, 18] for LBA production by *P. taetrolens*, but no additional information was found about protein changes during cultivation.

3.5. Inoculum Size

The final pH for sample $B50:50_b$ was 6.85 ± 0.19 and for $B50:50_a$ 6.58 ± 0.2, samples were inoculated with 30% v/v and 10% v/v of inoculum, respectively. Results showed that the final pH and *P. taetrolens* growth are dependent on the amount of inoculum added, as a result a lower amount of 1 M NaOH was used for pH regulation (Figure 4). A similar experiment was done by Alonso *et al*. (2011, 2013a) [9, 14], observing a pH rise from 6.40 to 7.36 in samples inoculated with higher inoculum (0.64 g/L compared to 0.035 g /L).

The sample $B50:50_b$ which was inoculated with 30% v/v inoculum showed significantly higher ($p<0.05$) LBA yield (59.9 ± 1.03%) compared to inoculum size – 10% v/v ($B50:50_a$). Higher inoculum size positively influences LBA production and final yield. Moreover, samples $B50:50_a$ and $B50:50_b$ showed the higher yield of LBA. The results indicate that combinations of acid and sweet whey in a 50:50 ratio could be suitable for lactose oxidation into LBA, compared to other combinations.

3.6. *pH*

pH of medium is one of the key values in production of LBA. Compared to Sarenkova's *et al*., (2019a) [17] shake–flask cultivation data with a bioreactor using acid whey (100%), current research results demonstrated a significantly ($p<0.05$) higher yield of LBA – 40.68 ± 1.56% for the acid whey substrate. This variation in yield could be as a result of maintaining pH at an optimal level during cultivation. However, other researchers established that LBA yield has been increased by prolonging cultivation time even without pH regulation. De Giorgi *et al*., (2018) [10] reported above 80% of LBA yield using the shake–flask method for 120 h of cultivation, but a similar yield can be achieved after 48 h in a bioreactor. pH control could help to decrease the time and costs of cultivation for higher LBA yields. Alonso *et al*. (2012) [15] have established that lactose conversion by *P. taetrolens* in sweet whey within pH 4.0 – 4.5 slows down the LBA production, while pH 6.0 – 6.5 enhances the proliferation of cells, resulting in faster LBA production.

The amount of LBA produced during the cultivation depends on the volume of 1 M NaOH used in the research. In the sample with a higher inoculum size, there has been an observed higher amount of 1 M NaOH addition for more intensive LAB production. De Giorgi *et al.* (2018) [10], and Alonso *et al*. (2012, 2013, 2015, 2017) [14, 15, 19, 20] also used NaOH for maintaining pH during LBA production, but data about the amount of NaOH used during the cultivation process was not presented.

The current study indicates that substrate composition has a significant influence on LBA yield. Changing proportions of whey (acid and sweet) and chemical composition (protein, minerals, lactic acid concentration) of the substrate have been possible to produce LBA, reaching an LBA yield of up to 59.9 ± 1%, while inoculum size also affects the lactose conversion rate into LBA.

4. Conclusion

The current study has demonstrated that the combination of acid and sweet whey in different proportions can affect the LBA production and yield. Higher protein and lactic acid concentrations in a substrate negatively affect lactose oxidation into LBA. Substrate pH is one of the key values in the production of LBA, but a high amount of NaOH used for pH adjustment can be crucial for *P. taetrolens* activity. Substrate composition (profile and concentration of salts; lactic acid concentration; and presence of bacteriocins) is significantly important for LBA production and final yield.

5. Declarations

5.1. Author Contributions

Conceptualization, J.Z. and V.R.N.; methodology, V.R.N. and J.Z.; software, V.R.N.; validation, J.Z., I.C. and I.S.; formal analysis, V.R.N.; investigation, V.R.N.; resources, V.R.N.; data curation, V.R.N.; writing—original draft preparation, V.R.N.; writing—review and editing, J.Z., I.S. and K.M.; visualization, V.R.N.; supervision, J.Z. and I.C.; project administration, I.C.; funding acquisition, J.Z. All authors have read and agreed to the published version of the manuscript.

5.2. Data Availability Statement

The data presented in this study are available in article.

5.3. Funding

The study was funded by the project No 19-00-A01612-000007 "*Economically justified processing of whey for new food and feed*" supported by the Ministry of Agriculture and Rural Support Service of the Republic of Latvia.

5.4. Ethical Approval

Not applicable.

5.5. Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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