Lactobionic Acid Production from Acid Whey under Different Fermentative Conditions

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***Abstract*—In the last years the production of lactobionic acid by bio-based synthesis have reached a high impulse and it could be supposed to reach the industrial level manufacturing in the near future. The aim of this work was to study the suitability of acid whey on lactobionic acid production by using shake-flasks and bioreactors. Whey has been fermented with *Pseudomonas taetrolens* LMG 2336 at 30°C in non-controlled pH and also controlling the pH at 6.5 during fermentation. The concentration of Lactobionic Acid (LBA) and Lactose (LAC) in the samples was determined using the high-performance liquid chromatography. Lactobionic acid purification was carried out initially by active carbon adsorption and further lyophilization. Results showed that the higher yield of LBA was achieved in sweet whey in bioreactor and controlled pH during fermentation. Using acid whey as a substrate for lactobionic acid production, the process is less productive due to the previous acidification of the substrate. pH lower than 6.5 diminishes *Pseudomonas taetrolens* lactose dehydrogenase activity. Results proved that pH adjustment around of 6.5 is necessary during fermentation. The study results will help to improve the efficiency of lactobionic acid production by microbial synthesis using acid whey.**

Index Terms*—acid whey, lactobionic acid, lactose oxidation, milk sugar, *Pseudomonas taetrolens

I. Introduction

Whey is a dairy by-product with 5–8% of total solids that contain around 60–80% of lactose. Lactose has been recognized as an attractive raw material for pharmaceutical and food products production. The interest on lactose from whey profiting is growing rapidly because whey is considered as a contaminant by-product [1]. Lactose is a source of several derivatives, including value added compounds such as lactobionic acid [2]. Whey causes substantial environmental problems if it is discarded without treatment, which is forbidden by law. Last years, several studies have been applied in the industrial field for efficient byproduct application [3], [4]. Low solubility (195g L^{-1} solubility in water), low sweetness (approximately 20-30% of sucrose) and the intolerance problems of some population limit the application of lactose in the food industry more-wider [5]. Lactose (4-O-β-D-galactopyranosyl- D-glucose) can be converted into high value-added products by chemical or biological processes in the industrial or laboratory scale. Compounds like lactobionic acid, are derived from lactose and can be used as alternative for the diversification of a potential industrial waste as the whey [6]. Lactobionic acid (4-O-β-D-galactopyranosyl- D-gluconic acid) is an aldonic acid naturally found in the "Caspian Sea yogurt" and chemically constituted from gluconic acid which is bonded with galactose. It is obtained through lactose oxidation, present in white crystallized powder form [5], [7]-[9]. The possibility of renewable material or waste utilization increases the interest of the biotechnological route to cost-effective and environmentally friendly production of lactobionic acid [10]. *Pseudomonas spp.* develop lactobionic acid through lactose oxidation pathway [8]. *Pseudomonas taetrolens* strains are suitable for production of lactobionic acid which further can be used in food products [11]. *Pseudomonas spp.* dehydrogenase catalyses lactose to lactobiono-δ-lactone using Flavin Adenine Dinucleotide (FAD) as an electron transfer. Lactobiono-δ-lactone is further hydrolysed by lactonase to lactobionic acid [5]. Whey is a cheap substrate for bioproduction of lactobionic acid by *Pseudomonas taetrolens*. Undoubtedly application of polluting byproduct as a very cheap source is advantageous [12].

Mostly accessible research articles refer to the use of sweet whey as substrate [12]-[15]. Acid whey (pH 4.2 – 5.0) usage has not been tested for lactobionic acid production yet. Lactobionic acid production via bio-based synthesis can be affected by many factors and it is important to choose appropriate methodology for lactose conversion to lactobionic acid. As *Pseudomonas taetrolens* are aerobic bacteria the fermentation process should provide the medium with oxygen, because dissolved oxygen represents decisive factors in aerobic systems displaying as limited oxygen supply may reduce or even inhibit cell growth, microbial biosynthesis and metabolism [8].

The aim of this study was to study the suitability of acid whey on lactobionic acid production by using shake-flask and bioreactor and comparing the results with sweet whey.

II. Materials and Methods

A. Microorganisms

Pseudomonas taetrolens LMG 2336 from the Belgian Coordinated Collection of Microorganisms (BCCM, Gent,

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Belgium), was maintained frozen (at - 18°C in 40% [v/v] glycerol). The strain was subsequently subcultured on Nutrient Broth agar plates (containing in 1L: 20g agar, 1g meat extract, 5g peptone, 2g yeast extract and 5g NaCl). Agar plates were incubated at 30°C for 48 h and used directly for preparation of inoculum.

B. Preparation of Inoculum

A loopful with 10µL capacity of *Pseudomonas taetrolens* LMG 2336 from a fresh Nutrient Broth agar plate was resorted to inoculate strains at 100 mL of nutrient broth liquid medium (containing in 1L: 5g peptone, 5g NaCl, 2g yeast extract, and 1g meat extract). These samples were stored at 30°C for 12h in an orbital shaker (New Brunswick Sci., NJ. USA) with agitation rate of 350 rpm. Biomass of *Pseudomonas taetrolens* LMG 2336 was separated at 10,000 rpm 10 min using centrifugation and further applied as a bulk starter.

C. Preparation of Sweet and Acid Whey

Acid whey (from an artisan cheesemaker Ca Sanchu, Asturias Spain) and sweet whey (from manufacturer ILAS S.A., Asturias, Spain) containing around 50-60g L^{-1} lactose were pasteurized at 90°C temperature for 30 min and filtered through cheesecloth, after that treated using a tangential microfiltration device equipped with a 0.22µm pore size Polyvinylidene Difluoride (PVDF) membrane-cassete (Millipore, Massachusetts, USA). Whey were used for further studies with following average composition: sweet whey: lactose 6.2±0.12%; fats 0.01±0.01%, proteins 0.12±0.03% and pH 6.34±0.01; acid whey: lactose 5.6±0.13%; fats 0.01±0.01%, proteins 0.23±0.08% and pH 4.67±0.01.

D. Fermentation Design

Shake-flask fermentation was processed in 500mL Erlenmeyer flasks containing 100mL of whey as a working volume. Samples were inoculated with 10% (v/v) of *Pseudomonas taetrolens* and cultivated 72 h at 30°C with an agitation rate of 350 rpm in an orbital shaker (New Brunswick Sci., NJ. USA). There were compared samples with two different pH conditions (controlled and non-controlled pH). In non-controlled pH runs, the pH was left uncontrolled during fermentation period. In controlled pH runs pH was maintained at 6.5 during fermentation period by adding manually 6M NaOH. Samples were withdrawn during fermentation at different times for monitoring cells weight of *Pseudomonas taetrolens*, bacterial growth, pH, lactose and lactobionic acid concentration in samples.

Fermentation was conducted in a 2L bioreactor (Bio Flo 110, New Brunswick Scientific, NJ, USA) with working volume of 1L for whey inoculated with 10% (v/v) of *Pseudomonas teatrolens* and cultivated 72h at 30°C with an agitation rate of 350 rpm. Foam formation was prevented by addition of diluted (1:10) Y-30 emulsion (SigmaAldrich, Germany). Bioreactor was equipped with pH meter (Mettler Toledo, Switzerland) and an electrode for polarographic dissolved oxygen (InPro 6830, Mettler Toledo, Switzerland) with monitoring of pH and Dissolved Oxygen Tension (DOT) values. There were compared the processes with two different pH conditions (controlled and non-controlled pH) as in the case of the shake-flasks. Non-controlled pH: leaving the pH free during fermentation. And controlled pH maintaining the pH at 6.5 during fermentation by automatically adding 6 M NaOH. Samples were also withdrawn during fermentation at different times for monitoring pH, cell weight of *Pseudomonas taetrolens*, bacterial growth, dissolved oxygen tension, lactose and lactobionic acid concentration. The notation of the samples is indicated in Table I.

TABLE I. SAMPLES ABBREVIATION

Samples	Type of whey	Equipment	pH
SF1	Sweet whey	Shake-flask	Non-controlled
SF2			Controlled
SB1		Bioreactor	Non-controlled
SB2			Controlled
AF1	Acid whey	Shake-flask	Non-controlled
AF2			Controlled
AB1		Bioreactor	Non-controlled
AB2			Controlled

After fermentation samples from bioreactor were treated using a tangential microfiltration device equipped with a 0.22µm pore size PVDF membrane-cassete (Millipore, Massachusetts, USA), further filtrated through carbon filter 3 times and concentrated till 40% of total solids. Concentrated samples were stored in freezer at -18°C. Frozen samples were lyophilizated in vacuum lyophilization equipment (Telstar cryodos - 80, Model 2007 year, Spain) till powdered white crystals. Protein, lactose and lactobionic acid content (0.4g crystals diluted in 100ml distilled water) were analysed in powdered product.

E. Analytical Methods

pH was determined using an InLab® Expert Pro-ISM pH electrode (METTLER TOLEDO, Switzerland).

Bacterial growth was detected spectrophotometrically at 600 nm (Thermo Scientific Helios Gamma UVG, England) after fermented samples centrifugation at 13,000 rpm for 10 min.

Pseudomonas taetrolens dry cells weight was determined using 1mL of cultivated medium. Samples were centrifuged for 10 min at 13,000 rpm, liquid was spilled out and precipitated cells were dehydrated by drying until constant weight.

Dissolved Oxygen Tension (DOT) was detected with a polarographic dissolved oxygen electrode (InPro 6830, Mettler Toledo, Switzerland).

The lactose and lactobionic acid concentration in cell free culture samples were determined by high performance liquid chromatography (Agilent 1200, Agilent Technologies Inc., CA, USA) equipped with a column (Coregel ION 300 Column). Sulphuric acid (pH 3.1, 0.450 Mm) was employed as the mobile phase with flow rate of 0.3mL min^{-1} and column temperature set at 75°C. Data acquisition and analysis were processed with ChemStation software (Agilent).

Protein content was determined using Kjeldahl method (ISO 8968-1:2014) in whey and in cell free samples after purification.

F. Data Analyses

Data analysis and acquisition were processed with Microsoft Excel 2010 program. All results are displayed as the average data from three independent tests. Statistical analyses were completed using t-test and Analysis of Variance (ANOVA) at significance level of $p<0.05$.

III. Results and Discussion

A. pH Changes in the Substrate during Fermentation Process

pH of the fermentative medium was analysed during 72h fermentation. Fig. 1A shows pH changes in sweet whey samples and 1B in acid whey samples during fermentation.

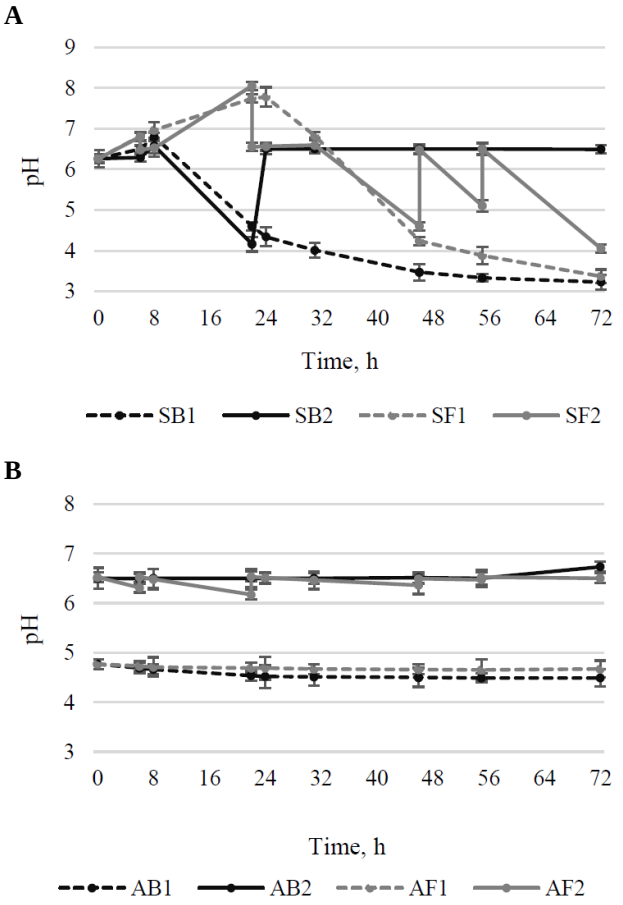
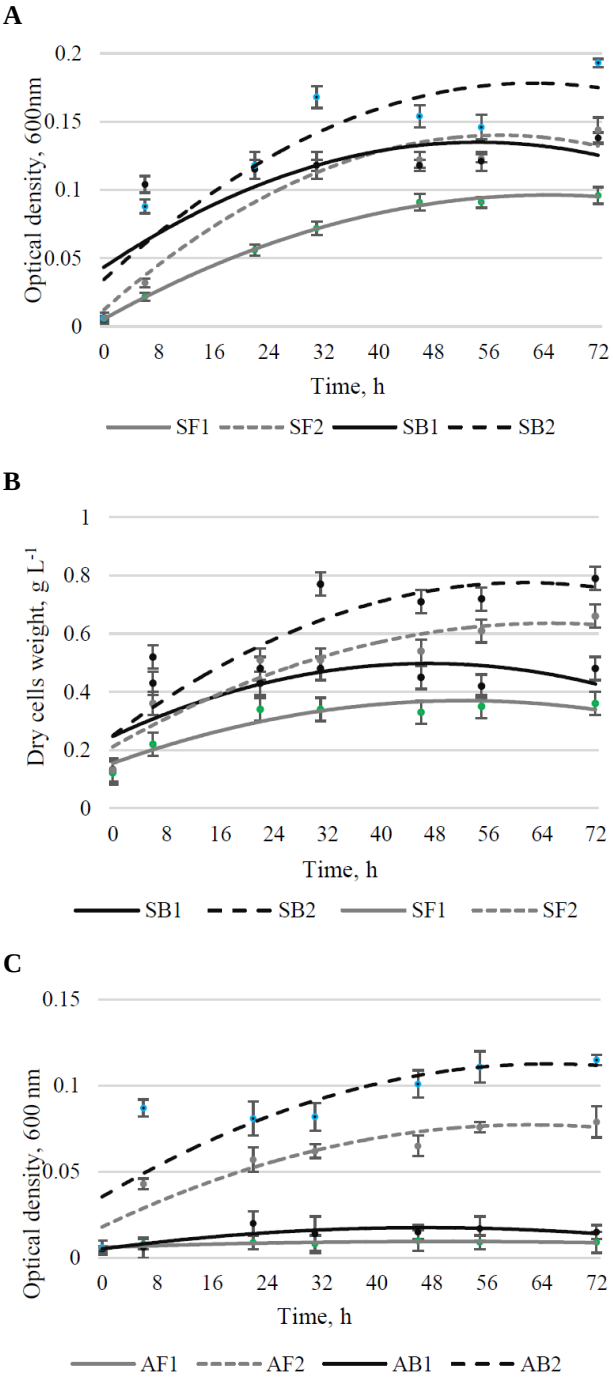


Figure 1. Time-course graphic of pH during fermentation with different substrates (A) sweet whey, (B) acid whey.

It can observed that an initially pH increases slightly in the sweet whey samples because bacteria have been adapted into the substrate, and then start to grow and oxidase lactose into lactobionic acid as the result decreasing of pH was observed. There was no significant difference between samples SB1 and SF1 at the end of fermentation ($p<0.05$). In Fig. 1B the pH of the samples changed slowly and apparently, bacteria could not adapt to the acidic medium. If pH is maintained manually time by time, we could observe much faster pH decreasing in sweet whey samples than in acid whey. At the end of the process significant differences ($p<0.05$) was not established between AB1 and AF1 samples, the pH changes in acid whey was similar in both equipment during fermentation.

B. Microorganisms Growth Monitoring by Optical Density and Dry Cells Weight in Fermentations

The amount of the converted lactose into lactobionic acid can be followed by the extent of optical density and dry cell weight amount. In sweet whey samples, *Pseudomonas taetrolens* present a higher growth than in acid whey samples (Fig. 2).



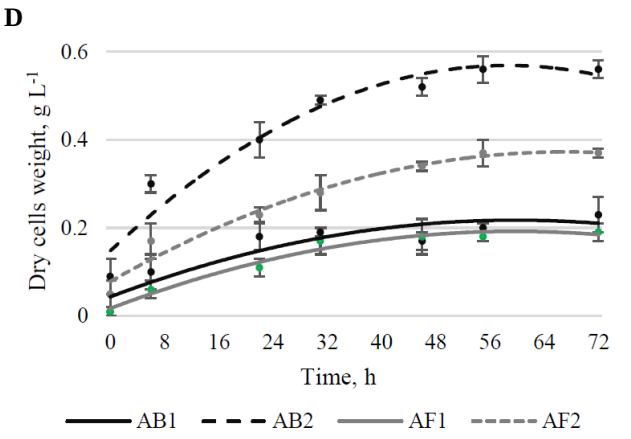


Figure 2. Time-course graphic of *Pseudomonas taetrolens* growth during fermentation in different substrates (A) sweet whey by optical density, (B) sweet whey by dry cells weight, (C) acid whey by optical density, (D) acid whey by dry cells weight.

The higher biomass production is obtained with adjusted pH using bioreactor. There were significant differences between AB1 and AF1 samples during fermentation process ($p>0.05$), in both samples bacterial growth was observed to be very low demonstrating that *Pseudomonas taetrolens* is stressed in acid medium.

Alonso *et al.* (2012) have reported that lactose oxidative bioconversion by *Pseudomonas teatrolens* in sweet whey medium with pH between 4.0 to 4.5 delaying onset of production, while with pH 6.0 to 6.5 contributed to enhance cellular proliferation, resulting in specific growth rate more than in another medium pH [14].

C. Oxygen Content in Substrate during Fermentation in Bioreactor

The dissolved oxygen amount in the substrate was strongly related to the growth stage, as observed in Fig. 3. In sweet whey samples (SB1 and SB2) noticeable exhaustion in dissolved oxygen concentration was found at the initial period of cells growth stage followed by a sudden quick increase to saturated values, representing the beginning of the production period.

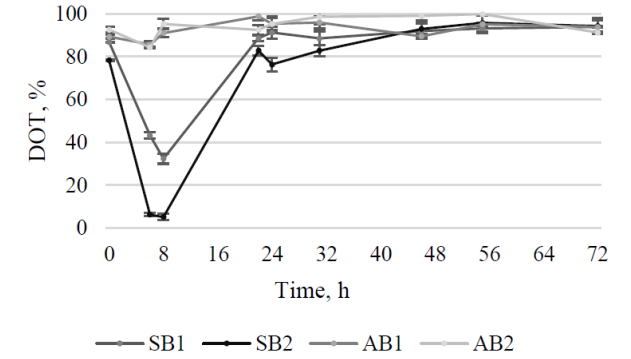


Figure 3. Time-course graphic of DOT during fermentation process by bioreactor in different substrates- sweet whey and acid whey.

Furthermore, there was a relevant difference in the minimum DOT level gained in the fermentation medium. A minimum DOT level of 6% was reached during the growth phase using a sweet whey with maintained pH 6.5 compared to the 85% gained when using an acid whey with non-controlled pH as a substrate. In Fig. 3. is observed that *Pseudomonas taetrolens* cells were barely consuming oxygen in acid whey samples (AB1 and AB2), since DOT values were very close to the saturation level. Under these circumstances in acid whey *Pseudomonas taetrolens* growth is restricted by some factors, like low pH and total compounds composition. *Pseudomonas taetrolens* growth is limited under these circumstances in acid whey.

D. Lactose Consumption and Lactobionic Acid Production During Fermentations

Fig. 4 shows differences between sweet whey (Fig. 4A) and acid whey (Fig. 4B) in lactose conversion into lactobionic acid.

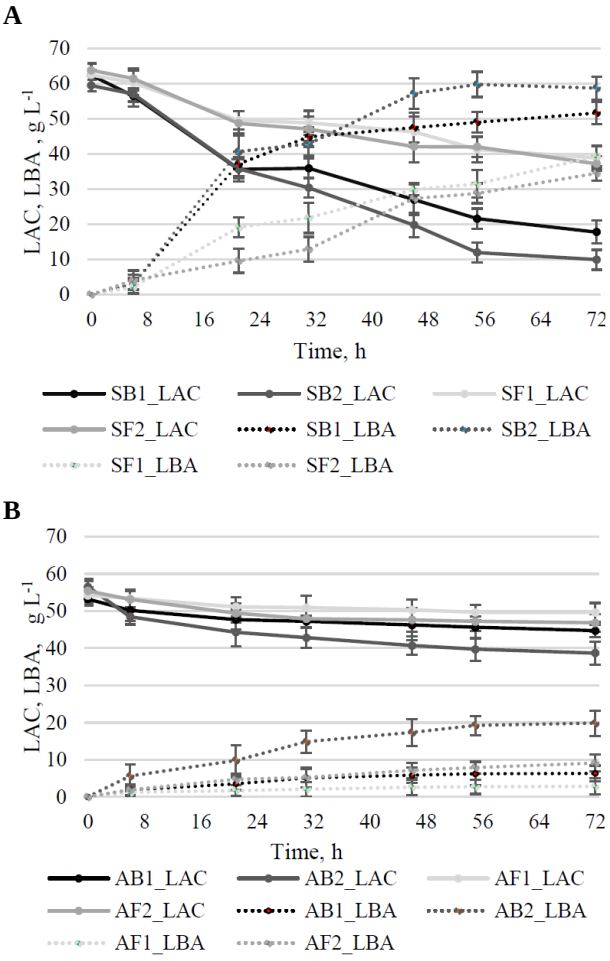


Figure 4. Time-course graphic of lactose (LAC) and lactobionic acid (LBA) during fermentation in different substrates (A) sweet whey, (B) acid whey.

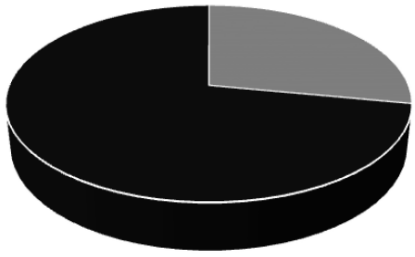
The higher conversion results were achieved (SB2 sample) using the bioreactor for sweet whey in controlled pH. Significant differences were established between acid whey and sweet whey fermentation. In acid whey samples, the lactose bioconversion process into lactobionic acid was very low. Significant differences were established between fermentation in bioreactor and shake-flask. Better results in lactose bio-conversation was obtained in bioreactor than

in shake-flask. The input of additional oxygen by agitation rate is necessary and it improves bacteria growth and production of lactobionic acid. Lactose is converted by lactose oxidase to lactobiono-δ-lactone and then by lactonase in lactobionic acid and as Alonso *et al.*, (2013) had mentioned where lactonase presents an optimum at pH 6.5-6.7 [15]. As well pH decreases in non-controlled pH samples during the fermentation process because of lactobionic acid production and it gives acid medium in the substrate which is not suitable for *Pseudomonas taetrolens* growth anymore and at the same time lactose conversion decrease too. For this reason, it is necessary to maintain pH around 6.5 during all fermentation period to make an optimal medium for *Pseudomonas taetrolens* cells growth, microbial biosynthesis and metabolism.

E. Purification Results

After fermentation, the products were purified via lyophilization as powdered white crystals. Then analysed to determine the purity. Lactose, lactobionic acid and proteins concentration was analysed (Fig. 5).

A



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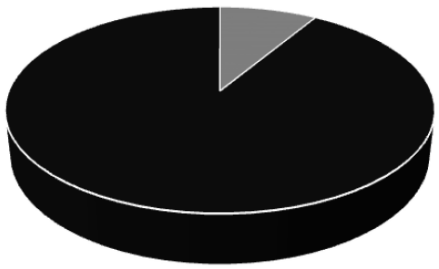

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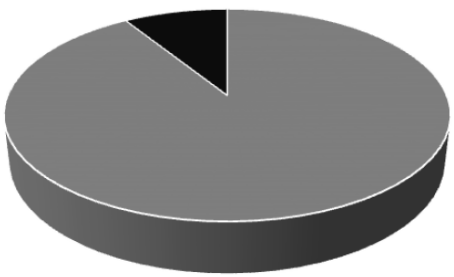

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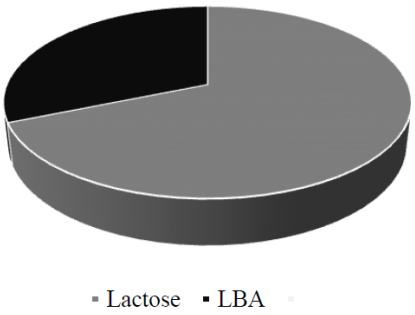

Figure 5. Yield of Lactobionic acid and Lactose in cell free crystal samples after 72 h of fermentation in bioreactor (A) sweet whey with non-controlled pH, (B) sweet whey with controlled pH, (C) acid whey with non-controlled pH, (D) acid whey with controlled pH.

In gained crystals were not detected proteins, they may be consumed during the fermentation. Gained crystals contained lactose and lactobionic acid mix. Results showed that lactobionic acid conversion yield is lower in acid whey samples. In samples were obtained 72.05% of lactobionic acid in SB1, 91.24% in SB2, 8.98% in AB1, 31.24% in AB2 and the significant differences were established among these samples ($p>0.05$). Significant amount of lactobionic acid was obtained in sweet whey samples, but in acid whey samples lactose was not effectively converted into lactobionic acid. Other researches with sweet whey substrates have gained results with 46% to 100% of lactobionic acid yield by *Pseudomonas taetrolens*. They have used different seed culture ages, feeding strategies, pH shifts and substrate supplements [8], [12]-[15].

More researches are needed to achieve an interesting lactose conversion to lactobionic acid using acid whey, and it may be improved by mixing sweet whey with acid whey, adding different supplements which is necessary for *Pseudomonas taetrolens* growth or adding bacterial biomass during fermentation process.

IV. Conclusion

The most suitable method for lactobionic acid production with *Pseudomonas taetrolens* is the bioreactor using sweet whey as a substrate in controlled pH conditions. Acid whey samples did not reach good results in lactose conversion into lactobionic acid, but this study proved that the use of acid whey as substrate for lactose conversation is better in bioreactor and maintaining the fermentative pH medium at 6.5 as best conditions. It is necessary to design new biotechnological strategies for acid whey fermentation to reach higher lactobionic acid yield. These strategies for acid whey use as substrate for lactobionic acid production with *Pseudomonas taetrolens* could include the mixing of sweet whey with acid whey or inoculate *Pseudomonas taetrolens* once the process begin to ensure bacteria growth and viability during all the fermentation period.

Conflict of Interest

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors had visited opening meeting about research and planned operating scheme. Inga Sarenkova, Manuel Rendueles, Inga Ciprovica conducted the research; Inga Sarenkova, Sara Saez Orviz operated in the laboratory; Inga Sarenkova, Sara Saez Orviz analyzed the data; Inga Sarenkova wrote the paper; All authors searched for methods and materials; Mario Diaz, Inga Ciprovica, Manuel Rendueles did paper revision and all authors had approved the final version.

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