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Repeated-Batch Fermentation of Cheese Whey for Semi-Continuous Lactic Acid Production Using Mixed Cultures at Uncontrolled pH

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Abstract: The paper investigates mixed-culture lactate (LA) fermentation of cheese whey (CW) in order to verify the possibility of using waste materials as feedstock to produce a product with high economic potential. The fermentation performance of two reactors operating in repeated-batch mode under uncontrolled pH conditions and various hydraulic retention time and feeding conditions was evaluated in terms of LA production. Five experimental phases were conducted. The hydraulic retention time (HRT) was varied from 1 to 4 days to verify its effect on the process performance. The best results, corresponding to the maximum LA concentration (20.1 g LA/L) and the maximum LA yield (0.37 g chemical oxygen demand $(COD)_{(LA)}$/g $COD_{(CW)}$), were reached by feeding the reactors with cheese whey alone and setting the HRT to 2 days. The maximum productivity of lactic acid (10.6 g LA/L/day) was observed when the HRT was decreased to 1 day.

Keywords: biorefinery; cheese whey; lactic acid; mixed culture; repeated-batch fermentation

1. Introduction

Waste biomass from food processing industries can be seen as an abundant source for biorefineries that aim at the industrial production of biofuels and value-added chemicals [1]. Dairy industries, for example, make an important contribution to the production of liquid effluents that are rich in organic substances characterised by significant contents of lactose (0.18–60 kg/m^3), protein (1.4–33.5 kg/m^3) and fats (0.08–10.58 kg/m^3) [2,3]. The generation of a liquid effluent, i.e., cheese whey (CW), is estimated at 0.8–0.9 L per litre of treated milk, or 9 kg per kg of produced cheese [2,4,5]. The total amount of produced CW worldwide is estimated to be around 180–190 million tons per year, and only half of this by-product is subsequently used for food or feed production [4]. On the other hand, around 100 million tons per year are typically discarded as a waste by-product in the environment, representing a significant issue for traditional wastewater treatment plants. Moreover, different functional proteins with high nutritional and therapeutic properties can be obtained from CW purification using a wide variety of separation techniques [5]. The chemical composition and the characteristics of the CW depend on the type of milk as well as on the adopted cheese production technique. However, generally, CW has a minerals content of about 0.46 ± 10%, and a concentration of total suspended solids that ranges between 0.1 and 22 g/L. Other typical characteristics are: pH in the range 3.3–9.0, a phosphorus content of 0.006–0.5 g/L, a total Kjeldahl nitrogen (TKN) of 0.01–1.7 g/L, chemical oxygen demand

(COD) values in the interval 0.8–102 g/L and biological oxygen demand (BOD) values in the range 0.6–60 g/L [2,3]. Therefore, while CW processing in conventional wastewater treatment plants can be quite challenging, this waste biomass could be conveniently used as valuable feedstocks for the production of biofuels and biochemicals.

A sustainable route for the production of biofuels and biochemicals is the development of biorefineries based on renewable biomass sources [6]. This is why anaerobic-fermentation-based bioprocesses, such as dark fermentation (DF) and anaerobic digestion (AD), have been widely tested and applied for the production of bio-hydrogen (H_2), bio-methane (CH_4) and several biochemicals, including high-value organic acids (i.e., acetate, lactate, and butyrate) [7]. Among others, lactic acid is probably one of the most interesting products of anaerobic fermentation, being widely used in food industries as a preservative compound and curing agent, and a flavoring agent in cosmetics and pharmaceuticals, such as skin care products. Moreover, lactic acid can be chemically treated for the production of biological plastics, a natural alternative to petrochemical plastics that represents, as is well-known, a significant environmental problem [7–9]. Even though most of the lactic acid that is used today is derived from biological routes, its production cost could be much more competitive if the used feedstock, which accounts for more than 70% of the production costs [10,11], is represented by organic wastes. For this, CW can serve as an excellent feedstock for the production of lactic acid. However, several previous studies have been carried out on pure cultures under axenic conditions [12–15]. To the best of our knowledge, only a few studies used a mixed-culture inoculum and complex substrates as a fermentation feedstock [16,17]. The operation and maintenance for axenic conditions and the use of a pH buffer may have significant cost implications for the lactic acid production economy [18]. Therefore, mixed-culture and real waste feedstock were adopted in this study, as they represent crucial elements in the development and application of a sustainable waste-based biorefinery [19–21].

The aim of this paper is to assess dark fermentative lactic acid production using CW mediated by mixed cultures. Among other biological conversion technologies, DF is considered to be particularly interesting, as it allows for the production of multiple biofuels and other platform chemicals from waste biomasses [10–14]. The study also aims at maximising the lactate yields along with identifying the optimal process stability at the natural pH of the substrate. Different management techniques have been adopted using two lab-scale fermentative bioreactors for lactic acid production from CW. The effect of digestate addition on lactic acid production and fermentation performance has been highlighted. The presented results constitute an interesting starting point for scaled-up applications in the field of CW valorisation and lactic acid production by mixed-culture fermentation.

2. Materials and Methods

2.1. Substrate and Inoculum

The substrate that was used in the present study was obtained from the dairy company La Perla del Mediterraneo, which is located in Capaccio (Salerno, Italy). The company exports a wide variety of products to different European and extra-European countries (i.e., the United States and Asia), being one of the larger companies in the area of Salerno. After sampling, CW was immediately frozen at −20° C to keep its characteristics as unaltered as possible.

The anaerobic digestate that was used as an inoculum for the DF was collected from the full-scale treatment plant of the same facility, and was adopted for the anaerobic co-digestion of CW and buffalo manure. The digestate was pre-treated at 105 °C for 1.5 h to inhibit methanogenic species, which are more sensitive to heat shocks than acetogenic and fermentative bacteria [22].

The main characteristics of the adopted substrate and inoculum were evaluated in triplicate and are reported in Table 1.

Table 1. Characteristics of the used cheese whey (CW) and inoculum.

	TS (g/L)	VS (g/L)	COD (gCOD/L)	pH (-)	Soluble Carbohydrates (g/L)
CW	47.1 ± 2.5	31 ± 2	41 ± 2	5.6	31 ± 2
Inoculum	51 ± 2	31 ± 2	41 ± 2	7.5	-

2.2. Experimental Setup and Operational Conditions

2.2.1. Dark Fermentation Bioreactor

The DF process was conducted using two 2 L glass reactors that were maintained under mesophilic conditions (35 ± 1 °C) and operated in repeated-batch mode aimed at semi-continuous lactic acid production. Constant stirring conditions of 250 rpm were adopted for both the reaction units. Differently from fed-batch reactors, the repeated-batch feeding mode ensures a constant reaction volume in bioreactors, which is more similar to the real-scale feeding strategy that is usually adopted in wet anaerobic treatment plants. The feeding strategy is deeply connected to the sampling strategy, as they are contextual at each feeding day. This allows for a semi-continuous production of lactic acid as the reactors are operated continuously for the entire experimental time. The reactors were equipped with three different ports. The first one was connected to an external tank, and used to feed the CW. The second one was utilised for effluent extraction. The last one, placed on the top of the reactor, was devoted to biogas extraction and to head-space gas analysis. A glass tube and gaskets were used for the junctions. Sealing joints were controlled by filling up each reactor, before use, with water and pressurised air.

2.2.2. Experimental Conditions

The experimental test was conducted for 136 days, with no pH correction, and was characterised by five distinct operative phases, as indicated in Table 2. Phase 1 (the start-up phase) was conducted in batch mode, assigning a substrate (Food) to inoculum (Microrganisms) ratio (F/M) equal to 1.9 gVS/gVS. The other phases, instead, were conducted in repeated-batch mode, varying, for each of them, the value of the hydraulic retention time (HRT). Four hundred millilitres (400 mL) of inoculum was added to the reactor at the beginning of phase 2 and phase 3, and after each HRT of these phases. No inoculum addition was performed before or during phase 4 and phase 5. This choice was adopted to simulate the management of a real-scale anaerobic digester operating under wet conditions. According to the assigned operating conditions, the Organic Loading Rate (OLR) increased during the experimental operations (Table 2). At selected times, small volumes of the influent and the effluent from the two reactors were sampled to check the pH value and to measure the concentration of organic acids (OAs) and ethanol (EtOH). Moreover, the characteristics of the used cheese whey were analysed daily to monitor their variation.

Table 2. Operating conditions of the different experimental phases.

Phase	1	2	3	4	5
OLR (kg VS/m^3/day)	batch	19.6	32.4	32.4	49
HRT (days)	batch	4	2	2	1
Time length (days)	18	31	11	53	10

2.3. Analytical Methods

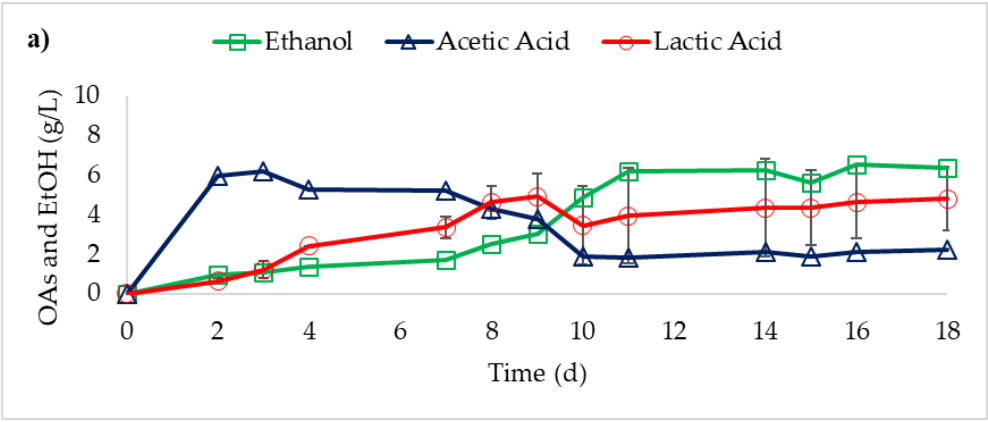
OAs and ethanol concentrations were determined by high-pressure liquid chromatography (HPLC). Samples were analysed using an LC 25 Chromatography Oven (Dionex, Sunnyvale, CA, USA) equipped with an Organic Acids column (Metrohom, Herisau, Switzerland) and a UVD 340U detector (Dionex, Sunnyvale, USA). A solution of 1 mM H_2SO_4 was used as an eluent and pumped at the rate of

0.7 mL/min by a GD 500 Gradient Pump (Dionex, Sunnyvale, USA). pH values were measured using an inoLab pH meter (WTW, Wheilheim, Germany). COD concentrations were measured through the optical density value by colorimetric analysis, according to the Standard Methods (APHA, 2005), using a Photolab Spektral spectrophotometer (WTW, Germany). According to other studies [1,23], Total Solids (TS) and Volatile Solids (VS) content was determined by oven drying at 105 °C and 550 °C, respectively. The composition of the biogas that was produced during the process was analysed using a Varian Star 3400 gas chromatograph equipped with a Shin-Carbon ST 80/100 column and a thermal conductivity detector, following the instructions reported elsewhere [24]. The separation was conducted using Argon as the carrier gas.

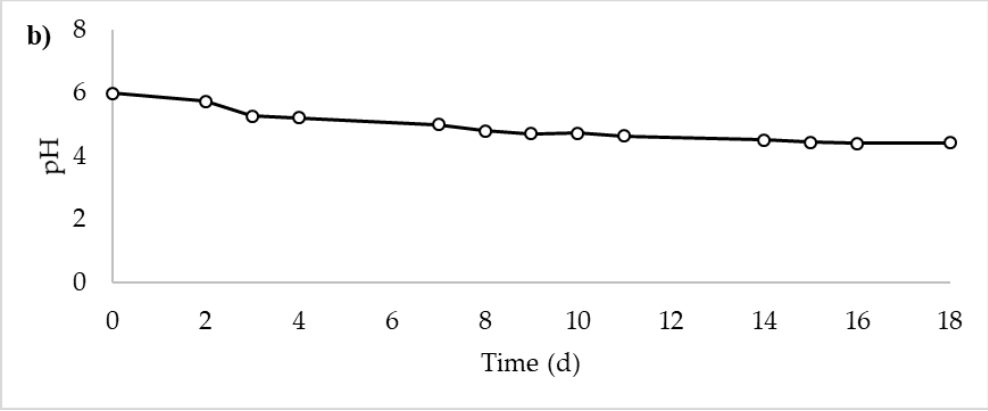
3. Results

CW fermentation was performed in two identical reactors operating under the same conditions and averaged values of all the investigated parameters are reported. Standard deviations between the two measures, corresponding to the variation range in this special case, are indicated by error bars and reported for lactic acid alone for clarity of representation.

During the start-up phase, a high F/M ratio and the anaerobic conditions led to an increase in organic acids (OA) production (Figure 1a) and a corresponding gradual decrease of pH from 6 to 4.5 (Figure 1b). Ethanol was detected as a significant fermentation by-product, and its concentration reached a maximum value of 5.5 g/L. It showed an increasing trend with a low concentration until Day 7. Acetic acid showed an opposite trend, reaching a stable low concentration (around 2 g/L) at Day 10. Moreover, a normalised hydrogen volume of 103 mL of H_2 was produced within 20 days of fermentation (data not shown).



(**a**)



(**b**)

Figure 1. Trends of the main organic compounds (**a**) and pH (**b**) during phase 1.

During phase 2 (Day 22–53), the adoption of a four-day HRT resulted in an increase in OA generation (Figure 2a). Increasing the lactic acid production led to lower pH values (Figure 2b). pH dropped from 4.5 to 3.8 at the end of phase 2. As shown in Figure 2a, acetic acid and ethanol were still present in the reactor. The amount of acetic acid increased until a constant concentration of around 4.5 g/L was reached, while, again, an opposite trend was exhibited by ethanol, whose concentration was very low during the final days of phase 2.

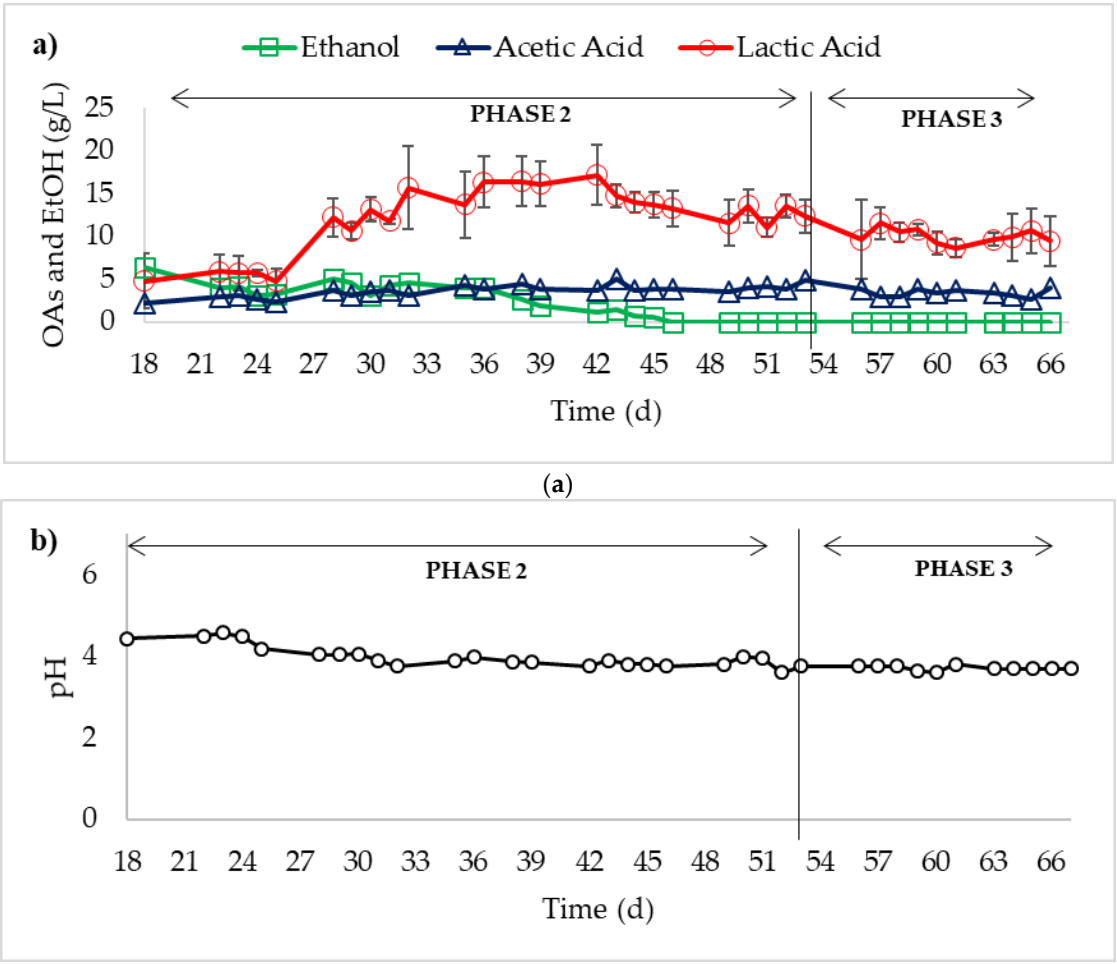


Figure 2. Process monitoring during phases 2 and 3, with inoculum addition: (**a**) organic acids (OAs) and ethanol (EtOH); (**b**) pH.

During phase 3 (Days 56–67), lactic acid and acetic acid were the main by-products, and their concentration remained unchanged. The HRT allowed for a fermentative process producing the maximum lactic acid concentration (11.6 g/L), which, nonetheless, was lower than the maximum value obtained during phase 2 (17.2 g/L). The acetic acid concentration remained constant (≈3 g/L) and no ethanol production was observed.

During phase 4 (Days 70–123), the digestate was no longer added, and the HRT was not varied compared to the previous phase (HRT = 2 days). In these conditions, the pH varied between 3.6 and 3.4, remaining almost stable for the whole phase's length, while the production of acetic acid slightly increased (Figure 3).

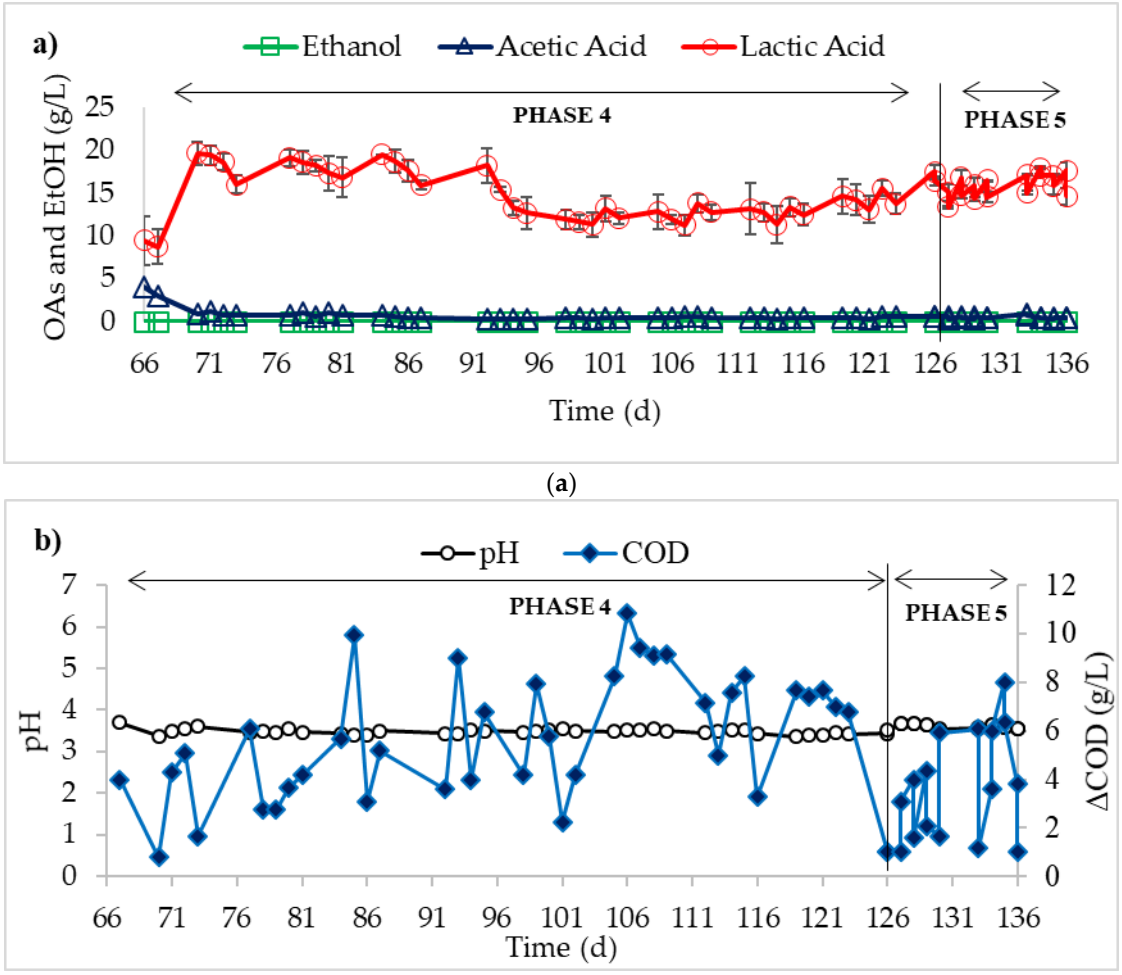


Figure 3. Process monitoring during phases 4 and 5, without inoculum addition: (**a**) OAs and EtOH; (**b**) pH (round indicators) and Δchemical oxygen demand (COD) (square indicators).

Finally, the minimum HRT (1 day) adopted in phase 5 (Days 126–136), corresponding to the maximum OLR of 32.43 g COD/L/day, had negligible effects on lactic acid accumulation within the effluent (Figure 3a). The pH value varied between 3.6 and 3.4 as in the previous phase (Figure 3b). A maximum lactic acid concentration of 17.6 g/L was reached, which was lower than those reached during phase 4.

For mass balance purposes, Figure 3b reports the trend of consumed soluble COD during phases 4 and 5. The reported values were calculated as the net soluble COD variation between the influent and the effluent. As can be seen, the maximum variation (ranging around 11 g/L) was registered during phase 4 at day 106.

Figure 4 shows, instead, the daily specific yield of lactic acid (Y^*) during the same operation phases, evaluated as mols of accumulated COD in the form of lactic acid by mols of net consumed soluble COD. The latter was calculated by removing the lactic acid contribution to the influent and effluent soluble COD value. The results showed a maximum and minimum yield of 0.33 (phase 4) and 0.10 (phase 5).

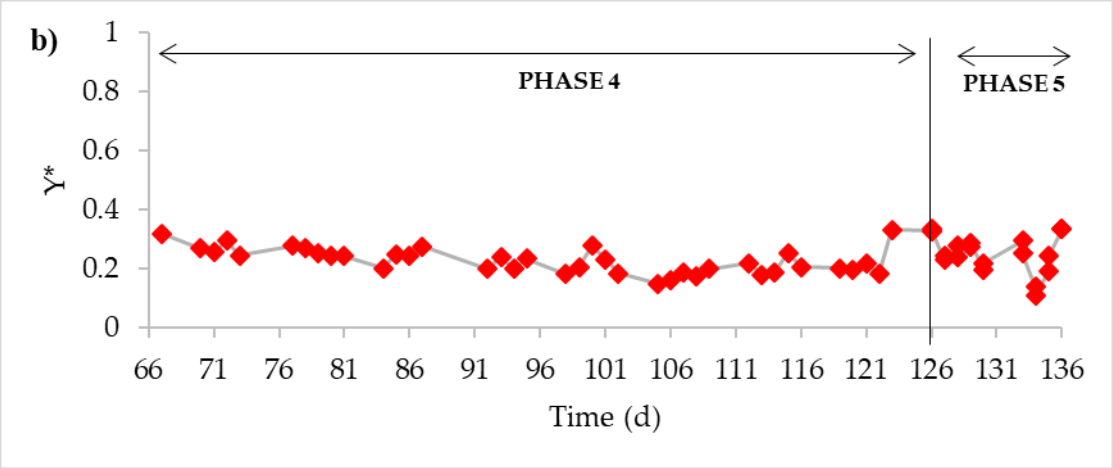


Figure 4. The daily specific lactic acid yields (Y*) during the final operational phases.

Hydrogen production was also detected during the initial phases of fermentation. The cumulative hydrogen generated during phases 1 and 2 reached a total normalised volume of 299 mL until Day 40 (data not shown). The hydrogen yields that were achieved during these phases were very low compared to other studies [22,25,26]. After that period, only CO_2 was generated. This result is in agreement with the observed trend of pH values. After phase 2, the pH value was always below 4, which generally corresponds to inhibiting conditions for hydrogen generation studies [24].

Finally, Figure 5 illustrates the average lactic acid yields obtained per unit of fed substrate in terms of soluble COD. Each bar corresponds to a single HRT. Different colours are used for different operation phases. The main results are further summarised in Table 3, which reports the average (Y) and the maximum (Y_{max}) value of the yield, and the average (P) and the maximum (P_{max}) lactic acid production rate (daily amount of lactic acid produced per litre). All shown values refer to the net converted soluble COD that was obtained by subtracting the influent lactic acid contribution already contained in the cheese whey. Standard deviations related to each phase (Table 3) refer to the averaged values of all the specified parameters during the different HRTs constituting a single phase.

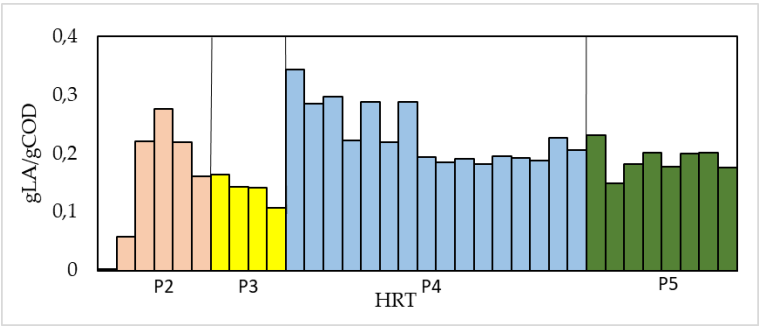


Figure 5. Lactic acid yields during the different operational phases (P1, P2, P3, P4 and P5 represent the five phases).

Table 3. Yield and production of lactic acid (LA) for the phases operated in repeated-batch mode.

Phase	Y ($gCOD_{(LA)}/gCOD_{(CW)}$)	P (gLA/L)	Y_{MAX} ($gCOD_{(LA)}/gCOD_{(CW)}$)	P_{MAX} (gLA/L)	Y^*_{MAX} %
2	0.191 ± 2.09	2.261 ± 2.017	0.33	3.75	-
3	0.141 ± 2.02	3.401 ± 2.287	0.2	4.55	-
4	0.241 ± 2.05	5.671 ± 2.626	0.37	8.55	0.33
5	0.201 ± 2.02	9.191 ± 2.289	0.23	10.6	0.33

It is evident that, after 16 cycles (during phase 4) conducted under different operational conditions, the reactors reached a stable performance until the end of the last phase (18 more HRT turnovers) even if an HRT change was adopted between phase 4 and phase 5. During phase 4, the maximum yield of 0.37 g $COD_{(LA)}$ g/$COD_{(CW)}$ was reached, while the maximum value of lactic acid productivity of 10.6 g LA/L/day was observed during the final phase at the HRT of 1 Day.

4. Discussion

The high organic carbon content and the prevalent rapidly biodegradable COD fraction of CW allow for a promising conversion of this liquid effluent in dark fermentative bioreactors [27–30]. During the start-up phase of continuous bioreactors, digestate from anaerobic digestion is normally used as an inoculum for DF experiments, as it contains fermentative acidogenic microbial consortia [31,32]. Different pre-treatment strategies have been introduced during the last few years [22] that lead to methanogenic activity inhibition by favoring acidogenic species [23]. In this study, the significant amount of digestate used as an inoculum for CW conversion had an important buffering effect during the start-up phase, and limited the pH drop (Figure 1b) due to acid generation [25]. Hydrogen production was low, while ethanol was the main fermentation by-product. This result was related to the fact that alcohol production processes (i.e., solventogenesis) are usually associated with the presence of acetic acid, which is produced by the Wood–Ljungdahl pathway using hydrogen as an electron acceptor [26]. However, the concomitant presence of ethanol and acetic acid could also have been due to the presence of heterofermentative bacteria. Heterofermentation, in fact, is one of the different pathways that lactic acid bacteria can follow, leading to lactic acid, carbon dioxide, ethanol, and acetic acid production [27,29]. As a consequence of the mentioned conversion processes, low lactic acid concentrations characterised phase 1. The maximum measured lactic acid concentration value, in fact, was below 5 g/L.

The lactic acid concentration clearly increased in the second phase of operation. The adoption of a repeated-batch feeding strategy, which was different from the start-up phase, strongly affected the lactic acid concentration and yields. In industrial-scale applications, lactic acid is generally produced in batch mode, and by using pure cultures, whose growth is optimised by the addition of a significant amount of chemicals [13]. Previous studies demonstrate that the adoption of a repeated-batch mode could give a higher yield than the adoption of a single-batch mode [10,23,30]. During the same phase, high concentrations of acetic and lactic acid were reached, and the pH profile indicated that the buffering capacity due to digestate addition did not affect the pH evolution.

During the third phase, the more stable OA concentrations suggested that the microbial community was more acclimated to the CW conversion. Although the lactic acid production rate was more stable, the higher ORL led to a lower substrate conversion rate, reducing the bioreactor's performance. These results were likely due to the reduced time for slower biological reactions contributing to lactic acid production during phase 2.

The acetic acid concentration, which varied between 2 and 4 g/L during the digestate addition phases (1, 2 and 3), drastically decreased to 1 g/L at the beginning of phase 4. Digestate is rich in acetogenic bacteria, which are able to convert organic compounds in acetic acid [31,32]. The lower presence of acetogenic micro-organisms in the feeding cheese whey led to their progressive washing-out during phases 4 and 5. Conversely, the lactic acid production considerably increased, and a maximum concentration of 20.1 g/L was obtained. This result was probably due to the prevalence of autochthonous lactic bacteria, which led to a higher lactic acid percentage compared to the previous phases [33]. Indeed, lactic acid bacteria are more acid-tolerant than other fermentative bacteria as they are able to regulate their intracellular pH [34–36]. Moreover, they are able to grow at extremely low pH [14,37]. In previous studies, acidic pre-treatments on fermentation inoculum were adopted to favor lactic acid bacteria growth and proliferation [14,38]. This procedure limited the production of undesirable compounds in the fermentation broth, increasing the purity of the produced lactic acid.

It is worth noting that the lactic acid concentration measured during phases 4 and 5 (without inoculum addition) did not reach the peak characteristics of phase 2 (operated with inoculum addition). Nonetheless, the concentration fluctuations were restrained (Figure 3a), suggesting that the process was more stable along the different feeding cycles.

As to the effect of the HRT variation, it could be observed that the adoption of lower values did not strongly affect the lactic acid concentration. As reported in a previous study [38], when low HRTs were adopted (12 h and 8 h), it was possible to reach a high extracted lactic acid mass in the presence of low lactic acid concentrations. HRT variations affected both the yield and the lactic acid productivity. The yield was higher when a higher HRT was adopted, while the production increased for lower HRT values. Therefore, it seems more convenient to use lower HRTs in order to extract a higher lactic acid amount in terms of daily mass.

Different studies demonstrate that the optimum pH value for lactic acid production is between 5.5 and 5.9 [13,39,40]. The adoption of lower values led to low lactic acid concentrations (below 5 g/L) [14,31,38]. In contrast, the results achieved in this study showed that it was possible to produce a high lactic acid amount (20.1 g/L) at low pH, under the adopted uncontrolled pH conditions. This result is highly relevant, as uncontrolled pH conditions are usually unfavorable for lactic acid production. Perez et al. [13], for instance, studied CW fermentation by *Lactobacillus helveticus* under uncontrolled pH conditions in batch mode. The maximum observed concentration value was about 15 g/L, while the same species was able to accumulate around 60 g/L at a fixed pH of 5.9, and 80 g/L with the supplementary addition of yeast extract. Liang et al. performed another example of uncontrolled pH fermentation in 2014 [16]. The study used potato peel waste as a substrate and mixed culture in batch mode. The maximum concentration of 14.7 g/L of lactic acid was observed. Wu et al. [41] studied acidogenic fermentation of fruit and vegetables wastes. In order to improve the lactic acid production, they varied the pH value of a Continuous-flow Stirred Tank Reactor (CSTR) from 4 to 5 by external addition of a NaOH solution. The maximum concentration of about 15 g/L was reached, which was again lower than the maximum concentration achieved in the present study. Regarding the lactic acid yields (Y and Y_{MAX} in Table 3), expressed as grams of produced lactic acid (in terms of COD) per gram of fed COD to the reactors, the higher Y_{MAX} value was reached by Choi et al., 2016 [38] using CW and performing the fermentation at a fixed pH of 5.5. When the same reactor was operated at pH 3, the average yields were below the value of 0.1. In this study, the values of Y ranged between 0.2 and 0.37, similarly to the case presented by Whu et al. in 2015 with different wastes [41].

Other authors have evaluated the yield in terms of mol of produced lactic acid per mol of consumed lactose, carbohydrates or soluble sugars consumed during the fermentation process [42–44]. This parameter adds qualitative information about the biological conversion and refers to the percentage of the feeding organic compound effectively converted into lactate. Ghaly et al. [42] studied the batch fermentation of cheese whey using pure cultures and nutrient supplementation. They reached yields (in terms of grams of lactic acid per gram of lactose) between 0.56 and 0.72. Different strains were tested by Joudeikiene et al. [43], who performed pure-culture cheese whey batch fermentation at 37 °C under stationary optimised conditions. The results showed significant lactic acid yields ranging between 0.33 and 0.065. In this study, the grams of lactic acid (in terms of COD) per gram of converted COD were evaluated under repeated-batch conditions. The maximum yield of 0.37 was achieved during phase 4, which was lower than the values reported under batch conditions using pure cultures.

No important pH variation was detected from the second phase to the end of the process, as the pH remained around the lactic acid pKa due to the prevalence of this compound in the culture medium. It was more convenient to feed the reactor with the cheese whey alone as it was possible to produce higher amounts of lactic acid, which had higher purity compared to the previous phases where other fermentation by-products were detected. The presented results demonstrate that mixed culture CW fermentation can be a promising alternative to the pure culture fermentation processes that are usually adopted for biological lactic acid production. This study represents a preliminary base for

subsequent higher-scale applications and for mathematical modeling of mixed-culture fermentation processes [32,45,46].

5. Conclusions

Semi-continuous lactic acid production from cheese whey under repeated-batch conditions was investigated using mixed microbial cultures under uncontrolled pH conditions. Two reactors were operated for five operational phases and 136 days. Different HRT and ORL values were tested to evaluate lactic acid yields and the fermentation performance. The results showed the maximum LA concentration of 20.1 g/L and the maximum yield of 0.37 g of lactic acid per g fed COD, which were achieved with the HRT of 2 days. Conversely, the maximum lactic acid productivity (10.6 g/L/d) was obtained when an HRT of 1 day was adopted. The obtained results represent an interesting base for the industrial application of the process due to the more realistic adopted conditions with respect to all previous studies.

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Conflicts of Interest: The authors declare no conflict of interest.

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