

Article

Conversion of Sweet Whey to Bioethanol: A Bioremediation Alternative for Dairy Industry

Laura Conde-Báez ^{1,*}, Cuauhtémoc F. Pineda-Muñoz ¹, Carolina Conde-Mejía ² , Elizabeth Mas-Hernández ³  and Antioco López-Molina ^{2,*} 

¹ Facultad de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero, Av. Lázaro Cárdenas, s/n, Ciudad Universitaria, Chilpancingo 39086, Guerrero, Mexico; cuauhtemoc.pineda@hotmail.com

² División Académica Multidisciplinaria de Jalpa de Méndez, Universidad Juárez Autónoma de Tabasco, Carretera Estatal Libre Villahermosa-Comalcalco Km 27 s/n, Ranchería Ribera Alta, Jalpa de Méndez 86205, Tabasco, Mexico; carolina.conde@ujat.mx

³ Facultad de Química, Universidad Autónoma de Querétaro, Cerro de las Campanas s/n, Col. de las Campanas, Santiago de Querétaro 76010, Querétaro, Mexico; elizabeth.mas@uaq.mx

* Correspondence: lcondebaz@gmail.com (L.C.-B.); antioco.lopez@ujat.mx (A.L.-M.)

Abstract: In many countries, whey from the dairy industry is an abundant waste that generates an important environmental impact. Alternative processes to use the whey and minimize the environmental impact are needed. This work considered six formulations with different ammonium sulfate and L-phenylalanine (L-Phe) concentrations to produce bioethanol in sweet whey fermentation by *Kluyveromyces marxianus*. The results showed a maximum bioethanol concentration equal to $25.13 \pm 0.37 \text{ g L}^{-1}$ ($p < 0.05$) for formulation F6, with 1 g L^{-1} of L-Phe and 1.350 g L^{-1} of ammonium sulfate (96 h). For these conditions, the chemical oxygen demand removal percentage (CODR%) was 67%. The maximum CODR% obtained was 97.5% for formulation F3 (1 g L^{-1} of L-Phe) at 96 h; however, a significant decrease in bioethanol concentration ($14.33 \pm 2.58 \text{ g L}^{-1}$) was observed. On the other hand, for formulation, F3, at 48 h of fermentation time, a bioethanol concentration of $23.71 \pm 1.26 \text{ g L}^{-1}$ was observed, with 76.5% CODR%. Based on these results, we suggest that the best conditions to obtain a significant bioethanol concentration and CODR% value are those used on the configuration F3 at 48 h.



Citation: Conde-Báez, L.; Pineda-Muñoz, C.F.; Conde-Mejía, C.; Mas-Hernández, E.; López-Molina, A. Conversion of Sweet Whey to Bioethanol: A Bioremediation Alternative for Dairy Industry. *Biomass* **2024**, *4*, 507–517. <https://doi.org/10.3390/biomass4020026>

Academic Editor: Amit K. Jaiswal

Received: 19 March 2024

Revised: 30 April 2024

Accepted: 14 May 2024

Published: 3 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Keywords: biofuels; COD removal percent; dairy waste; *Kluyveromyces marxianus*

1. Introduction

The residue derived from cheese production contributes, to some extent, to problems of waste generation and water and soil pollution, among others [1]. In this regard, the United Nations has established, as one of the sustainable development goals, the global reduction of food waste for 2030 [2]. It has been reported that the dairy industry produces around 400,000 million liters of residues. Sweet whey adds 50% of waste [3]. Considering its high production of about 180 million tons worldwide, sweet whey could be a renewable source to produce biofuels [4,5]. Regarding the production of bioethanol (BioEtOH), using sweet whey as a raw material has advantages over first- and second-generation materials due to its high content of carbohydrates, lactose, proteins, vitamins, salts, lipids, and minerals. In addition, it is a long-lasting and low-cost product [6]. Due to its quality, bioethanol produced from sweet whey can be used in food, drinks, and the pharmaceutical and cosmetic industries [3,7]. Annual industrial ethanol production of four million tons has been reported, of which 80% is produced by fermentation [8]. Therefore, sweet whey is an ideal raw material alternative to BioEtOH production, with a relatively easy fermentation process. It does not involve additional costs for the inoculum and does not require excessive energy consumption for the substrate pre-treatment [9].

The production of two types of BioEtOH has been reported depending on the raw material used. First-generation bioethanol (BioEtOH 1G) is obtained from sugarcane and corn starch as raw materials via biomass fermentation. However, using these sources has disadvantages, such as the competition for arable land, agricultural supplies for human or livestock consumption, and their scarce availability [10]. On the other hand, second-generation bioethanol (BioEtOH 2G) is obtained from non-food lignocellulosic biomass in a process with several stages: the pre-treatment that eases the separation of lignin, the hydrolysis through acids or enzymes to turn the polysaccharides into simple sugars, and a sugar fermentation stage to obtain the alcohol [11]. All these stages for producing BioEtOH 2G and its separation and purification sequence are long and complex. The advantages of using sweet whey as a raw source for making BioEtOH are that it does not require complex pre-treatment and that some yeast, like *Kluyveromyces marxianus*, can perform hydrolysis and fermentation processes together.

Nevertheless, the main disadvantage is biofuel's low concentration when obtained from raw or filtered whey [12]. Accordingly, whey powder is one alternative to increase the BioEtOH concentration because of its high lactose concentration. However, even in this case, the waste products have a high pollutant load expressed as the chemical oxygen demand (COD), and the lactose concentrate within the whey increases the production costs [13]. In this sense, an alternative for producing BioEtOH from sweet whey could be using sweet whey pre-treated only with pasteurization before fermentation but under the best process conditions to increase the product concentration.

Kluyveromyces marxianus is a fermentative breath yeast that generates energy through oxidative phosphorylation, the tricarboxylic acid cycle (TCA), or fermentation into ethanol [9]. Sufficient availability of nitrogen, phosphorus, traces, vitamins, and cofactors is important to secure the completion of the fermentation as fast as metabolically possible [7]. The production of BioEtOH with *K. marxianus* using whey as a substrate when permeated, pasteurized, and/or enriched was reported recently [14]. It has been reported that only 20% of the nitrogen source found in the sweet whey can be used for these kinds of yeast. Therefore, supplementing the sweet whey with a nitrogen source and an amino acid such as L-phenylalanine (L-Phe) would allow the galactose and the Adenosine triphosphate to interact allosterically with the first enzyme, acting on the Leloir pathway, and on the other hand, to produce the L-phenylalanine bioconversion via the Ehrlich pathway to produce other metabolites that also favor the production of BioEtOH [15,16]. Consequently, six formulations of raw sweet whey with the addition of ammonium sulfate and L-phenylalanine, using the yeast strain *Kluyveromyces marxianus*, were evaluated in this work. Along with the strain selection, these changes would permit (i) the increase in the production of BioEtOH and (ii) the reduction of the sweet whey pollutant load without any pre-treatment, reducing the environmental impact and the harmful effects on the population.

2. Materials and Methods

2.1. Sweet Whey Preparation and Microbiological Analysis

Samples were directly taken from the production of panela-type cheese. The sweet whey quality was evaluated by assessing aerobic mesophiles and thermophiles, *Salmonella* spp., *Staphylococcus aureus*, and psychrophilic microorganisms, according to the NOM-092-SSA1-1994 [17], NOM-114-SSA1-1994 [18], and NOM-115-SSA1-1994 [19] guidelines, respectively. The sanitary specifications were assessed according to the NOM-243-SSA1-2010 norm [20]. Afterward, the sweet whey was prepared via slow pasteurization at 63 °C for a 30 min Low-Temperature Long Time (LTLT) process. The protocol of Gómez-Aldapa et al. [21] was used to determine the pH and the proximate analysis of whey. In this study, we selected ammonium sulfate as an additional easily assimilable nitrogen (NFA) source because there are reports that indicate that only 25% of the nitrogen present in whey can be assimilated by *K. marxianus* [22]. At the same time, L-Phe was added as a precursor of the product that is taken up by the Ehrlich pathway, and in the breakdown of the cinnamate catabolic pathway of L-Phe, anaerobic pyruvate carboxylation contributes to

ethanol production [23]. The six formulations considered are shown in Table 1, with low, medium, and high concentration criteria [24].

Table 1. Sweet whey formulations at pH 4.8, 180 rpm/30 °C, and concentrations of (NH₄)₂SO₄ and L-Phe.

Sweet Whey Formulations	Ammonium Sulfate (g L ⁻¹)	L-phenylalanine (g L ⁻¹)
Formulation F1	0	0
Formulation F2	0	0.50
Formulation F3	0	1.00
Formulation F4	0.45	1.00
Formulation F5	0.90	1.00
Formulation F6	1.35	1.00

2.2. Strain Maintenance

The strain employed was *K. marxianus* ITD00262, isolated by Páez-Lerma et al. [25] from the alcoholic fermentation process of *Agave Duranguensis* during mezcal production in Durango. The culture media used and the methodology for the strain maintenance followed guidelines from Conde-Báez et al. [24].

2.3. Inoculum Preparation

The strain of *K. marxianus* was seeded in a petri dish in a lactose, peptone, and yeast extract (LPY) medium [24] and incubated at 28 °C/24 h (Arsa, Mexico City, Mexico). A culture sample with a wire loop was transferred to a 250 mL Erlenmeyer flask with 100 mL of LPY broth media at pH 4.8. The flask was incubated in stirring at 1 RCF (Relative Centrifugal Force) at 30 °C (Shaker Thermo® Scientific, Waltham, MA, USA) for 12 h at 28 °C. Afterward, an initial inoculum of 1 × 10⁶ cells mL⁻¹ was incorporated using the direct count technique under a microscope with a hemocytometer (Marienfeld). This inoculum was added to flasks with sweet whey at pH 4.8; the flasks were incubated with stirring (2 RCF) at 30 °C for 96 h. Sample storage and the yeast count were performed according to procedures reported by Conde-Báez et al. [24]. The reading of the samples was carried out in triplicate.

2.4. Quantifying the Bioethanol Production

Detection and quantification of BioEtOH were carried out from the filtering of the sweet whey samples with cellulose membranes (0.5 µm diameter), previously centrifuged at 9677 RCF (240 mm rotor radius) [Eppendorf™] for 6 min. The filtrate was analyzed using gas chromatography Perkin Elmer N931-6403 (Norwalk, CT, USA), equipped with a FID sensor, with a DB WAX J&W Scientifics column (60 m × 0.25 mm × 0.25 µm). Injector and detector temperatures were 250 °C and 300 °C, respectively. The furnace temperature was increased from 35 °C to 200 °C with a heating rate of 20 °C min⁻¹ for 10 min [14]. Nitrogen was used as the carrying gas at a flow rate of 1 mL min⁻¹ for all the samples. The extract of the injected samples was 2 µL. Compounds were identified and quantified by external calibration using reference compounds. Samples were injected in duplicate.

2.5. Evaluation of Reducing Sugar Content

The lactose content in the sweet whey samples was quantified via the 3,5-Dinitrosalicylic acid (DNS) method, as Hortsch et al. [26] reported. The DNS reactant was prepared by dissolving 10.6 g of 3,5-Dinitrosalicylic acid and 19.8 g of NaOH in 1416 mL of water and subsequently dissolved 306 g of potassium sodium tartrate. To analyze the samples in the Eppendorf™ tubes, they were centrifuged at 9677 RCF (240 mm rotor radius) for 3 min. Later, 100 µL of the sample was added in 25 mL sterile tubes with 1.5 mL of 3,5-Dinitrosalicylic acid for the hydrolysis polysaccharide. Finally, the tubes were maintained in water at 100 °C for 5 min. Lactose reduces one of the two nitro groups, leading to changes

in color from yellow to brown that were photometrically quantified at 540 nm (calibration with water with a shutter and lactose solution used as standard 4 g L^{-1}).

2.6. Determination of the Chemical Oxygen Demand (COD)

Chemical oxygen demand (COD) was determined through a standardized technique of closed reflux digestion [27]. The samples were centrifuged at 3360 RCF (240 mm rotor radius) [Eppendorf™] for 5 min; later, they were filtered using nylon membranes of $0.45 \mu\text{m}$ pore size. Sample reading was carried out with a spectrophotometer (Thermo Scientific BioMate 3S) at 600 nm. The supernatant was microfiltered with cellulose membranes with a pore diameter of $0.5 \mu\text{m}$. Following this was the addition of 1 mL of potassium dichromate with sulfuric acid and 1.5 mL of silver sulfate; this was placed on a digestion rack for two h at 150°C . Absorbance was determined by colorimetry at a wavelength of 620 nm. COD was measured every 24 h for four days in the batch systems of sweet whey. The chemical oxygen demand removal percent (CODR%) was calculated with the following equation:

$$\text{CODR}\% = \frac{(S_0 - S)}{(S_0)} \times 100 \quad (1)$$

where S_0 is the feed load of pollutant (COD in $\text{mg O}_2 \text{ L}^{-1}$), and S is the output pollutant load (COD in $\text{mg O}_2 \text{ L}^{-1}$).

2.7. Mathematical Models

The kinetic parameters ($\mu, Y_x(\text{biomass})/s(\text{lactose}), Y_p(\text{bioET})/s(\text{lactose}), r_p, -rs$) were determined using the Monod equation [28,29]. The experiments were carried out in a batch reactor, measuring the product (BioEtOH) and substrate concentrations with time. The analysis used the differential method, considering a constant volume exothermic reaction. For microbial growth, the equation used for Monod kinetics was

$$\mu = \frac{\mu_{\max} S}{K_s + S} \quad (2)$$

where μ is the specific growth rate (h^{-1}), S is the substrate degradation (g L^{-1}), μ_{\max} is the maximum specific growth rate (h^{-1}), and K_s is the saturation constant (g L^{-1}).

The following differential equation describes the substrate degradation rate:

$$\frac{dS}{dt} = -rs = -\frac{\mu}{Y_{x/s}} X \quad (3)$$

where t is time, rs is the substrate degradation rate, $Y_{x/s}$ is the biomass-substrate yield, and X is the biomass (g L^{-1}).

Moreover, the product formation is described with

$$\frac{dP}{dt} = rp = \frac{\mu Y_{p/s}}{Y_{x/s}} X \quad (4)$$

P is product formation, rp is the product formation rate, and $Y_{p/s}$ is the product-substrate yield.

The yield stoichiometric coefficient, g , was computed as follows:

$$g = \frac{1}{\pi} \left(\tan^{-1}(kY_{p/s}(Sf - S)) + \frac{\pi}{2} \right) \quad (5)$$

Here, k is the saturation constant (g L^{-1}), S is the initial substrate concentration (g L^{-1}), and Sf is the final substrate concentration (g L^{-1}).

Experimental data were independently fitted to the Gompertz equation, using non-linear regression according to Deseure et al. [30], with the Marquardt algorithm and the following equation:

$$y = a * \exp[-\exp(b - xc)] \quad (6)$$

The solution to Equation (6) as a function of A , μ_{max} , and λ is the following:

$$y = A * \exp\left\{-\exp\left\{\frac{\mu_{max}}{A}\right\}(\lambda - x) + 1\right\} \quad (7)$$

The values of the parameters a , xc , and b were obtained with OriginPro 8.1, using the least squares method, with a confidence interval of 95%. Using the values for a , xc , and b , for the model solution, the values for A , μ_m , and λ were calculated using Equation (7).

2.8. Statistical Analysis

Differences were considered significant when $p < 0.05$. An analysis of variance (factorial ANOVA) was performed with a post hoc Tukey test with a confidence level of 95% and a confidence interval of 95% using the software Statistica® 8.0.3. All the experimental analyses were performed in triplicate.

Figure 1 summarizes the methodology implemented to conduct the tests.

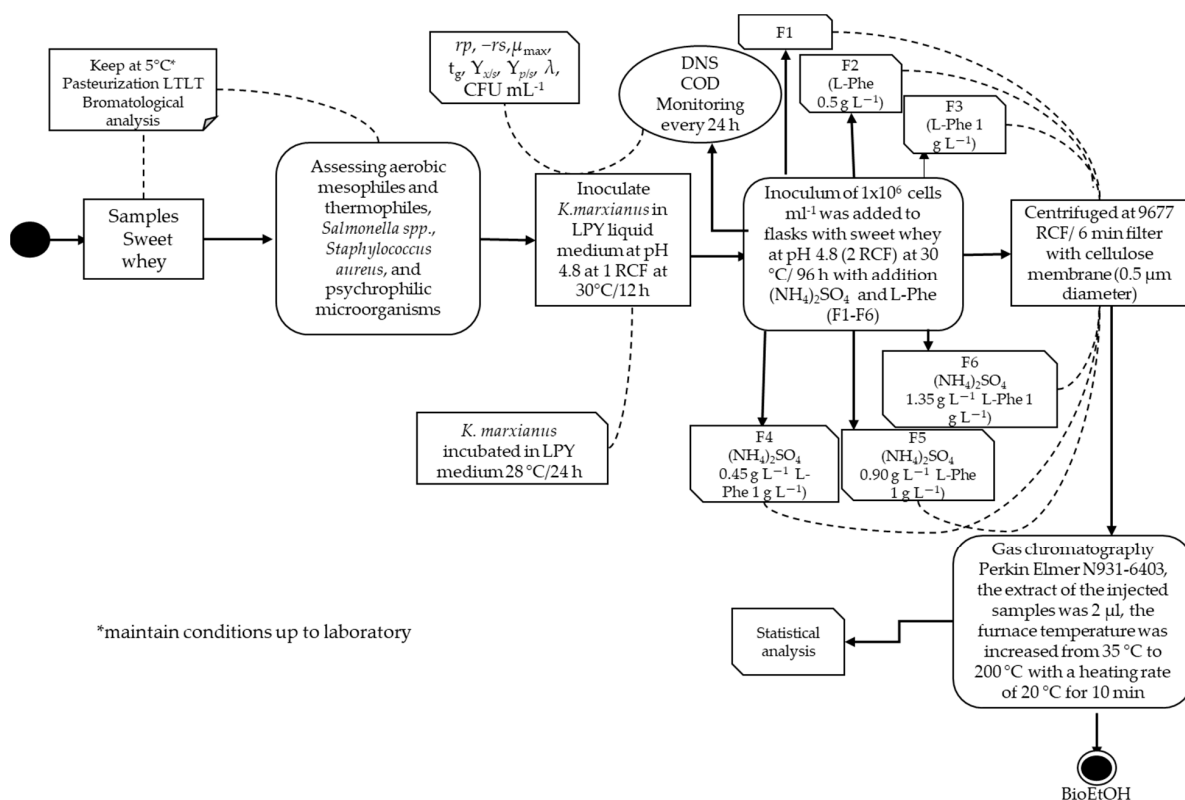


Figure 1. Graphic summary of the methodology.

3. Results and Discussion

The sweet whey microbiological analysis showed the absence of *Salmonella* spp., *Staphylococcus aureus*, aerobic thermophiles, and psychrophilic microorganisms. A total coliform concentration of 7 is the most probable number (MPN)/100 mL, and aerobic mesophiles of colony-forming units (CFUs) totaling 42 CFU mL⁻¹ were determined. These results are below the established limits in the NOM-243-SSA1-2010 guidelines (Table 2), which indicates an excellent microbiological quality of the sweet whey. The whey chemical

composition results without significant differences to it were reported by Gómez-Aldapa et al. [21].

Table 2. Maximum permissible limits for the microbiological analysis of sweet whey.

Parameter	Maximum Limits NOM-243-SSA1-2010	Sweet Whey Sample
<i>Salmonella</i> spp.	Absent in 25 mL	Absent
<i>Staphylococcus aureus</i>	≤ 10 UFC/mL	Absent
<i>aerobic thermophiles</i>	-	Absent
<i>psychrophilic microorganisms</i>	-	Absent
<i>total coliforms</i>	≤ 10 NMP	7 NMP/100 mL
<i>aerobic mesophiles</i>	≤ 250 UFC mL ⁻¹	42 UFC mL ⁻¹

BioEtOH was produced in the six formulations with concentration values ranging from 15.59 g L⁻¹ (F1) to 25.13 g L⁻¹ (F6) [Table 3]. Formulation F1 consisted of sweet whey in a system without precursors, with a biomass concentration of 1.13×10^9 CFU mL⁻¹ and a substrate consumption rate (*-rs*) of 0.62 g L⁻¹ h⁻¹ (R² = 0.97). This would indicate that BioEtOH produced through the mechanisms of imported lactose towards the cytosol needs easily assimilable nitrogen sources such as ammonium sulfate and/or other precursors that allow the specificity of predominant metabolic routes favoring the production of BioEtOH [10,12]. Adding L-Phe to the substrate increased the production of BioEtOH by almost 50% concerning raw sweet whey (F1), with a maximum concentration of 23.71 g L⁻¹ (F3). Meanwhile, the simultaneous incorporation of precursors (L-Phe and (NH₄)₂SO₄) managed to increase the BioEtOH concentration by 59.6% (F6). The L-Phe presence in the culture medium promoted the production of BioEtOH during the yeast's secondary metabolism, corresponding to the stationary growth phase [14]. Yeasts can transform amino acids into their respective fusel alcohols by the Ehrlich pathway. The amino acids are submitted to transamination, followed by decarboxylation and dehydrogenation to form the corresponding alcohol [31]. The lactose is then hydrolyzed to glucose and galactose, which are transformed into pyruvate by the glycolysis pathway. Then, pyruvate can be oxidized into BioEtOH and acetic acid by anaerobic fermentation or converted into organic acids by the metabolic pathway of the Krebs cycle [31]. It has been reported that only 25% of the nitrogen present in whey can be used by *K. marxianus* [22]. The (NH₄)₂SO₄ was used as a nitrogen source, showing that a suitable nitrogen source combined with aerobic conditions could increase the BioEtOH yield compared to the anaerobic fermentation or oxygen-limited conditions.

Production yield for BioEtOH over the substrate consumption did not show significant differences for F2 with the addition of L-Phe. However, with a higher L-Phe concentration in the medium (F3), significant differences were observed in the production with values of 23.71 g L⁻¹ (48 h). These concentration values are higher than those obtained by Zoppellari et al. [32], Hadiyanto et al. [33], and Boura et al. [34], with values of 17, 8, and 2 g L⁻¹, respectively, using lactose present in sweet whey. The conditions selected in this work considerably improve the bioethanol yield using lactose in whey as a fermentation substrate. During the experimental runs, the fit for the Monod and Gompertz models to determine the kinetic parameters that describe the BioEtOH production showed a good correlation with the experimental data, with R² values higher than 0.97 in all cases; see Figure 2.

Regarding the product formation rate (*rp*), the simultaneous addition of ammonium sulfate and L-Phe (F4 to F6) showed a significant decrease in *rp* compared to raw sweet whey (F1). In these formulations, the *rp* values varied from 0.24 to 0.34 g L⁻¹ h⁻¹, lower than when only L-Phe was added (F2 and F3); see Table 3.

The substrate consumption rate (*-rs*) increased with the presence of L-Phe because the assimilation of lactose in *K. marxianus* could have occurred through the lactose permease encoded by the LAC12 gene and hydrolyzed by intracellular β -galactosidase encoded by the LAC4 gene; catabolized by the Leloir pathway through an enzymatic reaction to

produce BioEtOH [10,35]. Concerning the substrate consumption rates ($-rs$) obtained with Monod's equation for F3 and F6 of 1.26 and $1.27 \text{ g L}^{-1} \text{ h}^{-1}$ (R^2 0.99), they are higher than those obtained by Hadiyanto et al., [33], Ozmihci et al., [36] and Lukondeh et al., [37] with values of 0.32 , 0.37 , and 0.16 g g^{-1} , respectively, in similar lactose concentrations (48 g L^{-1}) at 30°C . The results of $-rs$ for formulations F3 and F6 depend on the operation conditions, the nature of the substrate, the addition of precursors, and the used microorganism [30].

Table 3. Kinetic parameters in the sweet whey formulations at pH 4.8 30°C /180 rpm.

Parameters	F1	F2	F3	F4	F5	F6
rp ($\text{g L}^{-1} \text{ h}^{-1}$)	3.77 ± 0.68^a	0.72 ± 0.02^b	1.14 ± 0.13^c	0.32 ± 0.11^d	0.24 ± 0.09^e	0.34 ± 0.06^d
$-rs$ ($\text{g L}^{-1} \text{ h}^{-1}$)	0.62 ± 0.18^a	1.07 ± 0.24^b	1.26 ± 0.32^c	1.35 ± 0.16^a	1.34 ± 0.68^a	1.27 ± 0.34^f
μ_{max} (h^{-1})	0.72 ± 0.03^a	0.98 ± 0.01^b	0.27 ± 0.08^c	0.11 ± 0.07^b	0.19 ± 0.20^e	0.21 ± 0.07^f
tg (h)	0.41 ± 0.11^a	3.20 ± 0.42^a	1.11 ± 0.06^c	2.75 ± 0.25^d	1.52 ± 0.09^e	1.47 ± 0.41^f
$Y_{x/s}$ (g g^{-1})	0.12 ± 0.04^a	0.30 ± 0.08^b	0.22 ± 0.10^c	0.42 ± 0.03^d	0.28 ± 0.02^e	0.44 ± 0.06^a
$Y_{p/s}$ (g g^{-1})	0.65 ± 0.14^a	0.38 ± 0.24^b	0.52 ± 0.13^c	0.87 ± 0.53^d	0.26 ± 0.18^d	0.61 ± 0.18^f
λ (h)	0.32 ± 0.09^a	0.22 ± 0.04^a	0.22 ± 0.08^a	0.21 ± 0.17^a	0.20 ± 0.04^a	0.27 ± 0.14^f
Yeast count (CFU mL^{-1})	$1.13 \times 10^9 \pm 1.2 \times 10^6^a$	$2.19 \times 10^9 \pm 1.2 \times 10^6^b$	$1.12 \times 10^9 \pm 1.2 \times 10^6^c$	$2.4 \times 10^9 \pm 1.2 \times 10^6^d$	$2.1 \times 10^9 \pm 1.2 \times 10^6^e$	$2.7 \times 10^9 \pm 1.2 \times 10^6^c$
Initial lactose (g L^{-1})	54.10 ± 1.48^a	52.90 ± 3.87^b	47.37 ± 5.72^a	50.15 ± 0.73^c	54.08 ± 1.52^a	48.86 ± 0.93^d
Initial BioEtOH (g L^{-1}) *	ND	ND	ND	ND	ND	ND
Final BioEtOH (g L^{-1}) **	15.59 ± 1.92^a	18.96 ± 2.40^b	14.33 ± 2.58^c	22.69 ± 0.76^d	20.54 ± 2.06^e	25.13 ± 0.37^f
Residual lactose (g L^{-1})	12.70 ± 0.08^a	0.45 ± 0.072^a	0.30 ± 0.02^a	0.70 ± 0.03^a	0.81 ± 0.06^a	1.72 ± 0.058^a

tg: generation time; $Y_{x/s}$: biomass yield; rp : product formation rate; $-rs$: substrate consumption rate; $Y_{p/s}$: product yield; λ : lag phase; μ_{max} : maximum specific growth rate; ND not detected [* time 0 h, ** time 96 h]; a, b, c, d, e, and f: differences when $p < 0.05$; value \pm standard deviation.

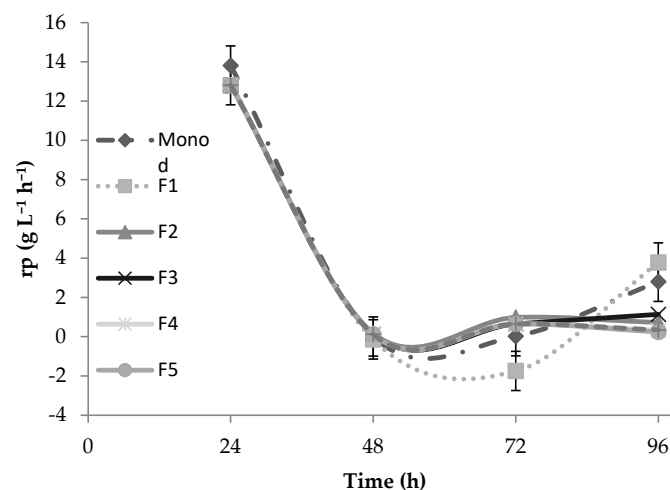


Figure 2. Adjustment of the Monod equation for the rate of product formation (rp) of sweet whey fermentation at pH 4.8, 30°C /180 rpm.

The results obtained for the maximum specific growth rate (μ_{max}) for F3 and F6, with values of 0.27 and 0.21 h^{-1} , respectively, where the maximum concentrations of BioEtOH were reached, indicate an efficiency on the conversion of the substrate towards BioEtOH, compared to the other formulations. For F6, a yield ($Y_{p/s}$) of 0.81 g g^{-1} was obtained. These results are higher than those reported by Hadiyanto et al. [33], Ozmihci et al. [36], and Lukondeh et al. [37] with BioEtOH yields of 0.21 , 0.54 , and 0.055 g g^{-1} . All the kinetic parameters are presented in Table 3. When L-Phe is added to the substrate, the rp decreases, the ethanol concentration increases, and the $-rs$ increases, allowing faster consumption and a more significant amount of substrate. This rise in the substrate consumption rate causes faster lactose consumption. Afterward, the yeast starts consuming other carbon sources, such as the BioEtOH, which could explain why, after 48 h, the ethanol concentration

decreases. On the other hand, adding L-Phe to the substrate could have accelerated Ehrlich's pathway, produced other metabolites, and reduced the concentration of BioEtOH in the medium.

The results for the CODR% values are presented in Table 4. Incorporating L-Phe favors the CODR%, which agrees with results obtained from residual lactose, increasing the substrate consumption rate. When ammonium sulfate is included (F5 and F6), the CODR% decreases, obtaining a concentration of residual lactose in these formulations of 0.81 and 1.72 g L⁻¹. Regarding the maximum CODR% of pollutant load, formulation F3 had a 97.59% (96 h), with an initial COD of 50,583 mgO₂ L⁻¹, a residual lactose content of 0.30 g L⁻¹, and a final COD of 1222 mgO₂ L⁻¹. The % COD removal obtained in F3 is higher than those reported in sweet whey samples by Venetsaneas et al. [38] and Comino et al. [39], with 95% and 82% values, respectively. Although, for F3 at 96 h, an important decrease in bioethanol concentration is observed.

Table 4. COD concentration in the sweet whey fermentation and final CODR% for the six configurations.

	F1	F2	F3	F4	F5	F6
Conditions	Stirring 180 rpm pH 4.8	Stirring 180 rpm pH 4.8 0.5 g L ⁻¹ (L-FE)	Stirring 180 rpm pH 4.8 1 g L ⁻¹ (L-FE)	Stirring 180 rpm pH 4.8 1 g L ⁻¹ (L-FE) 450 mg L ⁻¹ (NH ₄) ₂ SO ₄	Stirring 180 rpm pH 4.8 1 g L ⁻¹ (L-FE) 900 mg L ⁻¹ (NH ₄) ₂ SO ₄	Stirring 180 rpm pH 4.8 1 g L ⁻¹ (L-FE) 1350 mg L ⁻¹ (NH ₄) ₂ SO ₄
Time (H)	COD (mgO ₂ L ⁻¹)	COD (mgO ₂ L ⁻¹)	COD (mgO ₂ L ⁻¹)	COD (mgO ₂ L ⁻¹)	COD (mgO ₂ L ⁻¹)	COD (mgO ₂ L ⁻¹)
0	51,833 ± 3790.96 ^a	41,388 ± 994.32 ^b	50,583 ± 3033.21 ^a	37,027 ± 622.58 ^a	37,889 ± 2077.70 ^c	38,000 ± 2320.51 ^a
24	22,472 ± 273.57 ^a	15,388 ± 947.70 ^b	16,694 ± 569.04 ^c	18,027 ± 2793.42 ^d	19,666 ± 1083.92 ^e	21,055 ± 2025.81 ^f
48	19,055 ± 106.38 ^a	14,027 ± 662.60 ^b	11,888 ± 387.56 ^c	14,250 ± 1631.33 ^b	14,555 ± 1095.25 ^b	15,194 ± 1019.73 ^d
72	18,222 ± 115.64 ^a	11,916 ± 337.55 ^b	6500 ± 804.43 ^c	13,694 ± 1817.48 ^d	9500 ± 491.70 ^e	14,833 ± 476.83 ^f
96	16,472 ± 277.31 ^a	5083 ± 1184.063 ^b	1222 ± 45.36 ^c	10,750 ± 1784.34 ^d	8861 ± 620.92 ^e	12,277 ± 465.91 ^f
CODR% (96 H)	68.23 ± 39.67 ^a	87.72 ± 0.14 ^b	97.59 ± 0.86 ^c	70.97 ± 0.072 ^d	76.62 ± 0.16 ^e	67.70 ± 0.22 ^a

a, b, c, d, e, and f: differences when $p < 0.05$; value ± standard deviation.

Finally, Figure 3 shows the behavior between bioethanol production and CODR% in each formulation through fermentation time. The time is indicated in hours for formulations F3 and F6. The region with higher bioethanol production is enclosed in the blue oval. The analysis of this graph shows that the best value of bioethanol concentration was for the formulation F6 at 96 h (25.13 ± 0.37); however, the CODR% was not the best (67%). Configuration F3 at 48 h could represent the best conditions for ethanol production from sweet whey because the ethanol concentration (23.71 ± 1.26, F3, 48 h) is comparable with that obtained in formulation F6, and the CODR% for F3 (76%) is more significant compared to that in F6. Selecting the F3 conditions, the production and the CODR% could be satisfied.

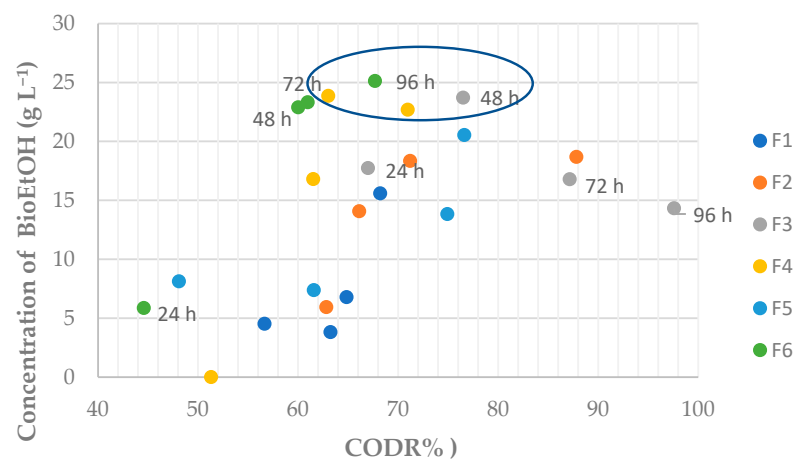


Figure 3. Bioethanol production and CODR% through fermentation. The blue oval shows the region with higher bioethanol production.

4. Conclusions

The study addresses the challenge of utilizing sweet whey, a byproduct of the dairy industry, by converting it into bioethanol as a sustainable and environmentally friendly alternative. Six formulations with varying concentrations of ammonium sulfate and L-phenylalanine were tested for bioethanol production using *Kluyveromyces marxianus*. Simultaneously, the chemical oxygen demand removal was measured. It was found that configuration F6, with a higher production of bioethanol ($25.13 \pm 0.37 \text{ g L}^{-1}$), does not present the best CODR% (67%). However, configuration F3, at 48 h of fermentation time, achieves a high bioethanol concentration ($23.71 \pm 1.26 \text{ g L}^{-1}$) with a high CODR% value (76%). The research successfully demonstrated the feasibility of producing bioethanol from sweet whey through fermentation with *Kluyveromyces marxianus*. The study identified conditions that maximize bioethanol production by optimizing nutrient concentrations while reducing dairy waste's pollutant load. This innovative approach not only offers a sustainable solution for utilizing dairy byproducts but also contributes to environmental remediation efforts in the dairy industry.

Author Contributions: Conceptualization, L.C.-B., C.C.-M. and A.L.-M.; data curation, L.C.-B., C.F.P.-M., C.C.-M. and A.L.-M.; formal analysis, C.F.P.-M., C.C.-M. and A.L.-M.; funding acquisition, C.C.-M. and E.M.-H.; investigation, L.C.-B., C.F.P.-M., C.C.-M. and E.M.-H.; methodology, L.C.-B. and C.F.P.-M.; resources, L.C.-B. and C.C.-M.; software, L.C.-B. and C.F.P.-M.; supervision, L.C.-B. and A.L.-M.; validation, L.C.-B., C.F.P.-M., C.C.-M. and A.L.-M.; visualization, E.M.-H. and A.L.-M.; writing—original draft, L.C.-B., C.C.-M., E.M.-H. and A.L.-M.; writing—review and editing, L.C.-B., C.C.-M., E.M.-H. and A.L.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within this article.

Acknowledgments: L.C.-B. thanks the Instituto Nacional de México, campus Durango for the facilities provided in the analysis of the samples. E.M.-H. acknowledges funding from ANID Fondecyt Postdoctoral project 3200839. C.C.-M. acknowledges funding from PRODECTI-2022-01/16 to pay the APC for this work.

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

References

1. Caldeira, C.; Vlysidis, A.; Fiore, G.; De Laurentiis, V.; Vignali, G.; Sala, S. Sustainability of food waste biorefinery: A review on valorisation pathways, techno-economic constraints, and environmental assessment. *Bioresour. Technol.* **2020**, *312*, 123575. [[CrossRef](#)] [[PubMed](#)]
2. PNUMA. *Integración del Consumo y la Producción Sustentable y la Eficiencia en el Uso de los Recursos en la Planificación del Desarrollo*; PNUMA: Nairobi, Kenya, 2009.
3. Asunis, F.; De Gioannis, G.; Dessì, P.; Isipato, M.; Lens, P.N.L.; Muntoni, A.; Poletti, A.; Pomi, R.; Rossi, A.; Spiga, D. The dairy biorefinery: Integrating treatment processes for cheese whey valorisation. *J. Environ. Manag.* **2020**, *276*, 111240. [[CrossRef](#)] [[PubMed](#)]
4. Conde Mejía, C.; Conde Báez, L. Chapter 11—Biorefinery, an integrated concept: Analysis of bioethanol and aromas production from whey. In *Biofuels and Biorefining*; Gómez Castro, F.I., Gutiérrez-Antonio, C., Eds.; Elsevier: Amsterdam, The Netherlands, 2022; pp. 447–471.
5. Warncke, M.; Kieferle, I.; Nguyen, T.M.; Kulozik, U. Impact of heat treatment, casein/whey protein ratio and protein concentration on rheological properties of milk protein concentrates used for cheese production. *J. Food Eng.* **2022**, *312*, 110745. [[CrossRef](#)]
6. Ginni, G.; Adish Kumar, S.; Mohamed Usman, T.M.; Pakonyi, P.; Banu, J.R. Chapter 13—Integrated biorefineries of food waste. In *Food Waste to Valuable Resources*; Banu, J.R., Kumar, G., Gunasekaran, M., Kavitha, S., Eds.; Academic Press: Cambridge, MA, USA, 2020; pp. 275–298.
7. Das, B.; Sarkar, S.; Maiti, S.; Bhattacharjee, S. Studies on production of ethanol from cheese whey using *Kluyveromyces marxianus*. *Mater. Today Proc.* **2016**, *3*, 3253–3257. [[CrossRef](#)]

8. Zafar, S.; Owais, M. Ethanol production from crude whey by *Kluyveromyces marxianus*. *Biochem. Eng. J.* **2006**, *27*, 295–298. [[CrossRef](#)]
9. Asunis, F.; De Gioannis, G.; Isipato, M.; Muntoni, A.; Poletti, A.; Pomi, R.; Rossi, A.; Spiga, D. Control of fermentation duration and pH to orient biochemicals and biofuels production from cheese whey. *Bioresour. Technol.* **2019**, *289*, 121722. [[CrossRef](#)] [[PubMed](#)]
10. Zou, J.; Chang, X. Past, Present, and Future Perspectives on Whey as a Promising Feedstock for Bioethanol Production by Yeast. *J. Fungi* **2022**, *8*, 395. [[CrossRef](#)]
11. Conde-Mejía, C.; Jiménez-Gutiérrez, A.; El-Halwagi, M.M. Assessment of Combinations between Pretreatment and Conversion Configurations for Bioethanol Production. *ACS Sustain. Chem. Eng.* **2013**, *1*, 956–965. [[CrossRef](#)]
12. Rao, R.; Basak, N. Optimization and modelling of dark fermentative hydrogen production from cheese whey by *Enterobacter aerogenes* 2822. *Int. J. Hydrogen Energy* **2021**, *46*, 1777–1800. [[CrossRef](#)]
13. Dessi, P.; Asunis, F.; Ravishankar, H.; Cocco, F.G.; De Gioannis, G.; Muntoni, A.; Lens, P.N.L. Fermentative hydrogen production from cheese whey with in-line, concentration gradient-driven butyric acid extraction. *Int. J. Hydrogen Energy* **2020**, *45*, 24453–24466. [[CrossRef](#)]
14. Hernández-Cruz, M.A.; Cadena-Ramírez, A.; Castro-Rosas, J.; Páez-Lerma, J.B.; Ramírez-Vargas, M.R.; Rangel-Vargas, E.; Romo-Gómez, C.; Lara-Gómez, A.B.; Conde-Báez, L.; Gómez-Aldapa, C.A. Comparative Analysis of Three Types of Whey as Substrate for Fermentation by *Kluyveromyces marxianus* and its Influence on Isoamyl Acetate Synthesis. *Waste Biomass Valorization* **2024**. [[CrossRef](#)]
15. Kong, S.; Pan, H.; Liu, X.; Li, X.; Guo, D. De novo biosynthesis of 2-phenylethanol in engineered *Pichia pastoris*. *Enzym. Microb. Technol.* **2020**, *133*, 109459. [[CrossRef](#)] [[PubMed](#)]
16. Kamthan, M.; Mukhopadhyay, G.; Chakraborty, N.; Chakraborty, S.; Datta, A. Quantitative proteomics and metabolomics approaches to demonstrate N-acetyl-d-glucosamine inducible amino acid deprivation response as morphological switch in *Candida albicans*. *Fungal Genet. Biol.* **2012**, *49*, 369–378. [[CrossRef](#)] [[PubMed](#)]
17. NORMA Oficial Mexicana NOM-092-SSA1-1994; Bienes y Servicios. Método Para la Cuenta de Bacterias Aerobias en Placa. Diario Oficial de la Federación: Mexico City, Mexico, 1994.
18. NORMA Oficial Mexicana NOM-114-SSA1-1994; Bienes y Servicios, Método Para la Determinación de Salmonella en Alimentos. Diario Oficial de la Federación: Mexico City, Mexico, 1994.
19. NORMA Oficial Mexicana NOM-115-SSA1-1994; Bienes y Servicios. Método Para la Determinación de *Staphylococcus aureus* en Alimentos. Diario Oficial de la Federación: Mexico City, Mexico, 1994.
20. NORMA Oficial Mexicana NOM-243-SSA1-2010; Productos y Servicios. Leche, Fórmula Láctea, Producto Lácteo Combinado y Derivados Lácteos. Disposiciones y Especificaciones Sanitarias. Métodos de Prueba. Diario Oficial de la Federación: Mexico City, Mexico, 2010.
21. Gómez-Aldapa, C.A.; Castro-Rosas, J.; López-Molina, A.; Conde-Mejía, C.; Pineda-Muñoz, C.F.; Jiménez-González, A.; Medina-Moreno, S.A.; Falcón-León, M.P.; Conde-Báez, L. Best Conditions for the Production of Natural Isopentyl Acetate (Banana Aroma) from Cheese Industry Waste: An Experimental Precursor Approach. *Processes* **2021**, *9*, 1880. [[CrossRef](#)]
22. Etschmann, M.; Bluemke, W.; Sell, D.; Schrader, J. Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 1–8. [[CrossRef](#)] [[PubMed](#)]
23. Wittmann, C.; Hans, M.; Bluemke, W. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. *Yeast* **2002**, *19*, 1351–1363. [[CrossRef](#)]
24. Conde-Báez, L.; López-Molina, A.; Gómez-Aldapa, C.; Pineda-Muñoz, C.; Conde-Mejía, C. Economic projection of 2-phenylethanol production from whey. *Food Bioprod. Process.* **2019**, *115*, 10–16. [[CrossRef](#)]
25. Páez-Lerma, J.B.; Arias-García, A.; Rutiaquiñones, O.M.; Barrio, E.; Soto-Cruz, N.O. Yeasts Isolated from the Alcoholic Fermentation of Agave duranguensis During Mezcal Production. *Food Biotechnol.* **2013**, *27*, 342–356. [[CrossRef](#)]
26. Hortsch, R.; Löser, C.; Bley, T. A Two-stage CSTR Cascade for Studying the Effect of Inhibitory and Toxic Substances in Bioprocesses. *Eng. Life Sci.* **2008**, *8*, 650–657. [[CrossRef](#)]
27. ALPHA. *Standard Methods for the Examination Water and Wastewater*, 19th ed.; American Public Health Association: Washington, DC, USA, 2005.
28. Muloiwa, M.; Nyende-Byakika, S.; Dinka, M. Comparison of unstructured kinetic bacterial growth models. *S. Afr. J. Chem. Eng.* **2020**, *33*, 141–150. [[CrossRef](#)]
29. Seekao, N.; Sangsri, S.; Rakmak, N.; Dechapanya, W.; Siripatana, C. Co-digestion of palm oil mill effluent with chicken manure and crude glycerol: Biochemical methane potential by monod kinetics. *Heliyon* **2021**, *7*, e06204. [[CrossRef](#)] [[PubMed](#)]
30. Deseure, J.; Obeid, J.; Willison, J.C.; Magnin, J.-P. Reliable determination of the growth and hydrogen production parameters of the photosynthetic bacterium *Rhodobacter capsulatus* in fed batch culture using a combination of the Gompertz function and the Luedeking-Piret model. *Heliyon* **2021**, *7*, e07394. [[CrossRef](#)] [[PubMed](#)]
31. Valdez Castillo, M.; Pachapur, V.L.; Brar, S.K.; Arriaga, S.; Blais, J.-F.; Avalos Ramirez, A. Effect of the concentration of L-Phenylalanine and lactose on 2-Phenylethanol production by whey fermentation using the yeasts *Kluyveromyces marxianus* and *Debaryomyces hansenii* under co-culture mode. *Bioresour. Technol. Rep.* **2022**, *18*, 100994. [[CrossRef](#)]
32. Zoppellari, F.; Bardi, L. Production of bioethanol from effluents of the dairy industry by *Kluyveromyces marxianus*. *New Biotechnol.* **2013**, *30*, 607–613. [[CrossRef](#)] [[PubMed](#)]

33. Hadiyanto; Ariyanti, D.; Aini, A.P.; Pinundi, D.S. Optimization of Ethanol Production from Whey Through Fed-batch Fermentation Using *Kluyveromyces Marxianus*. *Energy Procedia* **2014**, *47*, 108–112. [[CrossRef](#)]
34. Boura, K.; Kandyli, P.; Bekatorou, A.; Kolliopoulos, D.; Vasileiou, D.; Panas, P.; Kanellaki, M.; Koutinas, A.A. New generation biofuel from whey: Successive acidogenesis and alcoholic fermentation using immobilized cultures on γ -alumina. *Energy Convers. Manag.* **2017**, *135*, 256–260. [[CrossRef](#)]
35. Smithers, G.W. Whey-ing up the options—Yesterday, today and tomorrow. *Int. Dairy J.* **2015**, *48*, 2–14. [[CrossRef](#)]
36. Ozmihi, S.; Kargi, F. Ethanol production from cheese whey powder solution in a packed column bioreactor at different hydraulic residence times. *Biochem. Eng. J.* **2008**, *42*, 180–185. [[CrossRef](#)]
37. Lukondeh, T.; Ashbolt, N.J.; Rogers, P.L. Fed-batch fermentation for production of *Kluyveromyces marxianus* FII 510700 cultivated on a lactose-based medium. *J. Ind. Microbiol. Biotechnol.* **2005**, *32*, 284–288. [[CrossRef](#)]
38. Venetsaneas, N.; Antonopoulou, G.; Stamatelatos, K.; Kornaros, M.; Lyberatos, G. Using cheese whey for hydrogen and methane generation in a two-stage continuous process with alternative pH controlling approaches. *Bioresour. Technol.* **2009**, *100*, 3713–3717. [[CrossRef](#)]
39. Comino, E.; Riggio, V.A.; Rosso, M. Biogas production by anaerobic co-digestion of cattle slurry and cheese whey. *Bioresour. Technol.* **2012**, *114*, 46–53. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.