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Microbial Conversion of Cheese Whey to Polyhydroxybutyrate (PHB) via Statistically Optimized Cultures

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Abstract: The intended circular economy for plastics envisages that they will be partially replaced by bio-based polymers in the future. In this work, the natural polyester polyhydroxybutyrate (PHB) was produced by *Azohydromonas lata* using cheese whey (CW) as a low-cost substrate. Initially, CW was evaluated as the sole carbon source for PHB production; it was found to be efficient and comparable to PHB production with pure sugars, such as saccharose or glucose, even when mild (with dilute acid) hydrolysis of cheese whey was performed instead of enzymatic hydrolysis. An additional series of experiments was statistically designed using the Taguchi method, and a dual optimization approach was applied to maximize the intracellular biopolymer content (%PHB, selected as a quantitative key performance indicator, KPI) and the weight average molecular weight of PHB (M_w, set as a qualitative KPI). Two different sets of conditions for the values of the selected bioprocess parameters were identified: (1) a carbon-to-nitrogen ratio (C/N) of 10 *w*/*w*, a carbon-to-phosphorous ratio (C/P) of 1.9 *w*/*w*, a dissolved oxygen concentration (DO) of 20%, and a residence time in the stationary phase (RT) of 1 h, resulting in the maximum %PHB (61.66% *w*/*w*), and (2) a C/N of 13.3 *w*/*w*, a C/P of 5 *w*/*w*, a DO of 20%, and a RT of 1 h, leading to the maximum M_w (900 kDa). A final sensitivity analysis confirmed that DO was the most significant parameter for %PHB, whereas C/N was the most important parameter for M_w.

Keywords: polyhydroxybutyrate (PHB); polyhydroxyalkanoates (PHAs); *Azohydromonas lata*; cheese whey; biopolymer; molecular weight; Taguchi; fermentation; design of experiments (DoE)

Citation: Penloglou, G.; Pavlou, A.; Kiparissides, C. Microbial Conversion of Cheese Whey to Polyhydroxybutyrate (PHB) via Statistically Optimized Cultures. *Fermentation* **2023**, *9*, 624. https://doi.org/10.3390/fermentation9070624

Academic Editor: Nikos G. Chorianopoulos

Received: 29 May 2023
Revised: 26 June 2023
Accepted: 29 June 2023
Published: 30 June 2023

1. Introduction

Today, environmental pollution from plastics remains widespread. Most plastic products are traditionally made from fossil raw materials and usually contain harmful additives, such as plasticizers, stabilizers, coloring agents, etc., so they also pose a threat to human health [1]. Considering the long-appreciated advantages of synthetic/conventional plastics due to their excellent end-use characteristics, i.e., performance, flexibility, and durability—to name a few—in numerous applications, a rapid shift in awareness is currently underway in the plastics industry among various stakeholders. Their intention is to support circular economy approaches politically, technologically and financially while mitigating climate change [2]. It is time to establish a future agenda for plastics that includes essential actions for the circular economy and decarbonization, such as the provision for massive production of bio-based plastics [3]. Undoubtedly, the European (bio)plastics industry has already started to adopt policies and practices to address the above challenges, but this is not sufficient to fully achieve the goals of the Circular Plastics Alliance, the European Green Deal, and other similar initiatives [4,5].

Within this framework, it is widely recognized that both upstream (e.g., redesigning plastics, reducing consumption, and substituting with bio-based plastics) and

downstream (e.g., post-consumer recycling, composting, and degradation) practices related to plastics are complementary; to achieve maximum efficiency, they should be deployed together, as recently proposed by 'ReShaping Plastics' [6]. In addition, the replacement of synthetic plastics with bio-based, compostable, and/or biodegradable alternatives is already a well-known and widely accepted solution to the environmental problems associated with plastic wastes [7]. Although the production of bio-based plastics was first validated and demonstrated in the 1950s, far less than 1% of the total 360 Mtons of plastics produced in 2020 was bio-based. This limitation is because of their high production cost, lack of large-scale production facilities and supply chains, challenges in meeting the performance requirements of various applications, and the lack of relevant standards and regulations [8]. Overall, the production of bio-based plastics is expected to reach about 2.43 Mtons in 2024 [9] and 6.3 million Mtons in 2027 [10].

Among other bio-based polymers, polyhydroxyalkanoates (PHAs) and their most common and best-studied member—polyhydroxybutyrate (PHB)—are microbially synthesized polyesters formed intracellularly by numerous bacteria, fungi, yeasts, (micro)algae, plants, cyanobacteria, etc. [11]. Their production mechanism involves the formation and growth of intracellular biopolymer granules, which serve as carbon and energy stores, in response to specific stress conditions, such as nutrient limitations (e.g., nitrogen, phosphorous, and/or oxygen), pH control in basic or acidic conditions, temperature, and the type and availability of carbon sources [12]. In general, PHAs exhibit excellent characteristics, e.g., biodegradability and biocompatibility, as well as good physical, thermal, molecular, mechanical, and end-use properties, which make them a potential future replacement for synthetic polymers [13]. Most importantly, their biodegradability is fully aligned with the modern demand for a circular economy, as they provide a simple and sustainable end-of-life solution through biodegradation and a strong paradigm for reducing the carbon footprint of plastics use and disposal [14]. For example, PHAs degrade easily and primarily naturally in all types of landfills and disposal sites through microbial activity, enzymatic degradation, or composting, thereby reducing plastic wastes [11,12]. They are also produced from renewable resources and reduce dependence on non-renewable fossil-based plastics [13,14].

To date, PHAs have been produced by hundreds of different microbes and subsequently used in various applications, ranging from cosmetics and food packaging to specialized medical applications and agricultural solutions [15]. Despite the unanimous acceptance and constant growth of PHAs, their technology readiness level (TRL) is yet to be proven and competitive at the manufacturing scale [16]. Note that TRL is a common method used in research to evaluate the maturity of a technology or innovation; the TRL scale ranges from 1 to 9 and represents different stages of technological development [17]. The low TRL level of PHAs, as a whole class of bioplastics, is clearly due to their limited market, mainly because of their high production cost and price compared to the respective values of their synthetic counterparts [18]. The two major factors that contribute significantly to the high production cost of PHAs are the cost of purchasing and/or pretreating the carbon source as a substrate and the cost of the downstream processing (DSP) and separation of the biopolymer [19].

In the last decade, the microbial production of PHAs and PHB has shifted from pure substrates to renewable feedstocks and industrial/municipal wastes in search of cost-effective and environmentally friendly resources [20]. Therefore, the use of food industry by-products and/or agro-industrial wastes as carbon sources for the production of PHAs/PHB has been proposed as a promising solution with a dual advantage: it can minimize production costs while eliminating the economic burden of processing unwanted streams and residues [21]. In this direction, various wastes, such as municipal wastewater [22], food waste [23], molasses [24], olive mill waste [25], coffee waste [26], starch [27], glycerol from biodiesel production [28], used cooking oils [29], and cheese whey [30], have been used for the production of PHAs. Within the scope of this research focus, all these successful examples have already been discussed [31].

Specifically, in the case of cheese whey, the best-known and largest by-product of the dairy industry in terms of volume, many researchers recognized early that it is an efficient lactose resource and could potentially be used as a substrate for the production of PHAs [32]. Since then, several successful experiments with different microorganisms have been undertaken and described elsewhere [33]; among them, *Azohydromonas lata* has been used as a PHB producer [34]. In addition to the added value of biopolymer production from a low-cost substrate, PHAs production also addresses the shortcomings associated with the usual treatment of cheese whey: dairies deproteinize cheese whey by ultrafiltration, mainly with the aim of using the proteins as additives in food/feed [35]. However, the filtrate is still considered a 'problematic' waste because it contains lactose, which can be easily hydrolyzed and degraded, thus representing a serious contaminant, especially for aquatic environments. Thus, among the other components of cheese whey, lactose is a major environmental hazard for which a biotechnological use must be found [36].

The production of PHAs is somewhat competitive with other traditional (e.g., processing to produce whey powder, functional foods, beverages, and biogas) or modern (e.g., lactose recovery and utilization for lactic acid/PLA and bioethanol production, or whey protein utilization for edible films, coatings, and hydrogels) processes for the valorization of cheese whey [37,38]. All these approaches offer various environmental and techno-economic benefits, including wastes reduction and the minimization of the environmental impact associated with cheese whey disposal; resource efficiency due to the recovery of valuable nutrients, such as proteins, lactose, and minerals; energy savings and a reduction in dependence on fossil fuels; a reduction in greenhouse gas emissions (especially the methane released during the uncontrolled decomposition of cheese whey); and the prevention of water pollution [37,39]. Therefore, emerging approaches to convert cheese whey into valuable bio-based products, such as bioplastics and, more specifically, PHAs, are now being intensively promoted, especially considering their positive environmental balance in terms of carbon footprint and end-of-life options [40].

However, it has proven extremely difficult to find efficient PHAs producers from cheese whey because of the suboptimal composition of the growth medium, which significantly inhibits microbial growth and biopolymer accumulation, and the inability to directly utilize lactose as the sole carbon source [41]. The two solutions proposed in this case are the hydrolysis of cheese whey lactose into glucose and galactose before fermentation and the genetic modification of PHA-producing bacteria so that they are able to express lactose hydrolysis genes derived from other microorganisms [42]. The hydrolysis of cheese whey is performed primarily by enzymes and secondarily by chemical catalysts. The former is an efficient and selective, albeit costly, process, while the latter requires more stringent conditions in terms of acid concentrations and temperatures [39,43]. When considering alternative substrates such as cheese whey, it should be noted that the cost of the carbon source also corresponds to the pretreatment of the feedstock, which is carried out to increase the bioavailability and assimilation potential of the nutrients [44]. In addition, inefficient microbial growth and biopolymer accumulation, expressed as the biomass concentration and PHA content, respectively, and inadequate biopolymer quality, expressed by small values of the weight average molecular weight (M_w) of PHAs, are important performance criteria that affect the economics of downstream processing [45].

In this context, the main objective of the present study is to utilize cheese whey as the sole carbon source for PHB production. For this purpose, a wild-type bacterium, i.e., *A. lata*, is used after the treatment and/or hydrolysis of cheese whey. The aim is to facilitate the accumulation and recovery of PHB by improving the yield of the substrate to PHB, expressed as the intracellular biopolymer content, and maximizing the quality of PHB, expressed as the molecular weight of PHB. This two-way optimization strategy is carried out using well-designed experiments that follow the industrially relevant Taguchi design of experiments (DoE) approach. An additional goal is to provide ready-to-ferment cheese whey by replacing costly and time-consuming enzymatic hydrolysis with mild, dilute acid hydrolysis performed simultaneously with the autoclave/sterilization step.

Moreover, the common, though inefficient, batch cultivation of microbial cultures in Erlenmeyer flasks [46] is overcome a priori by using a feeding strategy based on previous studies on optimal PHB production using pure substrates [47,48]; thus, a simple fed-batch process is suitable for all cultures as the means to increase PHB production rates. Finally, considering the use of wild-type *A. lata* species for the fermentation of cheese whey, to the best of our knowledge, there are no previous studies that have equally focused on the amount of PHB produced and its molecular properties.

2. Materials and Methods

2.1. Fermentative PHB Production from Cheese Whey

The wild-type bacterial species selected for the fermentative production of PHB from cheese whey (CW) is *Azohydromonas lata* (DSM 1123, purchased directly from DSMZ in freeze-dried form). The strain was maintained in 2 mL of 1/1 *v*/*v* glycerol/nutrient broth (NB) stocks at –30 °C. A two-step preculture in sterile NB medium containing 5 g/L peptone and 3 g/L meat extract was used to prepare the seed culture (i.e., preculture) for each experiment.

All fermentation experiments were performed in 2 L Erlenmeyer flasks. The flasks were inoculated with an appropriate volume (approximately 33–55 mL) of a mature preculture (at optical density, OD = 1–1.5) to regulate the initial OD of the main culture to approximately 0.2 ± 0.05. Prior to inoculation, flasks were aseptically loaded into a laminar flow clean biological safety cabinet (Herasafe, ThermoFischer Scientific Inc., Waltham, MA, USA) with 250 mL of the cultivation medium (AL), which was adopted by previous studies [47,48] and modified accordingly: total sugars (as carbon source) 20 g/L; carbon-to-nitrogen mass ratio (C/N, expressed in *g*/*g* or *w*/*w* and defined as the total initial concentration of sugars divided by the initial concentration of $(NH_4)_2SO_4$) 6.6, 8, 10, or 13—see Section 2.2; carbon-to-phosphorous mass ratio (C/P, expressed in *g*/*g* or *w*/*w* and defined as the total initial concentration of sugars divided by the total initial concentration of $Na_2HPO_4 \cdot 12H_2O$ and K_2HPO_4, in the respective ratio 6/1 *w*/*w*) 0.8, 1.9, 3 or 5—see Section 2.2; $MgSO_4 \cdot 7H_2O$ 0.2 g/L; $CaCl_2 \cdot 2H_2O$ 0.01 g/L; citric acid 0.1 g/L; and trace element solution (TES) (identical to [47,48]) 1 mL/L. Note that a total of eight different sugars/carbon sources were used as substrates, i.e., saccharose (S), glucose (G), lactose (L), xylose (X), fructose (F), galactose (GL), a 1/1 *w*/*w* mixture of glucose and galactose (GG), and cheese whey (CW) (see also Table 1).

All sugars, nutrients, and chemicals were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany). Cheese whey was kindly provided by the Chelmos Ltd. dairy (Achaia, Greece) and had a (roughly) known composition: lactose 46.5 g/L, other sugars (mainly glucose) 3.4 g/L, proteins 6.4 g/L, lipids 4.4 g/L, inorganic salts 5.5 g/L, and lactic acid 1.4 g/L. CW from a single batch was used as the fermentation substrate after a simple pretreatment. First, pH was adjusted to 4.5 ± 0.25 (using a 3% *v*/*v* HCl buffer), and the solution was then centrifuged (at 10,000× *g* for 15 min in a Heraeus GmbH centrifuge, Hanau, Germany) to remove insoluble solids. The supernatant was subsequently neutralized to pH = 7 ± 0.25 (with a NaOH 3 M solution), sterilized (at 121 °C for 20 min in a Raypa AES-75 sterilizer, Barcelona, Spain), and then centrifuged (10,000× *g*, 15 min). Moreover, two additional experiments were performed with hydrolyzed cheese whey based on enzymatic and chemical treatments (Table 1). In the former case, enzymatically hydrolyzed cheese whey (CWE) was provided by the Department of Biology, National and Kapodistrian University of Athens (NKUA, Athens, Greece). The conditions used were as follows: 100 μL of commercial β-galactosidase from *Aspergillus oryzae* (10.9 units/mg solid; Sigma-Aldrich Co., St. Louis, MO, USA) were used per mL of cheese whey, and the suspension was incubated at 30 °C for 18 h, with stirring at 50 rpm. In the latter case, HCl was added at a final concentration of 1% *v*/*v* in order to facilitate the dilute acid hydrolysis of cheese whey (CWA) during sterilization in the autoclave (121 °C, 20 min). It should be noted that the experiment that was conducted with the mixture of glucose and

galactose (also referred to as GG in Table 1) aimed to mimic the type of carbon source that can be obtained during the hydrolysis of cheese whey lactose.

All flasks were incubated in a GFL 3033 (GFL GmbH, Burgwedel, Germany) orbital shaking incubator at 30 ± 0.1 °C and 210 rpm. In selected cases, the dissolved oxygen concentration (DO) was controlled at 5%, 10%, 20%, or 30% (see Section 2.2) by manually adjusting the agitation frequency in the range of 200-250 rpm. Before inoculation, culture pH was adjusted to 7 ± 0.05 by using appropriate base (NaOH 3 M) and acid (HCl 3% *v*/*v*) solutions. In each experiment, in order to gradually impose nitrogen-limiting conditions with excess carbon and thus stimulate PHB accumulation, a fed-batch procedure was applied, in which 250 mL of a nitrogen-free medium (AL^N) was used in three steps: 90, 80, and 80 mL at OD values of 8 ± 0.5, 12 ± 0.5, and 16 ± 0.5, respectively; AL^N was identical to AL but free of nitrogen. Each culture was stopped and harvested according to the selected stationary phase residence time value (RT), namely, 0, 1, 3, or 9 h (see Section 2.2). All experiments were performed in triplicate, and the values reported in this study are the respective averages.

2.2. Design of Experiments (DoE)

To systematically investigate the effects of the four selected bioprocess parameters, namely, C/N, C/P, DO, and RT, on the intracellular PHB content (%PHB) and the weight average molecular weight of PHB (M_w), a series of parallel experiments was performed for the fermentation of *A. lata*. The L_{16} Taguchi method of DoE and statistical design was used to determine the experimental conditions related to the four selected levels of the four parameters. In particular, based on previous studies [47,48] and preliminary optimization experiments, the following values were selected for the bioprocess parameters: (i) 6.6, 8, 10, and 13 *w*/*w* for C/N, (ii) 0.8, 1.9, 3, and 5 *w*/*w* for C/P, (iii) 5%, 10%, 20%, and 30% for DO, and (iv) 0, 1, 3, and 9 h for RT. It should be noted that the Taguchi method was selected due to its ease of use and the statistically significant results that it provides [49]. The implementation of DoE and the entire analysis of experimental results were carried out in Minitab 17 statistical software (Minitab Inc., State College, PA, USA). More specifically, two individual experimental designs were performed with the same set of selected parameters. The two selected output parameters/responses were %PHB (% *w*/*w*), a clear quantitative KPI, and M_w (kDa), a more qualitative KPI that can be correlated with the end-use properties of PHB [50]. Accordingly, a minimum number of 16 experiments (out of 256 potential runs) was determined (Table 2). It should be noted that after the initial comparison of different carbon sources (see Section 3.1), the two DoEs were performed using dilute-acid-hydrolyzed cheese whey.

2.3. Analytical Methods

Microbial growth was determined by measuring the optical density (OD) of 2 mL samples at 600 nm in a UV/Vis spectrophotometer (Hitachi U-1800, Tokyo, Japan). To calculate the biomass concentration (as dry cell weight, DCW), a 10 mL sample was successively centrifuged (10,000× *g*, 10 min), lyophilized overnight (in a ScanVac Coolsafe basic size 4 freeze drier, LaboGene Aps, Lillerød, Denmark), and weighed (in an XP105 Delta-Range scale, Mettler Toledo Ind., Columbus, OH, USA). The same dried sample was then used to determine the %PHB content in the microbial mass by FTIR analysis in a Frontier spectrometer (PerkinElmer Inc., Waltham, MA, USA) [47,48]. The biopolymer concentration (C_{PHB}) was calculated by simply multiplying the DCW by %PHB values. The supernatant obtained from the 10 mL culture sample was used to measure the total sugar concentration via the DNS colorimetric method and ammonium nitrogen using a SevenCompact pH/Ion meter (Mettler Toledo Ind., Columbus, OH, USA) equipped with a DX218 ion-selective electrode. Moreover, the Cellphase system (Fluorometrix Corp., Stow, MA, USA) was used for the online monitoring of DO in the flasks. In addition, upon culture harvesting, a 50 mL sample was processed for PHB recovery and purification using a previously established protocol that combines the disruption of cells with ultrasounds (Vibra

Cell VC-505, Sonics and Materials Inc., Newtown, CT, USA) and the extraction of PHB with chloroform [47,48]. Finally, the weight average molecular weight (M_w) of the extracted PHB was measured via gel permeation chromatography (PolymerLabs GPC, Varian Inc. Palo Alto, CA, USA).

3. Results and Discussion

3.1. Evaluation of Cheese Whey Conversion to PHB

Initially, a series of ten (10) experiments was performed to evaluate the ability of *A. lata* cells to metabolize untreated and/or hydrolyzed cheese whey as a carbon source, compared to other pure sugars. All cultivation conditions, including the feeding strategy, were identical in the ten flasks, except for the type of substrate. In particular, the C/N and C/P mass ratios were set at 10 *w*/*w* and 1.9 *w*/*w*, respectively, while the culture volumes were harvested when RT was equal to 1 h. The incubation frequency was constant at 210 rpm, so DO was not controlled in this series of experiments. As confirmed by the results in Table 1, *A. lata* is indeed able to grow on and utilize different sugar substrates, even if they are mono- or di-saccharides [51]. On the contrary, there are clear experiments, such as those with lactose (L), xylose (X), fructose (F), and cheese whey (CW), in which the bacteria could not fully metabolize the available carbon source and produce sufficient amounts of biomass and PHB. Specifically, for L, the *A. lata* cells did not metabolize this disaccharide, probably because of the absence of the enzyme β-galactosidase, as reported in a previous study [52] and similar to other PHA-producing microorganisms [53]. The same hypothesis was also made in another attempt to utilize cheese whey lactose in *A. lata* cultures; the low efficiency may be attributed to the only partial acid hydrolysis of lactose that occurs during the acidic pretreatment of cheese whey [54]. However, limited, albeit measurable, growth and PHB production were observed in the L and CW experiments. This was due to the partial thermal hydrolysis of lactose during autoclave sterilization. Moreover, the results of CW are slightly better than those of L, which is due to the presence of glucose and probably other sugars preferred by bacterial cells, even at lower concentrations than lactose. Overall, for the use of CW as a cost-effective and alternative substrate for PHB production, a hydrolysis step is mandatory for the selected species.

It should also be noted that one of the less successful experiments corresponds to the only case involving a pentose (i.e., xylose, X). In contrast, three specific experiments using pure sugars (i.e., saccharose, S; glucose, G; and a mixture of glucose and galactose, GG) measured sufficiently high biomass and PHB concentrations, all corresponding to %PHB values above 55% *w*/*w*. Similar efficiency was observed when both enzymatically (CWE) and acid-hydrolyzed (CWA) cheese whey was used (>55% *w*/*w*). Compared with the GG experiment, these two cultures showed that the hydrolysis of CW lactose to glucose and galactose was successful and effective. Furthermore, it is clear that the enzymatic hydrolysis of cheese whey can be easily replaced by dilute acid hydrolysis, without a significant loss of efficiency. This can overcome both the large cost of producing and separating or purchasing enzymes and the extended duration of enzymatic hydrolysis [55,56].

As expected, the largest %PHB values were measured in the experiments with the most common and typical substrates, i.e., S and G (>60% *w*/*w*). However, both experiments with hydrolyzed cheese whey were efficient and showed sufficiently high PHB concentrations (>3.2 g/L) in cultures with a high cell density (>5.8 g/L), considering the flask scale of these cultures. In all experiments, the consumption of carbon and nitrogen sources was significant, however, with marked differences. Only in the most favorable cases was the nitrogen source almost exhausted (e.g., in culture G, the final C_N was equal to 0.04 g/L), which was more conducive to the accumulation of PHB in the intracellular granules due to nitrogen limitation [57]. Moreover, the carbon source was never nearly depleted due to the successive feeding steps of the AL^N medium.

Regarding the molecular properties of PHB, no large variations were observed among the molecular weights obtained in all experiments. The total M_w values were in the

narrow range of 785–867 kDa (Table 1). Considering the large variations in M_w values found in the open literature for different systems [58–60], it can be concluded that, in the present study, the substrate type (or the selected sugar type) did not have a clear influence on the molecular properties of the biopolymer. In addition, the M_w obtained in this series of experiments is high enough to support the decision for further optimization and exploitation [61]. It should be emphasized that the limited accumulation of PHB during the CW experiment did not allow its effective separation from the bacterial cells; therefore, no measurement of M_w was possible for this particular experiment.

Looking specifically at the results of the CWA experiment, it is clear that the final DCW (5.88 g/L) and PHB (3.25 g/L) concentrations are comparable to the corresponding values of the pure substrates. This observation can be easily extended to all the qualitative and quantitative characteristics, including the weight average molecular weight of PHB (789 kDa). Accordingly, *A. lata* exhibits its well-known and excellent characteristics, such as rapid biomass growth, efficient growth-associated biopolymer accumulation, and versatile ability to metabolize various carbon sources, especially sugars [62]. Therefore, the dilute acid hydrolysis of cheese whey can be expected to be sufficient and effective; for the following DoE experiments, it is possible to use this simple hydrolysis procedure for the treatment of cheese whey. It should also be emphasized that the present study aims to demonstrate that PHB production can be considered as a complement to the already-established options for cheese whey utilization as a renewable substrate without demanding treatment [37,38]. This need arises from the significant environmental and economic benefits of food waste recovery, which have been discussed in previous studies [39,40,44].

Table 1. Experimental results of the fermentative PHB production by *Azohydromonas lata* utilizing different pure sugars and processed cheese whey as carbon sources/substrates.

Sugar/ Substrate	OD [1] (@ 600 nm)	DCW [2] (g/L)	C_{PHB} [3] (g/L)	%PHB [4] (% *w*/*w*)	C_N [5] (g/L)	C_S [6] (g/L)	M_w [7] (kDa)
Saccharose (S)	22.3 ± 2.7	6.52 ± 0.7	4.11 ± 0.4	63.1 ± 5.3	0.12 ± 0.04	2.57 ± 0.6	852 ± 84
Glucose (G)	25 ± 2.6	6.25 ± 0.7	4.06 ± 0.5	65 ± 4.9	0.04 ± 0.02	1.76 ± 0.4	803 ± 81
Lactose (L)	15.4 ± 1.9	2.95 ± 0.6	1.09 ± 0.3	36.9 ± 4.4	0.37 ± 0.02	4.32 ± 0.7	826 ± 66
Xylose (X)	16.4 ± 1.7	3.15 ± 0.5	1.32 ± 0.4	41.9 ± 3.9	0.26 ± 0.01	5.34 ± 0.5	867 ± 74
Fructose (F)	19.1 ± 1.5	4.75 ± 0.6	2.42 ± 0.6	50.9 ± 5.1	0.13 ± 0.02	2.73 ± 0.5	795 ± 53
Galactose (GL)	20.3 ± 2.1	5.43 ± 0.8	2.41 ± 0.7	54.4 ± 4.7	0.11 ± 0.01	2.48 ± 0.6	812 ± 65
Glucose + Galactose (GG)	22.4 ± 2.0	6.11 ± 0.7	3.46 ± 0.6	56.6 ± 3.6	0.09 ± 0.03	1.54 ± 0.4	811 ± 58
Cheese Whey (CW)	18.3 ± 2.2	3.24 ± 0.5	1.33 ± 0.5	41 ± 4.7	0.44 ± 0.08	7.43 ± 0.3	–
Enzymatically Hydrolyzed CW (CWE)	21.6 ± 2.3	6.02 ± 0.6	3.33 ± 0.5	55.3 ± 6.2	0.14 ± 0.06	1.78 ± 0.6	801 ± 59
Acid-Hydrolyzed CW (CWA)	20.1 ± 2.4	5.88 ± 0.6	3.25 ± 0.3	55.3 ± 4.1	0.17 ± 0.03	1.89 ± 0.3	789 ± 66

[1] OD: optical density as an indication of microbial growth. [2] DCW: dry cell weight as a measurement of biomass concentration. [3] C_{PHB}: concentration of produced PHB. [4] %PHB: PHB content in the dried cells. [5] C_N: concentration of residual nitrogen source. [6] C_S: concentration of residual carbon/substrate source. [7] M_w: weight average molecular weight of PHB.

3.2. DoE, Optimization, and Analysis of Variance

After comparing and evaluating the different substrates, the Taguchi L_{16} series of experiments (see Table 2) was carried out to analyze the effects of the four selected bioprocess parameters (C/N, C/P, DO, and RT) on the two output variables/responses, i.e., %PHB and M_w. The measured mean values for both responses are also reported in Table 2. It can be seen that the measured values of %PHB and M_w vary significantly depending on the selected experimental conditions. The minimum (46.6%) and maximum (56.9%) values of %PHB were measured in experiments #16 and #10, respectively, while the corresponding values of M_w (668 kDa min and 892 kDa max) were determined in experiments #1 and #14. These ranges indicate the significant combined effect of the four selected parameters and the scope of further optimization.

Table 2. Experimental conditions and results for fermentative PHB production by *Azohydromonas lata* utilizing dilute-acid-hydrolyzed cheese whey, based on L_{16} Taguchi DoE.

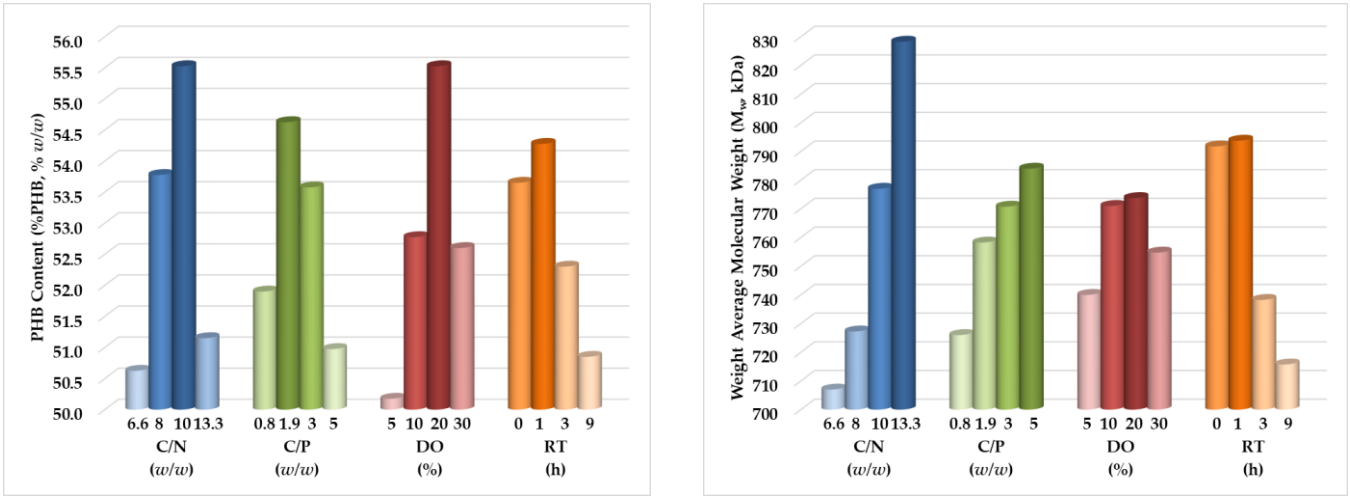
Run #	C/N [1] (*w*/*w*)	C/P [2] (*w*/*w*)	DO [3] (%)	RT [4] (h)	%PHB [5] (% *w*/*w*)	Mw [6] (kDa)
1	6.6	0.8	5	0	48.2 ± 4.3	668 ± 69
2	6.6	1.9	10	1	54.3 ± 5.3	742 ± 79
3	6.6	3	20	3	53.3 ± 5.4	717 ± 81
4	6.6	5	30	9	46.7 ± 4.9	701 ± 82
5	8	0.8	10	3	52.4 ± 4.7	702 ± 74
6	8	1.9	5	9	50.7 ± 5.2	669 ± 73
7	8	3	30	0	55.6 ± 6.1	756 ± 82
8	8	5	20	1	56.4 ± 5.9	782 ± 76
9	10	0.8	20	9	55.8 ± 4.9	704 ± 68
10	10	1.9	30	3	56.9 ± 5.1	732 ± 73
11	10	3	5	1	55.2 ± 5.2	821 ± 82
12	10	5	10	0	54.2 ± 5.7	851 ± 85
13	13.3	0.8	30	1	51.2 ± 5.5	830 ± 78
14	13.3	1.9	20	0	56.6 ± 5.1	892 ± 85
15	13.3	3	10	9	50.2 ± 4.9	789 ± 68
16	13.3	5	5	3	46.6 ± 4.7	802 ± 73

[1] C/N: carbon-to-nitrogen mass ratio, defined as the total initial concentration of sugars divided by the initial concentration of $(NH_4)_2SO_4$. [2] C/P: carbon-to-phosphorous mass ratio, defined as the total initial concentration of sugars divided by the total initial concentration of $Na_2HPO_4{\cdot}12H_2O$ and K_2HPO_4 in the respective ratio 6/1 *w*/*w*. [3] DO: dissolved oxygen concentration. [4] RT: residence time in stationary phase. [5] %PHB: PHB percentage content in dried cells. [6] M_w: weight average molecular weight of PHB.

From the analysis of variance (ANOVA) of the experimental data in Table 2, the individual effects of the four parameters on the two selected responses (%PHB and M_w) were derived and are shown in Figure 1. In addition, the optimal values of the four parameters that maximized the percentage accumulation of PHB and the associated M_w are presented in Table 3. An examination of the two main effect plots in Figure 1 confirms the significant variation in %PHB and M_w as a function of the four levels of the same in number parameters. In fact, the intensity of the effect of each parameter varies due to the large range of variation. Moreover, the optimal value of each parameter that maximizes %PHB and M_w can be determined; at the same time, the effect of each parameter can be studied individually. Accordingly, the optimal values of the parameters (also presented in Table 3) are:

- Carbon-to-nitrogen mass ratio (C/N): 10 *w*/*w* for %PHB and 13.3 *w*/*w* for M_w.
- Carbon-to-phosphorous mass ratio (C/P): 1.9 *w*/*w* for %PHB and 5 *w*/*w* for M_w.
- Dissolved oxygen concentration (DO): 20% for both %PHB and M_w.
- Residence time in stationary phase (RT): 1 h for both %PHB and M_w.

It is clear that for both DO and RT, the optimal values for maximizing %PHB and M_w are identical (i.e., 20% and 1 h, respectively). On the contrary, the values of C/N and C/P that maximize %PHB (i.e., 10 *w*/*w* and 1.9 *w*/*w*, respectively) are within the experimental study range, while the respective values that maximize M_w (i.e., 13.3 *w*/*w* and 5 *w*/*w*) correspond to the largest selected level for each parameter. Consequently, it is not possible to identify a global set of optimal conditions that maximizes both responses simultaneously. Giving priority to the quantitative KPI (i.e., %PHB), the selection of the optimal conditions follows the indications in Figure 1a (the levels that maximize %PHB are colored darker): C/N = 10 *w*/*w*, C/P = 1.9 *w*/*w*, DO = 20%, and RT = 1 h. Moreover, the nearly linear correlation between M_w and the C/N and C/P mass ratios suggests that further optimization can be performed to maximize the molecular weight, but at the expense of %PHB.



(a) (b)

Figure 1. Main effect plots of carbon-to-nitrogen mass ratio (C/N), carbon-to-phosphorous mass ratio (C/P), dissolved oxygen concentration (DO), and residence time in stationary phase (RT) for maximizing the (**a**) intracellular PHB content (%PHB, % *w*/*w*) and (**b**) weight average molecular weight of PHB (M_w, kDa) during the production of PHB using dilute-acid-hydrolyzed cheese whey. Descriptive statistics: (**a**) for %PHB variable, number of experimental data (N) = 16, mean (M) = 52.77%, standard error mean (SEM) = 0.87%, standard deviation (SD) = 3.48%, minimum (Min) = 46.6%, quarter 1 (Q1) = 50.33%, median (Me) = 53.75%, quarter 3 (Q3) = 55.75%, and maximum (Max) = 56.9%; (**b**) for M_w variable, N = 16, M = 759.9 kDa, SEM = 16.7 kDa, SD = 66.9 kDa, Min = 668 kDa, Q1 = 702.5 kDa, Me = 749 kDa, Q3 = 816.3 kDa, and Max = 892 kDa.

It is important to note that high values of C/N and/or C/P correspond to nitrogen and/or phosphorus limitation and also result in high values of M_w. On the other hand, the C/N and C/P values that maximize %PHB are identical to those discovered in previous studies that used the same species with saccharose as the carbon source [47,48]. The relative ranking of the four parameters and their percentage contributions to the total variance of %PHB and M_w are reported in Table 3. As can be seen, DO is the most important parameter for %PHB, followed by C/N (31% and 28% contribution to variation, respectively). The two remaining parameters (i.e., C/P and RT) are less important, with, however, a considerable contribution to the %PHB variance (≥20%). In contrast, C/N is the most important parameter for M_w (42%), followed by RT (26%); between the other two parameters, only the impact of C/P is considerable (20%).

Overall, the importance of DO during PHB production by *A. lata* can be linked to the aerobic central carbon metabolism of sugars to PHB in a growth-associated mechanism and also to the intracellular polymerization mechanism of PHB within certain biopolymer granules [63]. The importance of C/N and in extend of nitrogen-limiting conditions is already known, especially for maximum %PHB values [64]. A similar effect can be described for the balance of the two reactions/mechanisms responsible for the initiation and propagation of biopolymer chains: under nitrogen limitation, the latter mechanism is favored (increased propagation simply corresponds to larger molecular weights). Overall, it is crucial to carefully control the aeration conditions of the culture while applying a nutrient limitation strategy favoring nitrogen over phosphorous with excess carbon. In addition, the residence time in the stationary phase should be limited to avoid the intracellular degradation of biopolymer chains from the bacterial cells, since they serve as carbon and energy sources [65]. The effect of this 'self'-depolymerization mechanism is primarily noticeable in the M_w values and secondarily in the %PHB values.

Table 3. Statistical analysis results based on L_{16} Taguchi DoE in order to maximize %PHB and M_w during PHB production using dilute-acid-hydrolyzed cheese whey.

Scenario	Parameter	C/N [1]	C/P [2]	DO [3]	RT [4]
Maximize %PHB [5]	Optimal Level	10 *w*/*w*	1.9 *w*/*w*	20%	1 h
	%PHB Range of Variation (Delta, % *w*/*w*)	4.9%	3.65%	5.35%	3.42%
	Significance Ranking	2	3	1	4
	Contribution to Variance (%)	28%	21%	31%	20%
Maximize M_w [6]	Optimal Level	13.3 *w*/*w*	5 *w*/*w*	20%	1 h
	M_w Range of Variation (Delta, kDa)	121.3	58	33.8	78
	Significance Ranking	1	3	4	2
	Contribution to Variance (%)	42%	20%	12%	26%

[1] C/N: carbon-to-nitrogen mass ratio, defined as the total initial concentration of sugars divided by the initial concentration of $(NH_4)_2SO_4$. [2] C/P: carbon-to-phosphorous mass ratio, defined as the total initial concentration of sugars divided by the total initial concentration of $Na_2HPO_4 \cdot 12H_2O$ and K_2HPO_4, in the respective ratio 6/1 *w*/*w*. [3] DO: dissolved oxygen concentration. [4] RT: residence time in stationary phase. [5] %PHB: PHB percentage content in dried cells. [6] M_w: weight average molecular weight of PHB.

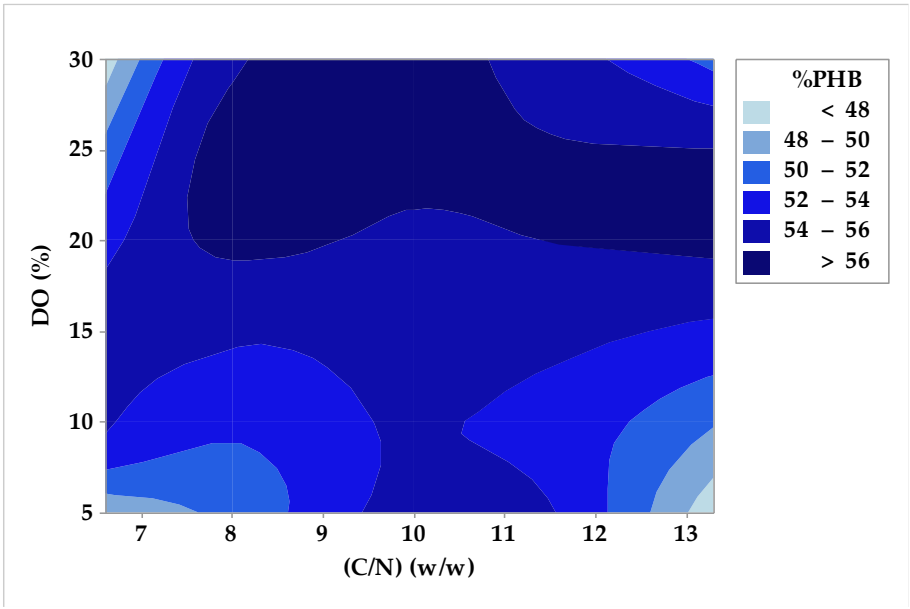
Note that the two sets of parameter values that potentially maximize %PHB or M_w were not assigned by Minitab as part of the original DoE (Table 2). This means that the maximum values were not determined for either %PHB or M_w. In principle, these values can be derived by the automatic predictions of the simple statistical-empirical models created by Minitab 17 during the processing of statistical data:

- For C/N = 10 *w*/*w*, C/P = 1.9 *w*/*w*, DO = 20%, and RT = 1 h, the maximum estimated biopolymer content value is %PHB = 61.66% *w*/*w*; this value was verified with minimal deviation (2%) by an additional experiment.
- For C/N = 13.3 *w*/*w*, C/P = 5 *w*/*w*, DO = 20%, and RT = 1 h, the maximum estimated weight average molecular weight value is M_w = 900 kDa; this value was also verified with a small deviation (5%) by a second additional experiment.

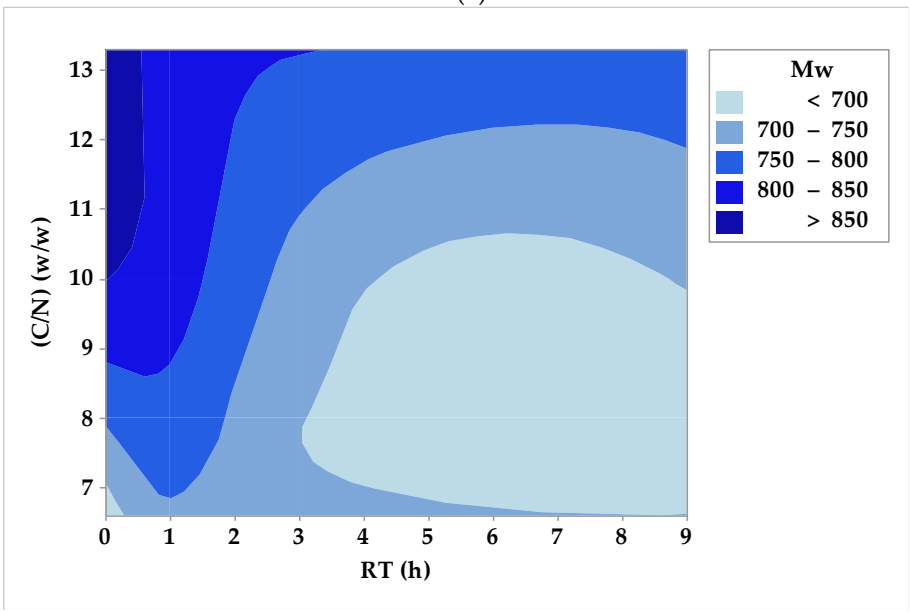
In order to better visualize the optimal operating windows that maximize %PHB and M_w, two contour plots (see Figure 2) were developed based on the interactions between the two most important parameters for each response (see also Table 3). Accordingly, the following conclusions regarding the maximization of %PHB and M_w can be made, respectively:

- DO–C/N (Figure 2a): medium to large values of both DO and C/N maximize the value of %PHB.
- C/N–RT (Figure 2b): medium to large values of C/N, combined with very small values of RT, maximize the value of M_w; the degradation of PHB and the decrease in M_w take place at larger RT values.

Interpretation of the detailed information obtained by the previous analysis revealed a very strong interaction between pairs of parameters for both responses, i.e. DO and C/N for %PHB and C/N and RT for M_w. Undoubtedly, these parameters should be the subject of primary optimization when maximizing the intracellular content or weight average molecular weight of PHB is set as the main objective.



(**a**)



(**b**)

Figure 2. Contour plots for the combined effect of (**a**) dissolved oxygen concentration (DO)—carbon-to-nitrogen mass ratio (C/N) on PHB content (%PHB, % *w*/*w*) and (**b**) C/N—residence time in stationary phase (RT) on weight average molecular weight of PHB (M_w, kDa).

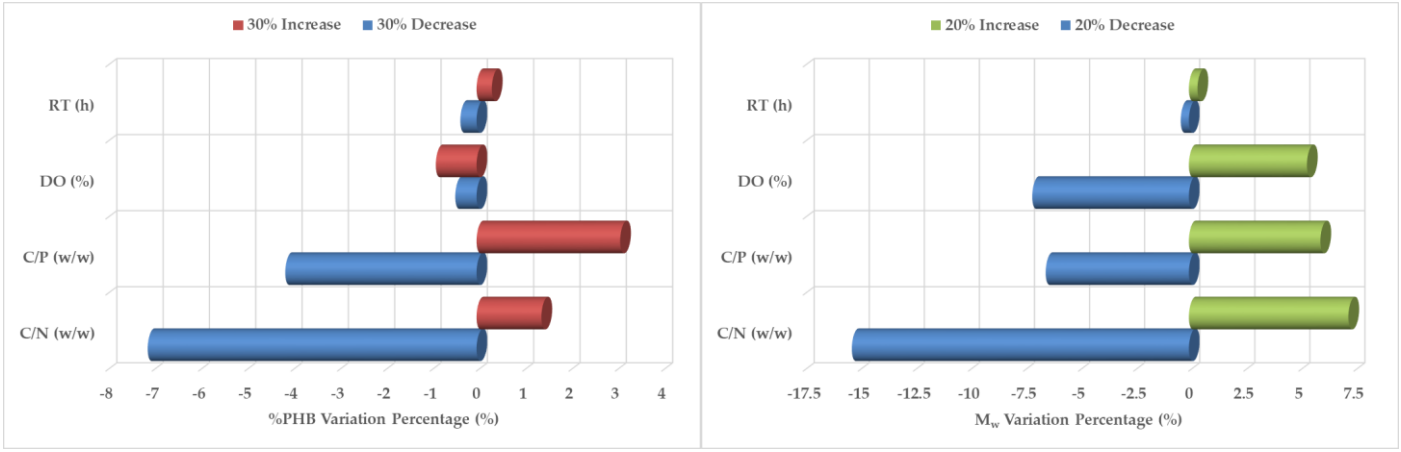
3.3. Regression Modeling and Sensitivity Analysis

To further quantify the effects of the four selected parameters on %PHB and M_w, two simple regression models were derived using Minitab 17. For this purpose, the 'fit regression model' option of the software was used. The models consist of polynomial terms up to the second order of the four parameters. Note that the parameter related to C/N^2 in Equation (1) and the parameter related to RT^2 in Equation (2) were omitted from the models, because their confidence intervals included the value zero. Recognizing that their use should be limited only to the study areas of the selected parameters, the models have the following expressions (with R-sq of 95.19% for %PHB and 92.30% for M_w):

$$\%PHB = 5.44 + 8.29 \cdot C/N + 3.19 \cdot C/P + 0.98 \cdot DO - 0.55 \cdot RT - 0.6 \cdot C/P^2 - 0.025 \cdot DO^2 - 0.021 \cdot RT^2 \quad (1)$$

$$M_w = 475 + 28.1 \cdot C/N + 35.6 \cdot C/P + 7.08 \cdot DO - 23.11 \cdot RT - 0.47 \cdot C/N^2 - 3.83 \cdot C/P^2 - 0.19 \cdot DO^2 \quad (2)$$

Subsequently, the above equations were used to perform a sensitivity analysis for the two responses around their optimal conditions. The two central scenarios were defined by the two sets of parameter values found (in Section 3.2) to maximize %PHB and M_w, namely, C/N = 10 *w*/*w*, C/P = 1.9 *w*/*w*, DO = 20%, RT = 1 h, and C/N = 13.3 *w*/*w*, C/P = 5 *w*/*w*, DO = 20%, and RT = 1 h, respectively. Specifically, the sensitivity for each response was examined with two additional scenarios: (1) For %PHB, the values of the four parameters were autonomously increased and decreased by 30% from their optimal value, while the other three parameters were kept constant in the central scenario. The results are shown in Figure 3a and correspond to the percentage change (increase or decrease) in %PHB from the so-far maximum value of 61.66% *w*/*w*. (2) For M_w, the respective autonomous change in each parameter was set to 20% from the central scenario that led to the optimal value (900 kDa). The percentage changes shown in Figure 3b correspond to the percentage change in M_w from this value.



(**a**) (**b**)

Figure 3. Sensitivity analysis of (**a**) intracellular PHB content (%PHB, % *w*/*w*) and (**b**) weight average molecular weight of PHB (M_w, kDa), with respect to 30% and 20% variations, respectively, in C/N, C/P, DO, and RT, during the production of PHB using dilute-acid-hydrolyzed cheese whey.

From the examination of Figure 3a, it is evident that there are unexplored scenarios that can further increase %PHB. In particular, increasing C/N and C/P, i.e., by limiting the concentration of each nutrient under carbon sufficiency, increases PHB accumulation, but only to a limited extent (about +3%). On the other hand, a potential decrease in the values of these two parameters has a much stronger effect on biopolymer accumulation, since the decrease in %PHB is more than twice as large (in absolute terms) as the previous increase (approximately –7%). Moreover, it should be taken into account that any change in DO has a clear impact on %PHB in the direction of lower biopolymer accumulation efficiency. Considering that the consecutive reduction in %PHB is really small (<1%), the importance of DO is limited to the already discovered absolute optimal value of the central scenario (20%); further optimization of %PHB by manipulating DO cannot be considered. Thus, the original goal of identifying and directly optimizing the most important parameter has been achieved. Further optimization should be based on the remaining parameters, with a clear focus on nitrogen and phosphorous sources concentrations. Finally, the contribution of RT in not important, since the changes in %PHB do not exceed ±1%.

Specifically, for M_w, the individual increase in C/N or C/P also increases M_w to a limited extent (approximately +7%). On the other hand, the opposite change (decrease) in the values of these two parameters has a much stronger effect on the molecular weight: the M_w value decreases significantly (about –15%). Similar conclusions, but with much smaller effects, resulted from the study of the other two parameters, DO and RT. Nevertheless, it

is clear that the set goal of optimizing the most significant parameters for M_w has been accomplished for the time being. Further optimization can be carried out, but only to a limited extent with respect to the expected M_w increase. Overall, it is equally, if not more, important to keep the selected parameter values close to the optimal cases, as the impact on the undesired reduction in %PHB and M_w is significant, as shown in Figure 3.

4. Conclusions

Research on the microbial production of PHAs and PHB remains predominately on low TRLs, as there are only a few examples of industrialization. Almost without exception, the high production cost of this class of biopolymers is cited as the major obstacle, with the carbon source being the largest contributor to this cost. At the same time, this technology is multi-parametric, so there are many optimization issues to be addressed. Therefore, an important goal is to successfully optimize the bioprocess for the production of PHAs/PHB when a low-cost and alternative substrate is used instead of a model sugar.

In this study, the production of PHB by the wild-type bacterium *Azohydromonas lata* using cheese whey (CW) as the sole carbon source was demonstrated in response to the above two challenges. Moreover, the major drawback of costly and time-consuming enzymatic hydrolysis of cheese whey was overcome by using simple hydrolysis with dilute acid. An additional advantage of the proposed solution was that no CW pretreatment steps were required other than the standard autoclave sterilization. In addition, the fed-batch conditions promoted PHB accumulation by the bacteria, since nitrogen-limiting conditions existed with excess carbon. In this sense, the systematic evaluation of four selected parameters (C/N, C/P, DO, and RT) and their effects on the quantitative and qualitative KPIs of the system (%PHB and M_w responses, respectively) was carried out using Taguchi statistical DoE. It is worth highlighting that two sets of conditions corresponding to the optimal parameter values were identified using the DoE results, as well as ANOVA and sensitivity analysis. In fact, one set of values was found to maximize the percentage accumulation of PHB, and another set was found to maximize the weight average molecular weight of PHB. This is due to the different influence that each parameter has on the two responses and also to the complementarity with the other parameters and to their interactions. This clear inability to identify a global set of parameters that simultaneously maximize %PHB and M_w can possibly be explained by two competing mechanisms during PHB accumulation. In principle, it can be proposed that microbial PHB production is controlled by two mechanisms with a clear trade-off: (1) the first mechanism occurs during nitrogen sufficiency and is responsible for the formation of numerous new intracellular PHB granules; it can be referred to as an initiation mechanism that controls the amount of biopolymer produced through the development of different biopolymer chains; (2) the second mechanism is activated at full spread under nitrogen limitation and is responsible for the growth of existing (already initiated) biopolymer chains within the formed granules; it is rather a propagation mechanism that controls the length of PHB chains and, to a large extent, their molecular weight.

Since the role and importance of the parameters change between %PHB and M_w, it is not safe to propose a single set of parameter values for optimization. Instead, a choice between the two identified sets that explicitly maximize %PHB or M_w can only be made based on prioritizing the responses, i.e., %PHB over M_w in this case. Furthermore, there is only a narrow range around these parameter values for further optimization. Thus, the optimal operational windows, focusing on the two most important parameters (DO and C/N), are clear. Nevertheless, the stability of the system should also be ensured, especially if possible disturbances can move the parameter values from the optimal conditions. It may be possible in the future to create a wider operational window for maximum efficiency by overcoming all relevant metabolic constraints during sugar metabolism and PHB accumulation. Accordingly, a tailored genetically engineered strain could excel in both quantitative and qualitative KPIs. In this way, %PHB could be maximized without

compromising the value of M_w and vice versa; both responses could eventually be maximized in a single bioprocess.

It is also important to emphasize that the selected strain showed remarkable tolerance to dilute-acid-hydrolyzed cheese whey, among other substrates tested. In almost all cases and with many different sugars, *A. lata* was able to activate the Entner-Doudoroff carbon pathway (a variant of glycolysis), regardless of the presence of mono- or di-saccharides. Therefore, it can be considered as an 'industrial' bacterial strain with the prospect of being applied in scalable cultivations. Using this species and depending on the prescribed nutritional conditions and operational parameters, it is possible to efficiently produce PHB samples with different molecular properties and thus different biopolymer grades. This result sets a clear objective for the future, namely, the validation of the proposed bioprocess in an operational environment while achieving the goal of zero substrate cost. It is clearly a promising future scenario that will be evaluated in a continuation of this study, first on a larger scale and then from a techno-economic point of view. In this sense, it is the authors' intention to directly transfer the most promising scenario for PHB production from cheese whey to the bioreactor scale and gradually validate the present study in higher TRLs. Moreover, clear synergies of similar approaches with plastic production, upcycling, and recycling, as well as waste management, which inevitably focus on low-cost substrates, could provide a new perspective on the economic and environmental potential of bio-based plastics, including their production, usage, and disposal.

Author Contributions: Conceptualization, G.P. and C.K.; methodology, G.P. and A.P.; validation, G.P. and A.P.; formal analysis, G.P., A.P. and C.K.; investigation, G.P. and A.P.; data curation, G.P. and A.P.; writing—original draft preparation, G.P. and A.P.; writing—review and editing, G.P., A.P. and C.K.; visualization, G.P. and C.K.; supervision, G.P. and C.K.; project administration, G.P. and C.K.; funding acquisition, G.P. and C.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (Project name: Bioconversion of Food Industry Wastes to Biopolymers for Packaging Applications in a Biorefinery Concept—Wastes-to-Biopolymers; project code T1EDK-02822).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors would like to sincerely thank Aspasia Koroniou of the Chelmos Ltd. dairy industry, Achaia, Greece, for providing characterized cheese whey, and Dimitris Hatzinikolaou of the Department of Biology, National and Kapodistrian University of Athens (NKUA), Greece, for performing the enzymatic hydrolysis of cheese whey.

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

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