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Transformation of Enzymatic Hydrolysates of *Chlorella*–Fungus Mixed Biomass into Poly(hydroxyalkanoates)

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Abstract: The production of poly(hydroxylalkanoates) (PHA) is limited by the high cost of the feedstock since various biomass wastes look attractive as possible sources for polymer production. The originality of this present study is in the biotransformation of mixed *Chlorella*-based substrates into PHAs. The synthetic potential of *Cupriavidus necator* B8619 cells was studied during the bioconversion of algae biomass in mixtures with spent immobilized mycelium of different fungi (genus *Rhizopus* and *Aspergillus*) into PHAs. The biomass of both microalgae *Chlorella* and fungus cells was accumulated due to the use of the microorganisms in the processes of food wastewater treatment. The biosorption of *Chlorella* cells by fungal mycelium was carried out to obtain mixed biomass samples (the best ratio of "microalgae:fungi" was 2:1) to convert them by *C. necator* B8619 into the PHA. The influence of conditions used for the pretreatment of microalgae and mixed types of biomass on their conversion to PHA was estimated. It was found that the maximum yield of reducing sugars (39.4 ± 1.8 g/L) can be obtained from the mechanical destruction of cells by using further enzymatic hydrolysis. The effective use of the enzymatic complex was revealed for the hydrolytic disintegration of treated biomass. The rate of the conversion of mixed substrates into the biopolymer (440 ± 13 mg/L/h) appeared significantly higher compared to similar known examples of complex substrates used for *C. necator* cells.

Keywords: poly(hydroxyalkanoates); *Cupriavidus necator*; *Chlorella vulgaris*; fungi; sorption capacity; mixed biomass; enzymatic hydrolysis

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1. Introduction

Traditionally, the biomass of *Chlorella* cells attracts attention as a raw material that can be transformed into various products: liquid fuel [1], organic acids (lactic, fumaric, succinic, lactic, aspartic) [2], organic solvents [3], biogas [4,5], polysaccharides (bacterial cellulose, xanthan, pullulan) [6]), etc. At the same time, both the biomass of the whole cells of these microalgae [7] and the individual fractions (lipids, defatted part) of this biomass can undergo conversion [8].

In parallel with this, issues related to the ubiquitous presence of particles of synthetic poorly biodegradable microplastics, which form serious environmental problems, have been increasingly discussed recently [9]. In this regard, the development of processes for the production and use of biodegradable polymers and composite materials is becoming increasingly relevant [10,11]. At the same time, among biodegradable polymers, the maximum attention is attracted not so much by materials synthetically obtained from various bioresources (polymers and copolymers of lactic acid, polycaprolactone, poly(butylene succinate), etc.), but naturally synthesized; for example, poly(hydroxyalkanoates) (PHA) [7,12]. In the case of PHA, there is no need to obtain monomers for their use in polymerization processes and the special development of chemical catalysts for such reactions and polymer production. The synthesis of PHA occurs directly in the cells of many bacteria [7], allowing the extraction of a ready-made polymer from the biomass of cells. According

to their characteristics, these polymers are close to synthetic ones, but they are biocompatible and completely biodegradable in carbon dioxide and water. At the same time, *Cupriavidus necator* cells turned out to be the most widely used as an active producer of PHA, capable of converting various raw materials having complex biochemical compositions into this natural polymer (Table 1, [13–28]).

Table 1. Production of PHA by *C. necator* from various complex substrates.

Substrate [Reference]	Dry Cell Weight (DCW) (g/L)	PHA Concentration (g/L)	Intracellular PHA Content (%)	* Q_{PHA} (mg/L/h)
Broken rice waste [13]	14.0	47.3	6.5	67.8
Palm oil [14]	4.7	4.0	83.7	83.3
Desert date oil [15]	9.0	40	3.6	75.0
Date molasses and date seed oil combination [16]	6.9	51.0	3.5	72.9
Sludge palm oil [17]	9.7	74.0	7.2	150.0
Carboxylic acids obtained by the anaerobic fermentation of cheese whey [18]	15.0	71.0	10.0	192.3
Polystyrene waste pretreated with thermal oxidation processes [19]	3.6	48.0	1.7	35.8
Grape winery waste [20]	8.3	63.0	5.2	176.3
Food waste-derived volatile fatty acids [21]	2.2	75.3	1.6	44.4
Digestate of chicken manure combined with waste sunflower oil [22]	6.1	75.1	4.6	47.9
Laminaria japonica biomass acid hydrolysate [23]	5.0	32	1.6	26.3
Sargassum sp. biomass enzymatic hydrolysate [24]	5.4	74.4	3.9	109.2
The hydrolysis liquid from inedible rice [25]	7.0	68.6	4.8	66.9
Soybean hull enzymatic hydrolysate with waste glycerol [26]	6.7	39.0	2.6	27.1
Waste glycerol [27]	10.5	69.0	7.3	183.0
Acid hydrolysate of defatted *Chlorella* biomass [8,28]	10.0	75.4	7.5	62.6

* Q_{PHA}—average rate of PHA accumulation in *C. necator* cells. This characteristic was calculated on the basis of experimental data presented in the cited references.

All resources used for the biosynthesis of PHA are usually pre-subjected to physical–chemical disintegration. Among the many substrates used for PHA production, *Chlorella* biomass is of undoubted interest since it can more easily be disintegrated in comparison with cellulose-containing raw materials [29]. Interestingly, that biomass can be accumulated as a result of *Chlorella* cells' use for nature-like wastewater treatment [30]. The use of lipid-containing substrates (Table 1, [17,18,21,27]), as well as grape winery waste [20], provides an accumulation of the maximum amount of PHA (Table 1). When searching for advantageous substrates for the production of PHA, one should focus on those that can be obtained as a result of waste transformation and contain either high concentrations of lipids and/or reducing sugars (RS). In the analysis of the present data (Table 1), as mentioned in a number of investigations, the preferences in choosing methods of pretreatment of complex substrates were given to enzymatic techniques [24,26]. However, before disintegration and treatment, such a biomass of *Chlorella* cells should be initially concentrated from any aqueous medium.

Recently, more publications have appeared, demonstrating the results of effective collections of *Chlorella* biomass in various aquatic environments by sorption of these cells

on the mycelial biomass of various filamentous fungi [31]. This is done precisely in order to provide a cheap option for harvesting *Chlorella* cells without using traditional technological energy-consuming (separation, centrifugation) or less efficient (cell sedimentation) technological stages. At the moment, two main approaches to harvesting microalgae cells from various media using sorption on fungal mycelium can be distinguished in the literature (Table 2, [32–41]): spore- and pellet-assisted harvesting. Various researchers are studying the possibility of collecting different microalgae (M) cells by means of their biosorption on the surface of biological sorbents; the biomass of mycelial fungi seems to be the most interesting and highly effective. Today, it is known from the literature [32–41] that the mycelium of fungi (F) of the genera *Mucor*, *Aspergillus*, and *Penicillium* are used for such purposes, which most easily enter into such sorption interactions with chlorella cells, the biomass of which is of particular interest for its subsequent processing. The left part of this table contains specific examples from experimental articles in which the authors presented specific pairs of "Microalgae/Fungi" used to study the process of the sorption of microalgae biomass on mycelium of different fungi. In the center of the table are the conditions under which the sorption process itself was carried out (the initial ratio of biomass of both types of microorganisms, the time of the sorption process, the mixing rate of the mixed biomass to avoid its sedimentation, and the concentration of glucose in the medium necessary to maintain the viability of fungal cells). The table on the right shows the result of the efficiency of the sorption of the biomass of microalgae cells on mycelial biomass (the so-called "harvesting efficiency"). This is a parameter that authors use in different studies to compare their results. "Spore-assisted harvesting", in which fungal spores and inoculate of microalgae cells are introduced into the same medium (this is reflected in the magnitude of the mass ratio between "Microalgae/Fungi" (M/F) (Table 2, [32–38]).

Table 2. Results of sorption harvesting of microalgae *Chlorella* cells by mycelial biomass of filamentous fungi.

Microalgae/Fungi (M/F) [Reference]	Process Conditions	Harvesting Efficiency, %
	Spore-assisted harvesting	
C. pyrenoidosa/*Mucor circinelloides* [32]	Ratio M/F = 333:1, 24 h, 150 rpm, 1.5 g/L glucose	~100
C. vulgaris/*Aspergillus niger* [33]	Ratio M/F = 300:1, 72 h, 150 rpm, 2 g/L glucose	>90
C. vulgaris/*A. niger* [34]	1×10^4 spore/mL, 72 h, 150 rpm, 15 g/L sucrose	~100
Chlorella sp./*Penicillium* sp. [35]	1×10^4 spore/mL, 28 h, 160 rpm, 5 g/L glucose	99
C. vulgaris/*Aspergillus* sp. [36]	Ratio M/F = 100:1, 4 h, 80 rpm, molasses wastewater	97
C. pyrenoidosa/*A. oryzae* [37]	Ratio M/F = 4000:1, 72 h, 150 rpm, 5.5 g/L total sugars	~100
C. vulgaris/*M. circinelloides* [38]	Ratio M/F = 300:1, 180 h, 140 rpm, 2 g/L glucose	95
C. vulgaris/*M. circinelloides* [38]	Spore-assisted harvesting with support matrix Ratio M/F = 300:1, 180 h, 140 rpm, 2 g/L glucose, cotton patches (2.5 cm × 2.5 cm) as support	~100
	Pellet-assisted harvesting	
Chlorella sp./*Penicillium* sp. [34]	Ratio M/F= 2:1, 2.5 h, 160 rpm	98
C. vulgaris/*Aspergillus* sp. [36]	2 g dry weight fungal biomass/L, 4 h, molasses wastewater	97
C. vulgaris/*A. niger* [39]	Ratio M/F = (4.8×10^{10} cells/mL): (4 fungal pellets (each ~1 cm)/10 mL), 24 h, 120 rpm	93
C. pyrenoidosa/*A. fumigatus* [40]	Ratio M/F = 5:1, 3 h, 100 rpm	99
Chlorella sp./*Aspergillus* sp. [41]	Ratio M/F = 1:3, 5 h, 100 rpm, 6 g/L dextrose	>90

Next, *Chlorella* cells and fungi are co-cultured, as a result of which fungal spores germinate, a mycelium is formed on which microalgae cells are sorbed, and thus pellets of mixed biomass are formed [32–38].

The peculiarity of this approach to collecting *Chlorella* cells is that sorption takes quite a long time (24–180 h) when fungal spores are used for this process and requires the presence

of a nutrient medium, which is necessary for the growth of fungus cells. According to the indicated M/F ratios, if fungal spore material is initially used for the sorption of microalgae cells [32–38], then the concentration of *Chlorella* biomass introduced for sorption most often significantly exceeded the concentration of fungal biomass. In addition, during the growth process, mycelial fungi exhibit metabolic activity and secrete hydrolytic enzymes that negatively affect the cell integrity of harvested microalgae biomass.

A modified version of the described approach is "pellet-assisted harvesting", which consists of introducing an additional matrix into the medium for simultaneous sorption of cells of two different microorganisms (both microalgae and fungi) on it [38]. In this case of using previously formed mycelium, the ratio between the biomass of microalgae and fungi changed significantly [34,40,41]; pre-grown mycelium, owing to its morphology, quickly accumulated microalgae biomass. The sorption process in this case was much less prolonged (2.5–5 h), and the initially introduced amounts of microalgae and fungi biomass already turned out to be comparable. At the same time, it is noted that the sorption process of *Chlorella* cells is carried out as efficiently as possible using viable fungal biomass, rather than fungal cell debris [42].

Along with the absence of significant differences in the efficiency of harvesting of *Chlorella* cells, which in many cases is close to 100% for both approaches mentioned (Table 2), the use of already formed mycelium for biosorption of accumulated microalgae biomass seems more attractive. The reason is that, for the purposes of collecting microalgae cells, it is possible to use not specially grown fungus biomass, but what remains from processes in which fungi are used as the main producers in some biotechnological processes [43]. So, obtaining a mixed biomass of fungi with microalgae can also be attractive as a way of recycling spent fungus biocatalysts; however, the special accumulation of fungal biomass for microalgae sorption or fungi co-cultivation with microalgae, in any case, leads to the expenditure of resources. There are a number of biotechnological processes, as a result of which, the developed fungal mycelium remains as a waste [2]. Since then, the wastes can be interesting for use in the biosorption of *Chlorella* cells.

In any case, as a result of the highly efficient sorption of *Chlorella* cells on mycelial biomass, actually mixed biomass is obtained, and then the question arises about its subsequent processing into some target products. Of course, it is interesting, as well as relevant and important, from a scientific and practical point of view, to study the possible conversion of such mixed biomass into PHA. In this regard, this work aimed to study the feasibility of the biosynthesis of PHA from the hydrolyzed mixed biomass of *Chlorella* and fungal mycelium obtained as a result of the sorption of microalgae cells on fungus biomass. At the same time, the *Chlorella vulgaris* C-1 biomass itself was accumulated during the wastewater treatment of milk plant [30], and the fungal biomass used for the biosorption of microalgae was performed by spent immobilized living mycelial fungal cells, which were used for a long time in the corresponding processes: *Rhizopus oryzae* F1032 cells synthesized fumaric acid [2], *R. oryzae* F814 cells were used in the food wastewater treatment [44], and *Aspergillus terreus* F728 cells produced hydrolytic enzymes [45]. Particular attention was paid to the comparative evaluation of the results of the sorption of microalgae cells on the mycelium of different fungi and the comparison of the results of PHA biosynthesis by *C. necator* B-8619 cells in media with pretreated mixed biomass. The biomass was subjected to hydrolytic disintegration under different conditions to prepare it for *C. necator* cultivation. Before switching to the use of disintegrated mixed biomass of whole fungal cells with sorbed microalgae in the synthesis of PHA, the influence of different methods of pretreatment of whole *Chlorella* cells on the yield and rate of PHA accumulation in *C. necator* cells was initially evaluated in this work. This study generally contributes to the implementation of criteria for the sustainable economic development of modern biotechnological industries, access to zero-waste processes, and rational use of raw materials. In fact, it represents another step toward the implementation of economically and technologically feasible nature-like processes (phototrophic growth, biosorption) with the participation of

biocatalysts, both fungal because it is a way of processing waste of fungal biomass and bacterial because it will reduce the cost of the process of obtaining PHA from waste.

2. Results

2.1. Comparison of the Effectiveness of Various Disintegration Methods of Microalgae C. vulgaris Biomass and Subsequent Conversion of the Obtained Hydrolysates into PHA

To accumulate the biomass of *C. vulgaris* C-1 microalgae cells, an immobilized inoculum of this culture was used, and cultivation was carried out on food wastewater under previously described conditions [30]. The accumulated *Chlorella* biomass separated from the medium was subjected to disintegration using various (physical, chemical, and enzymatic) methods, as well as their combinations (Table 3).

Table 3. Influence of different conditions of biomass disintegration of microalgae *C. vulgaris* cells on the main characteristics of the process at different initial concentrations of biomass (g of dry cell biomass weight) in the reaction medium (C_{DCW}).

Conditions of Biomass Disintegration	* C_{RS}, g/L	C_{GLU}, g/L	Q_{RS}, g/L/h	Q_{GLU}, g/L/h
C_{DCW} = 20 g/L				
Acid hydrolysis combined with thermolysis (1.2 M HCl, 0.5 h, 121 °C, 1 atm)	6.9 ± 0.3	2.3 ± 0.07	13.8 ± 0.6	4.6 ± 0.2
Acid hydrolysis combined with thermolysis (1.0 M H_2SO_4, 0.75 h, 121 °C, 1 atm)	7.7 ± 0.3	3.4 ± 0.08	10.2 ± 0.5	4.5 ± 0.2
Mechanical destruction (4 min) and further acid hydrolysis combined with thermolysis (1.0 M H_2SO_4, 0.75 h, 121 °C, 1 atm)	8.5 ± 0.3	4.6 ± 0.1	11.3 ± 0.5	6.1 ± 0.3
Enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	3.0 ± 0.1	1.4 ± 0.04	0.2 ± 0.01	0.07 ± 0.01
Thermolysis (0.5 h, 108 °C, 0.5 atm) and further enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	8.4 ± 0.3	5.9 ± 0.2	0.4 ± 0.02	0.3 ± 0.01
Treatment by ionic liquid [Bmim]Cl (1 h, 120 °C) and further enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	5.7 ± 0.2	3.8 ± 0.1	0.3 ± 0.01	0.2 ± 0.01
Mechanical destruction (4 min) and further enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	9.8 ± 0.3	7.8 ± 0.2	0.5 ± 0.02	0.4 ± 0.01
C_{DCW} = 30 g/L				
Mechanical destruction (4 min) and Enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	14.7 ± 0.8	11.6 ± 0.6	0.7 ± 0.03	0.6 ± 0.02
C_{DCW} = 50 g/L				
Mechanical destruction (4 min) and Enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	24.2 ± 1.2	19.1 ± 0.9	1.2 ± 0.06	1.0 ± 0.04
C_{DCW} = 70 g/L				
Mechanical destruction (4 min) and Enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	33.1 ± 1.6	25.0 ± 1.2	1.7 ± 0.08	1.3 ± 0.06
C_{DCW} = 100 g/L				
Mechanical destruction (4 min) and Enzymatic hydrolysis (20 h, 37 °C, pH 5.5) **	39.4 ± 1.8	28.3 ± 1.4	2.0 ± 0.09	1.4 ± 0.07

* C_{RS}, C_{GLU} and Q_{RS}, Q_{GLU} are the concentrations (C) and average rates of accumulation (Q) of reducing sugars (RS) and glucose (GLU), respectively, determined in the soluble part of reaction media with treated biomass. ** Enzymatic hydrolysis was conducted in presence of a mixture of Cellulases (8 mg/g dry cell biomass weight) with α-Amylase (2 mg/g dry cell biomass weight) containing reagents.

Since, by the beginning of this work, the results of previously conducted hydrolytic treatments of *Chlorella* cells under the action of various inorganic acids [8,28] as well as thermolysis of microalgae biomass [3] were known, similar techniques were also applied among the methods of processing whole microalgae cells (Table 3). In addition to sulfuric (H_2SO_4) and hydrochloric (HCl) acids, variants of combining thermolysis and acid hy-

drolysis, mechanical disintegration of biomass, and its subsequent enzymatic treatment were investigated.

For comparison, experiments were conducted using ionic liquid 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) for the disintegration of *Chlorella* biomass, which previously showed good results in the disintegration of cellulose-containing raw materials [46] prepared for subsequent biocatalytic transformation under the action of cellulases.

It should be noted that, unlike known studies in which the treatment of defatted *Chlorella* cells was carried out for further PHA obtaining [8,28], in these experiments, whole cells of microalgae were used and treated for subsequent application in the PHA biosynthesis. The analysis of the efficiency of biomass destruction was focused on the control of RS and glucose (GLU) accumulating in the liquid phase of the reaction medium in dissolved form.

It can be seen from the data obtained (Table 3) that when acid hydrolysis was used, the destruction of cellular biomass occurred quite quickly with the release of controlled sugars into the solution. The subsequent addition of this treatment by thermolysis and, at the same time, by the preliminary mechanical destruction of cells gave the maximum result of this kind of hydrolytic effect on the *Chlorella* biomass.

Enzymatic hydrolysis of biomass, according to the data obtained, is not of interest as an independent method of disintegration of *Chlorella* cells, since they do not allow obtaining significant amounts of RS in the reaction medium; however, the use of this type of hydrolytic treatment in combination with preliminary thermolysis, and especially with the preliminary mechanical disintegration of microalgae cells, provides the best results in RS and GLU release among all investigated samples of reaction media (Table 3).

Interestingly, the use of an ionic liquid, even in combination with subsequent enzymatic hydrolysis in the case of *Chlorella* cells, did not give a result that would preserve any hopes for further prospects for the development of such an approach. At the same time, the combination of enzymes and conditions of its use, which we selected empirically, based on the results of the analysis of the biochemical composition of microalgae cells (Table 4) and known data on the structure of typical biopolymers that are involved in the cell wall structure of *Chlorella* cells [47], allowed us to obtain results with indicators confirming the high efficiency of the disintegration of the biomass. In these experiments, varying the concentration of *Chlorella* biomass (20–100 g dry cells weight (DCW)/L), which was successively subjected to both mechanical and enzymatic treatment, made it possible to obtain media with different concentrations of RS and GLU and to use them for further cultivation of *C. necator* cells and the accumulation of PHA in bacterial biomass (Figure 1). This polymer accumulation was conducted using pre-grown *C. necator* cells, because the cell growth and PHA accumulation are usually divided into two stages of bacterial cultivation.

Table 4. Characteristics of the PHA accumulation by *C. necator* cells in media with hydrolyzed *C. vulgaris* biomass used in different initial concentrations for disintegration.

Initial Concentration of *C. vulgaris* Biomass, Used for Mechanical–Enzymatic Disintegration, g DCW/L	**30**	**50**	**70**
Duration of process, h	10	15	20
Intracellular PHA content, %	51 ± 1.3	60 ± 1.8	59 ± 1.7
Average rate of cell biomass accumulation, $Q_{CB,}$ mg DCW/L/h	396 ± 10	440 ± 13	320 ± 9
Average rate of PHA accumulation, $Q_{PHA,}$ mg/L/h	373 ±10	405 ± 12	294 ± 8

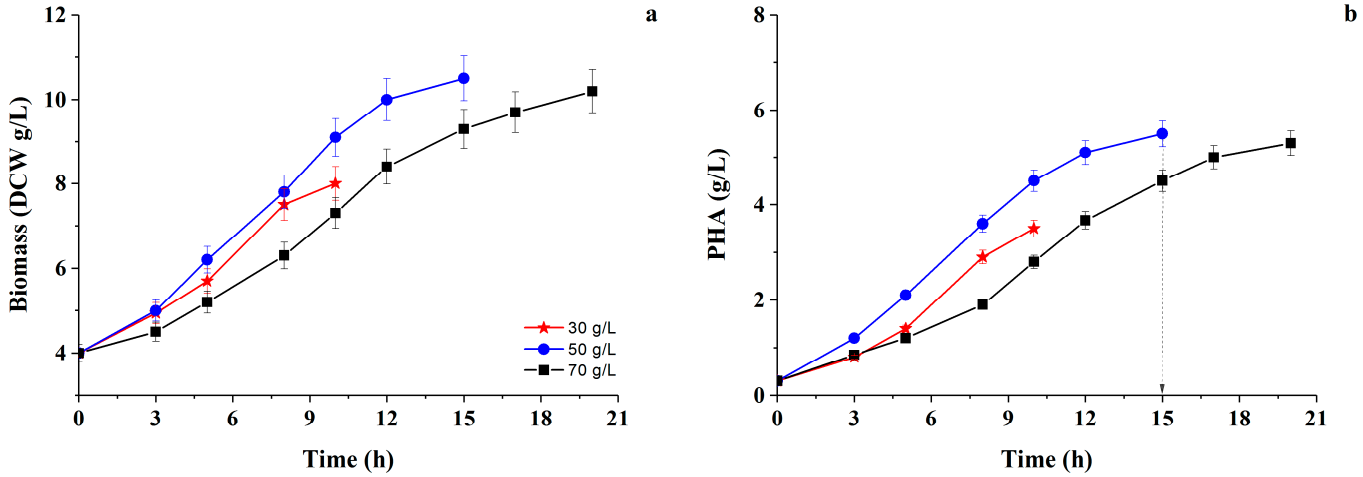

Figure 1. Biomass increase (**a**) observed due to PHA accumulation (**b**) in *C. necator* cells during their cultivation in the medium containing enzymatic hydrolysates of *C. vulgaris* biomass taken for the disintegration in various initial concentrations (C_{DCW}): 30 (★), 50 (●), 70 (■) (g dry cell weight of biomass/L).

The medium obtained with the lowest concentration of microalgae biomass (20 g DCW/L) was not already introduced in this investigation, whereas the results were obtained using media that were formed during mechanical–enzymatic processing of *Chlorella* biomass taken at concentrations of 30, 50, and 100 g DCW/L. The data obtained allowed us to conclude that, with an increase in the hydrolysates of the initially injected *C. vulgaris* biomass from 30 to 50 g DCW/L, an increase in the total concentration of PHA by 1.5 times in the biomass of *C. necator* cells was revealed. An increase in the specific concentration of the accumulating polymer in bacterial cells (Table 4) and the specific rate of PHA accumulation was observed with an increase in the hydrolyzed biomass of *C. vulgaris* from 30 to 50 g DCW/L in the medium.

The use of the hydrolysate obtained by the treatment of 100 g DCW/L microalgae biomass for its disintegration did not lead to an increase in the expected concentration of PHA in cells and the rate of polymer accumulation in *C. necator* cells. Conversely, this was accompanied by the appearance of a 3–4 h lag phase at the beginning of *C. necator* cell cultivation, indicating a clear inhibitory effect on the bacterial producer of some components that were present in this hydrolysate used.

Thus, the introduction of hydrolysate obtained from the biomass of *C. vulgaris* microalgae taken for mechanical–enzymatic disintegration at an initial concentration of 50 g DCW/L proved to be the most expedient in the process under study.

2.2. Biosorption of Microalgae Cells by the Spent Fungal Mycelium, Its Characterization, and Disintegration

For the sorption of microalgae cells, immobilized mycelial fungi were used in this work after their application in the corresponding processes: accumulation of fumaric acid [2], purification of food wastewater [44], and the production of hydrolytic enzymes [45]. At the same time, two of the three samples of fungal mycelium were formed by cells from the different strains of filamentous fungi of the same genus *Rhisopus*, and the third sample was presented by *Aspergillus* cells (Figure 2).

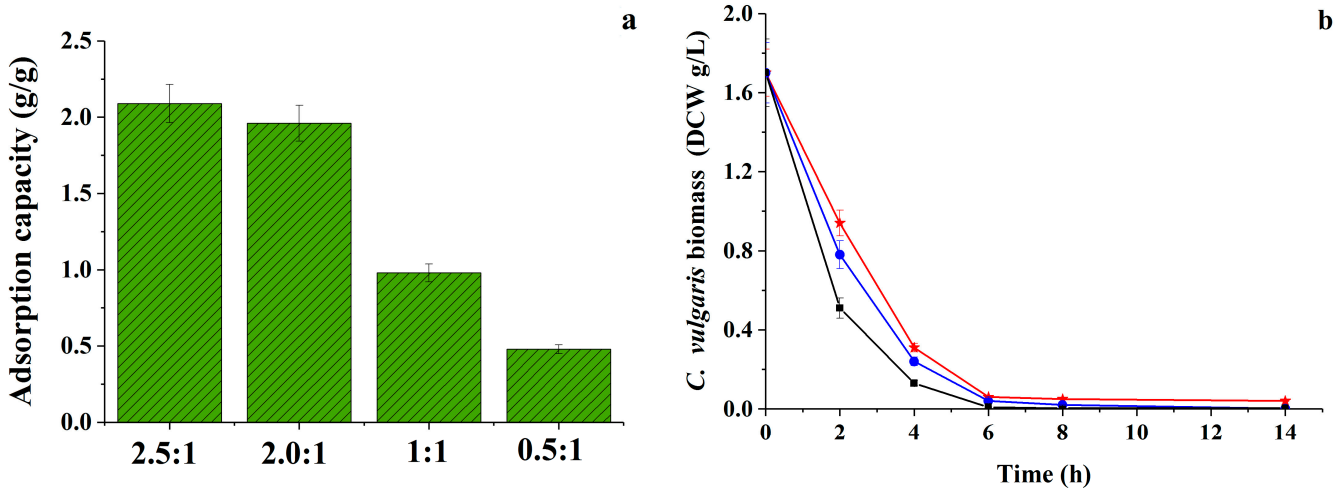

Figure 2. (**a**) Adsorption capacity of *R. oryzae* F1032 depending on the mass ratio M/F between the *C. vulgaris* C-1 and *R. oryzae* biomasses. (**b**) Concentration of *C. vulgaris* C-1 biomass in the medium during cell sorption by *R. oryzae* F1032 (■), *A. terreus* F728 (■) and *R. oryzae* F814 (★) cells, when the ratio M/F was 2:1.

It should be noted here that the use of "spent" free fungal mycelium in the processes of fumaric acid production for the biosorption of microalgae cells was practically impossible due to the fact that, by the end of the process of acid obtaining from various renewable sources of raw materials, lysis and partial destruction of non-immobilized fungal mycelium was observed. In this regard, for an adequate comparison of different fungi, the immobilized forms of all types of fungal mycelial cells were used. To do this, a suspension of the biomass of whole *Chlorella* cells was introduced into the contact with fungus pellets for their joint exposition, and both the decrease of concentration of microalgae cells in the exposure medium and the change in the weight of mycelial biomass with sorbed microalgae were monitored (Figure 2).

Initially, using the example of only one fungus strain *R. oryzae* F1032, an appropriate mass ratio (M/F) between biomasses of the microalgae cells of the fungi, which can be used in the sorption process, was evaluated. It was shown that the ratio of 2:1 can be considered the best (Figure 2a) because a further increase in the sorption capacity of 1 g of fungal cells was not obtained. Since then, the ratio was used in further experiments to study the sorption of *Chlorella* cells on other types of mycelium (Figure 2b). The main process of the sorption of *C. vulgaris* microalgae cells was actually completed within 6 h for all the studied media variants.

An estimation of the sorption capacity of immobilized mycelium after 14 h of the process showed that the most effective use for these purposes was the usage of pellets of the filamentous fungi *R. oryzae* F1032 (after use in fumaric acid production), which may have been due to the individual characteristics of its mycelium. However, the general difference in the sorption capacity of mycelium between different fungus cells did not exceed 3% by 6 h. The photos of *Chlorella* suspension and pellets of fungus *R. oryzae* F814 cells before and after sorption of microalgae cells are shown in Figure 3.

It should be noted that empty granules (without mycelial fungus cells) of poly(vinyl alcohol) (PVA) cryogel itself, which was initially used as support to obtain immobilized fungal spores and subsequent mycelium germination of all three used variants of fungi, are capable of sorbing *C. vulgaris* algae cells from their suspension; however, the sorption capacity in this case was caused solely by the mycelium itself, the dense packing of which in the volume of PVA cryogel granules was repeatedly demonstrated earlier [48].

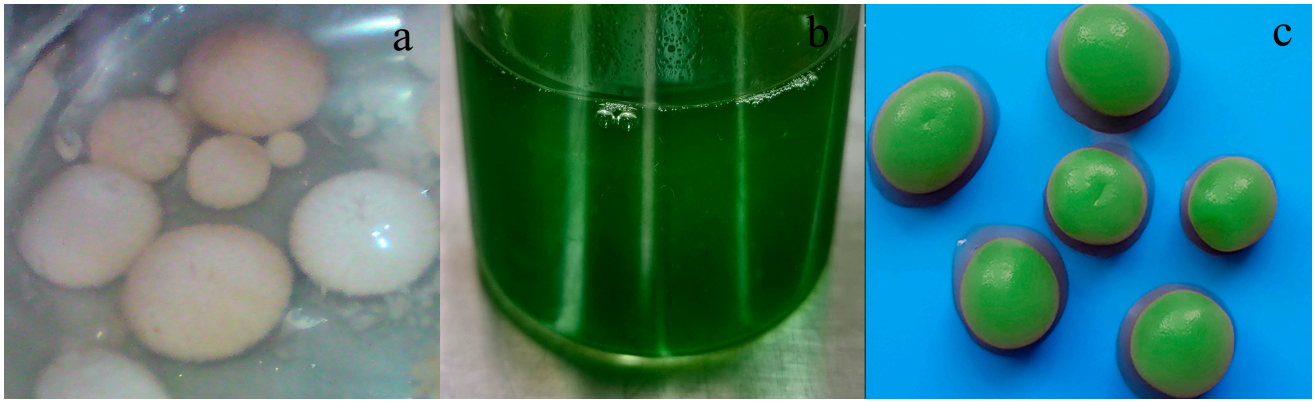

Figure 3. Pellets of spent immobilized fungal *Rhizopus* F814 (**a**) cells and suspended *C. vulgaris* C-1 (**b**) cells before and after sorption of the microalgae on mycelium of the filamentous fungi (**c**).

Before using the obtained three samples of mixed biomass in disintegration to prepare the media necessary for the biosynthesis of PHA, their biochemical analysis was performed and the total concentrations of proteins, lipids, and carbohydrates were determined (Table 5). It was obvious that such mixed biomass samples differed greatly in their characteristics from the biochemical composition of *Chlorella* itself and between each other, and then it was interesting to see the possible effect of such changes on the biosynthesis of PHA.

Table 5. Biochemical characteristics of various individual and mixed types of biomass.

* Type of Biomass	Lipids, %	Proteins, %	Carbohydrates, %
Biomass of individual cultures			
Biomass of *C. vulgaris* cells	17.1 ± 0.9	9.9 ± 0.5	55.5 ± 2.5
Immobilized mycelium of *R. oryzae* F1032 after fumaric acid production	8.0 ± 0.8	22.1 ± 1.1	46.8 ± 2.3
Immobilized mycelium of *R. oryzae* F1814 after food wastewater treatment	46.1 ± 2.3	16.1 ± 0.8	24.1 ± 1.2
Immobilized mycelium of *A. terreus* F728 after enzyme production	5.1 ± 0.2	37.2 ± 1.8	39.7 ± 1.9
Mixed biomass			
Immobilized mycelium of *R. oryzae* F1032 with sorbed biomass of *C. vulgaris*	13.8 ± 0.6	13.6 ± 0.6	52.3 ± 2.6
Immobilized mycelium of *R. oryzae* F814 after food wastewater treatment with sorbed biomass of *C. vulgaris*	25.7 ± 1.2	10.7 ± 0.5	44.7 ± 2.2
Immobilized mycelium of *A. terreus* F728 fungi after enzyme production with sorbed biomass of *C. vulgaris*	12.5 ± 0.6	18.4 ± 0.9	49.7 ± 1.9

* The biomass was after mechanical disintegration of biomass (4 min) with further enzymatic hydrolysis (20 h, 37 °C, 200 rpm, pH 5.5) catalyzed by the mixture of G (6 mg/g dry biomass), A (3 mg/g dry biomass), L (3 mg/g dry biomass) and P (3 mg/g dry biomass), where G—Glucanex, A—α-Amylase, L—Lipase, P—Protease; Ratio M/F = 2:1.

It should be noted that to analyze the content of mixed biomass samples and to prepare media for the production of PHA based on these biomasses, the disintegration of obtained variants of fungus mycelium with sorbed microalgae cells was required. It was carried out taking into account the previously obtained results, when the evaluation of treatments of *Chlorella* cell biomass by various methods was undertaken and where the mechanical–enzymatic method turned out to be the most successful among others (Table 3). For the treatment of mixed biomasses, we experimentally selected enzymatic combinations and doses of the active hydrolytic enzymes in these combinations, which allowed us to obtain

RS and GLU yields (Table 6) comparable to those achieved for one *Chlorella* biomass when its concentration used for the treatment was 50 g DCW/L (Table 3).

Table 6. Results of mechanical–enzymatic treatment of mixed biomass (C_{DWB} = 50 g DCW/L) containing *C. vulgaris* C-1 microalgae cells, sorbed during 6 h on spent immobilized mycelium of various filamentous fungi (M/F = 2:1).

Medium with Biomass Content	* C_{RS}, g/L	C_{GLU}, g/L	Q_{RS}, g/L/h	Q_{GLU}, g/L/h	COD, g/L
Immobilized *R. oryzae* F1032 after the fumaric acid production with sorbed biomass of *C. vulgaris*	21.91 ± 1.09	13.02 ± 0.65	1.10 ± 0.05	0.65 ± 0.03	55.3 ± 2.7
Immobilized *R. oryzae* F814 after use in food wastewater treatment with sorbed *C. vulgaris* cells	17.59 ± 0.87	8.79 ± 0.43	0.88 ± 0.04	0.44 ± 0.02	70.2 ± 3.51
Immobilized *A. terreus* F728 fungi after production of enzymes with sorbed *C. vulgaris* cells	19.56 ± 0.97	9.27 ± 0.46	0.98 ± 0.04	0.46 ± 0.02	53.4 ± 2.67

* Characteristics were detected after treatment under conditions mentioned in footer of Table 5. C_{RS}, C_{GLU} and Q_{RS}, Q_{GLU} are the concentrations (C) and average rates of accumulation (Q) of reducing sugars (RS) and glucose (GLU), respectively, determined in the soluble part of reaction media with treated biomass.

The analysis of the obtained information about the efficiency of disintegration of the three samples of treated mixed biomass revealed the "champion" among them, which appeared to be *R. oryzae* F814 with sorbed *Chlorella* cells. To disclose this leader, the chemical oxygen demand (COD) was additionally determined for all prepared samples (Table 6).

Due to the presence of lipids in twice higher concentrations in the mixed biomass of the "champion" as compared to other samples, the COD value in this variant was significantly higher. Since it is known (Table 1) that *C. necator* can transform both sugars and fatty acids to PHA, so directly the disintegrated sample of the "champion" with the highest COD value was further used for the cultivation of *C. necator* and biosynthesis of PHA.

2.3. Biosynthesis of PHA from the Mixed Biomass and Characterization of the Polymer

Further, the biosynthesis and accumulation of PHA in the *C. necator* cells were conducted using a hydrolysate of *R. oryzae* F814 cells with sorbed *C. vulgaris* cells for 15 h (Figure 4).

The following characteristics of the process were obtained: intracellular PHA content–58%, average rate of biomass increase (Q_C)–476 ± 8 mg DCW/L/h, average rate of PHA accumulation (Q_{PHA})–440 ± 13 mg/L/h.

Comparison of these results with those previously obtained for the biomass of single *Chlorella* under the same conditions (accumulation of PHA for 15 h in medium with hydrolysate of 50 g/L microalgae biomass) (Table 4) showed that the usage of mixed biomass gave a slightly better effect.

The characterization of PHA isolated from the bacterial cells after their cultivation in the medium with disintegrated mixed biomass of the "champion" was undertaken. Analyzing obtained the FTIR spectrum of the polymer (Figure 5a); it was revealed that mostly poly(hydroxybutirates) (PHB) were synthesized by *C. necator* under the investigated conditions. Additionally, the chemical structure of PHA extracted from the bacterial cells was also confirmed as PHB by 1H NMR analysis (Figure 5b).

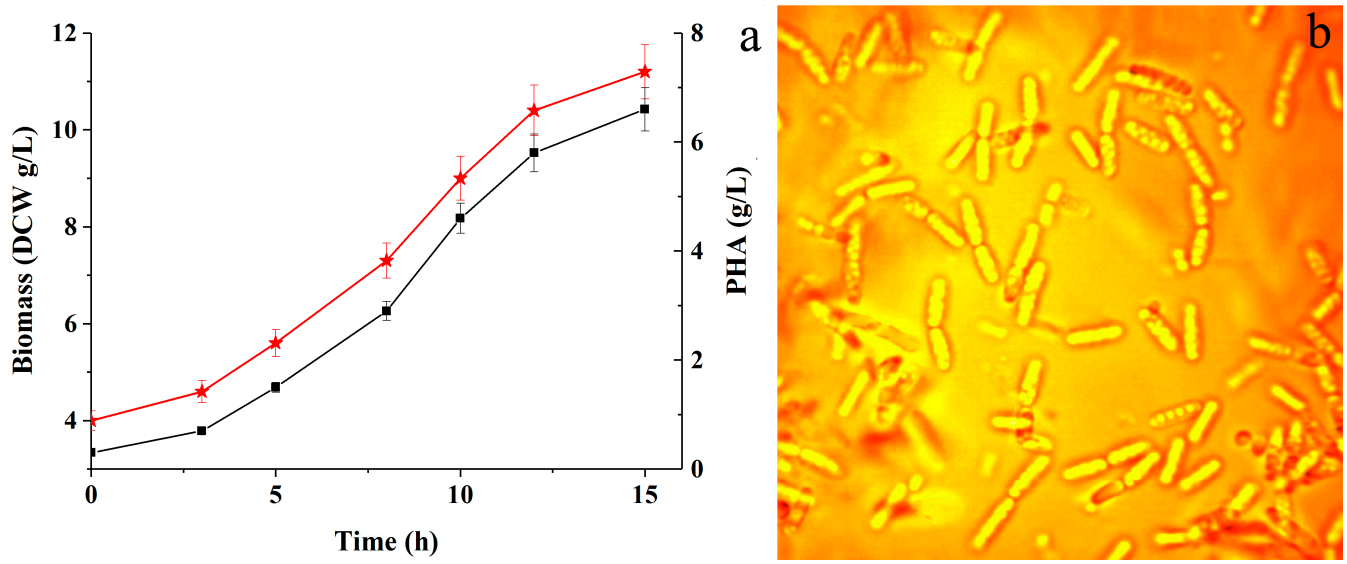

Figure 4. (**a**) Biomass increase of *C. necator* cells (★) and PHA accumulation (■) in the bacteria during their cultivation in the medium containing mechanical–enzymatic disintegrate of mixed biomass initially containing *C. vulgaris* and fungi *R. oryzae* F814 (50 g/L). (**b**) Optical microscopic image of *C. necator* cells containing polymer accumulated in the medium with the mixed biomass.

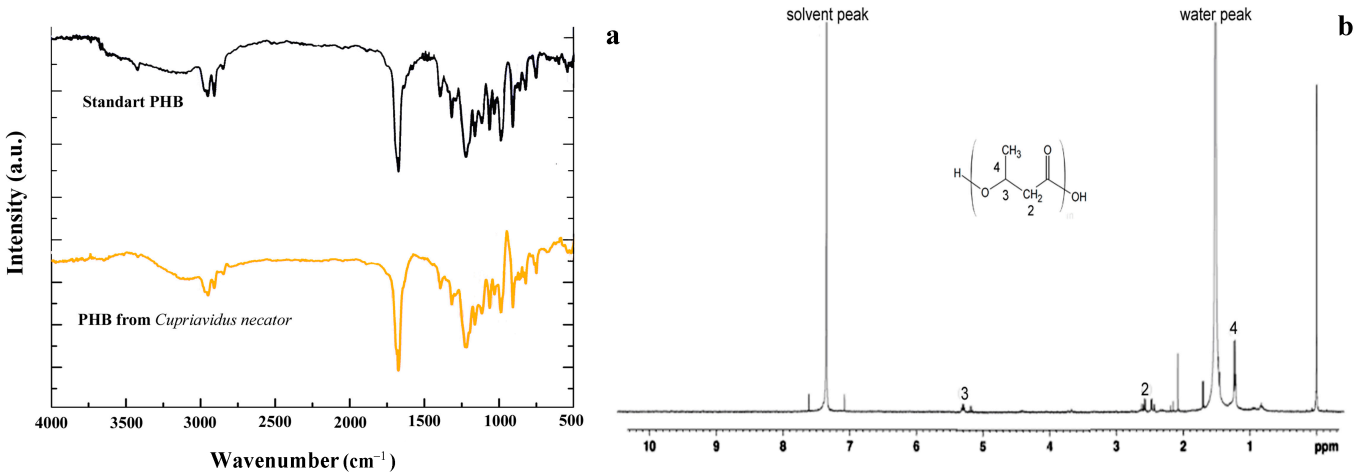

Figure 5. (**a**) FTIR spectra of commercial PHB used as reference standard and of PHB produced by *C. necator* in the medium with enzymatic hydrolysate of mixed biomass (*C. vulgaris* sorbed by the *R. oryzae* F814 mycelium). (**b**) 1H NMR spectra of extracted PHB sample from *C. necator* cultivated in the medium with enzymatic hydrolysate of mixed biomass of *R. oryzae* F814 mycelium with sorbed biomass of *C. vulgaris*. The signal showed CH_3 proton at δ 1.3 ppm, CH_2 proton at δ (2.3–2.6) ppm, and CH proton at δ 5.3 ppm.

3. Discussion

PHA-based bioplastics are biocompatible and biodegradable materials, so interest in them is continuously increasing. The synthesis of polymer materials in the form of PHA is carried out by the biotechnological method directly in the cells of microorganisms after they have already grown. In fact, cells synthesizing PHA thus accumulate reserves of carbon source in their biomass as the main nutrient. The study of the possibility of involving the waste of various biomasses in the processes of PHA obtaining [13–28] seems relevant (Table 1). One such waste, allowing both *Chlorella* biomass and filamentous fungi biomass accumulated during the treatment of different wastewaters to be processed into a commercially significant product, was the mixed biomass of these microorganisms, which could be obtained by the sorption of microalgae on the mycelia of the fungi. The possibility

of such sorption was known prior to this work (Table 2), and the results of the study only expanded the range of fungi [32–42] that can be used to harvest *Chlorella* cells and confirmed the probability of using spent fungal cells being in an immobilized form.

The difference between this work and the previously published results [8,28] is that *Chlorella* cells were not fractionated before their involvement in the disintegration process and lipids were not separated, but whole cells containing lipids, carbohydrates, and proteins were used. Since it is known that not only carbohydrates but also lipids can be converted by *C. necator* bacteria into PHA [14–17], it was expected that in the studied process, thus, it would be possible to increase the concentration of the polymer accumulated in the cells of the producer in comparison with the data known from the literature. It was interesting to compare the rate of polymer accumulation in *C. necator* cells in a medium for the preparation of which whole *Chlorella* cell biomass was used (Table 4) with the rate that was shown for defatted biomass and other substrates (Table 1). The interest was satisfied; moreover, comparing the results obtained on the basis of even mixed media from biomass with previously known ones (Table 1), it became clear that a significant acceleration of polymer accumulation in *C. necator* cells is also possible.

Key data were identified (Table 3), allowing for a comprehensive comparative analysis of the main results from the point of view of obtaining hydrolysates with a maximum concentration of RS and GLU from the carbohydrate components of the biomass of the used *C. vulgaris* microalgae. Some of the indicators allowed us to evaluate the effectiveness of processes from a kinetic point of view by comparing the values of Q_{RS} and Q_{GLU} obtained with different variants of cell processing. From the point of view of the maximum hydrolysis rate, minimizing time costs, and obtaining sufficiently high yields of RS, the best results corresponded to acid hydrolysis (with simultaneous thermolysis) of polysaccharides of *C. vulgaris* biomass. The maximum values of the main parameters were achieved with the preliminary mechanical destruction of cells in many variants of further treatment. With preliminary mechanical disintegration (within 4 min), the duration of the biomass hydrolysis process was reduced, and the final concentrations of RS increased. However, acid hydrolysis is not without drawbacks, since acids as catalysts can simultaneously participate in the reaction of the RS formation and the reactions of their subsequent transformation into products such as furfural, oxymethylfurfural, etc. [49,50]. It is known that these substances have an inhibitory effect on microorganisms and thereby can reduce the effectiveness of the subsequent transformation of sugars into target products, including PHA.

Discussing the reasons for the use of biosorption of microalgae cells on fungal mycelium as a method for harvesting microalgae biomass, it should be noted that the size of *Chlorella* cells is quite small (usually in the range of 2–10 μm), and they do not perform sedimentation very well, so it is not easy to collect them technologically. The cost of this process is 20–30% of the total cost of biomass production [51]. Microalgae cells sorbed on the mycelium of filamentous fungi can be easily separated from the medium, significantly simplifying, speeding up and reducing the cost of collecting free *Chlorella* cells.

The comparison of the results obtained (Table 4) with the data available in the literature (Table 1) for the processes of PHA accumulation in *C. necator* cells, when using various hydrolysates and waste products as feedstock, allowed us to conclude that hydrolysates of *C. vulgaris* microalgae biomass may be of commercial interest for the production of biodegradable polymers. The analysis of the obtained characteristics (Table 4) showed that the enzymatic hydrolysis of *Chlorella* biomass with or without mixing with the biomass of filamentous fungi is a worthy alternative to acid hydrolysis, despite the fact that enzymatic catalysis requires more time for processing biomass (Table 3); however, it is carried out without elevated temperature and pressure and provides a faster accumulation of PHA in *C. necator* cells in the case of the use of enzymatic hydrolysates of raw materials.

The composition of the enzyme complex selected by us for the hydrolysis of mechanically pretreated *Chlorella* biomass and mixed biomass has shown good results in the disintegration of raw materials in this work and can be taken into account by other researchers conducting investigations in this field.

The solutions investigated in this article for the PHA production process can have a significant positive impact on the environment: due to the implementation of an integrated approach, all raw materials used to produce PHA (microalgae and fungal biomass) are not only renewable but also associated with waste recycling and their involvement in deep processing. During their growth, microalgae cells metabolized both carbon dioxide, reducing its content in the air (decarburization), and utilized the organic components from the applied medium (wastewater). Spent biocatalysts of biotechnological processes, which are subject to disposal, were applied as the fungal biomass in this study. The rational use of these types of biomass in the form of mixed waste materials for the production of biodegradable plastics (PHA), which is a commercial product with a significantly higher cost than the raw materials, is undoubtedly a significant step towards forming the foundation for an environmentally beneficial process. The potential costs of energy and raw materials for obtaining such raw materials within the production of PHA actually are zero (wastes). Unlike cellulose-containing substrates, both microalgae and fungal biomass do not contain lignin and do not require complex sample preparation before biotransformation, which also saves resources, and there is no lignin at the end of the process, which further treatment, as well as phenol residue coming from the waste, raise new problems. PHA is a promising biodegradable commercially significant polymer that, after targeted application, does not cause environmental problems, such as contamination of soil, water, and air with microplastics.

4. Materials and Methods

4.1. Microorganisms and Their Cultivation

The work used cells of the green microalgae *Chlorella vulgaris* C-1, obtained from the Collection of microalgae and cyanobacteria IP RAS (Moscow, Russia, https://ippras.ru/, accessed on 25 November 2022); strains of mycelial fungi *Rhizopus oryzae* F1032, *R. oryzae* F814, and *Aspergillus terreus* F728; and bacteria *Cupriavidus necator* B8619 obtained from All-Russian Collection of Industrial Microorganisms (Moscow, Russia, https://vkpm.genetika.ru/, accessed on 25 November 2022).

To accumulate the biomass of free microalgae cells of *C. vulgaris*, an immobilized inoculum of this culture was used, the cultivation of which was carried out in wastewater from a milk processing plant (OOO Ostankinsky Molochny Kombinat, Moscow, Russia) under the previously described conditions [30]. To determine the concentration of microalgae biomass in the medium, a calibration graph of the dependence of the concentration of *C. vulgaris* cells on OD_{540} was used.

The biomass of immobilized mycelial fungal cells for the sorption of chlorella cells was obtained after their application in the corresponding processes: *R. oryzae* F1032 cells after accumulation of fumaric acid [2], *R. oryzae* F814 cells after wastewater treatment [44], and *A. terreus* F728 cells after use for the production of hydrolytic enzymes [45].

Biosorption of *C. vulgaris* microalgae cells from the medium was carried out by immobilized mycelial fungal cells repeatedly used in target processes. The process was carried out at 22 °C under aerobic conditions with constant stirring (100 rpm) for 14 h.

The accumulation of *C. necator* bacterial cells was carried out on a medium of the following composition: glucose–20, $(NH_4)_2SO_4$–4, KH_2PO_4–13.3, $MgSO_4 \times 7H_2O$–1.2, citric acid–1.7, a solution of trace elements–10 mL. The solution of trace elements contains (g/L in 0.1 M HCl): $FeSO_4 \times 7H_2O$–10, $ZnSO_4 \times 7H_2O$–2.25, $CuSO_4 \times 5H_2O$–1.0, $MnSO_4 \times 4H_2O$–0.5, $CaCl_2 \times 2H_2O$–2.0, H_3BO_4–0.3, and $(NH_4)_6Mo_7O_{24}$–0.1. Cultivation was carried out under aerobic conditions by constant stirring (180 rpm) at 28 °C. To maintain a pH of 7.0 ± 0.2, a 2 M KOH solution was used. Further, such biomass was used to accumulate PHA in media based on the hydrolysates of *Chlorella* biomass and three types of mixed biomass.

4.2. Disintegration of Biomass and Its Use for PHA Biosynthesis

The main conditions used for biomass disintegration are presented in Table 3 and in the footer of Table 5.

For acid hydrolysis, *C. vulgaris* microalgae biomass was used after its separation from the cultivation medium by centrifugation (8000 rpm, 10 min). The precipitate was suspended in solutions of H_2SO_4 or HCl, then the resulting suspensions were subjected to thermolysis. Thermolysis of *C. vulgaris* biomass was carried out in a 50 mM Na-acetate buffer (pH 5.5) at 108 or 121 °C and a pressure of 0.5 or 1 atm, respectively, as mentioned in Table 3. At the end of acid hydrolysis, the pH was neutralized with 2.5 M NaOH solution, and samples were taken from the reaction mixture to determine the concentration of RS and GLU.

The preliminary mechanical disintegration of *C. vulgaris* biomass was carried out in a Mini-BeadBeater-24 ball mill (glass beads size 0.5 mm, rotor speed 3000 rpm).

The degree of disintegration of biomass was estimated by a microbiological method and calculated as a percentage of the difference in the number of whole cells in a sample of a certain volume before and after disintegration for a certain period of time to the initial number of cells. The biomass of *C. vulgaris* thus disintegrated was further subjected to acidic or enzymatic hydrolysis.

Enzymatic hydrolysis of microalgae *C. vulgaris* biomass was carried out in 50 mM Na-acetate buffer (pH 5.5). The following enzymatic preparations were used in the work: Glucanex, α-Amylase, Lipase of *Rhizopus niveus* Protease of *Rhizopus* sp., and Cellulases of *Trichoderma viride* from Sigma (St. Louis, MO, USA).

Pretreatment of *C. vulgaris* cells using [Bmim]Cl was carried out by adding ionic liquid to the suspension of microalgae biomass so that the mass content of this biomass in the mixture with [Bmim]Cl was 4% by dry cell weight. After the treatment, [Bmim]Cl was removed as follows: the working mixture was resuspended in distilled water and subjected to centrifugation (10,000 rpm, 3 min), after which a repeated procedure was carried out with the sediment until the concentration of [Bmim]Cl in the washing waters was no more than 0.2 g/L (the concentration of [Bmim]Cl was determined spectrophotometrically by the optical density of the solution at a wavelength of 210 nm).

The transformation of enzymatic hydrolysates of microalgae and mixed biomass into PHA under the action of *C. necator* bacterial cells was carried out at 28 °C under aerobic conditions with constant stirring (180 rpm) for 10–20 h. The initial concentration of bacterial *C. necator* cells was 4 g DCW/L (Figures 1 and 4).

Extraction of PHA from *C. necator* biomass included the following stages [52]: extraction of PHA using $CHCl_3$ by stirring on a thermoshaker at 37 °C for 12 h; separation of PHA solution from cellular disintegrate by filtration; separation of PHA from $CHCl_3$ solution by precipitation with isopropyl alcohol; subsequent multiple dissolution of PHA in $CHCl_3$ and precipitation with isopropyl alcohol; drying at 60 °C to a constant weight.

4.3. Analytical Methods

Glucose concentration was controlled by using the standard Impact reagent kit (Impact, Moscow, Russia). The Shomodyi–Nelson method was applied to determine the concentration of RS [46]. To determine the DCW, the previously described method was used [28]. Potentiometric measurements were conducted using a Corning Pinnacle 530 pH meter (Corning Incorporated, Corning, NY, USA).

The biochemical composition analysis of microbial biomass was done using published techniques [3]. The COD concentration was determined by the standard bichromate method described in [53].

Optical microscopic analysis of *C. necator* cells was performed using a Bioptic CS-200 microscope with digital camera (Biomed-Service, Moscow, Russia).

Characterization of PHA was conducted in terms of Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR) on PHA isolated from the bacterial cells. FTIR spectrum was analyzed using Tenzor 27 spectrometer (Bruker, Billerica, MA, USA). The FTIR spectrum was recorded in the range of 4000 to 500 cm^{-1}.

For determination of the organic functional groups of PHB samples, the 1H NMR analysis was used. A sample of PHB (20 mg) was dissolved in deuterated chloroform (1 mL) and analyzed using a Bruker AVANCE 400 spectrometer (Bruker, Billerica, MA, USA).

4.4. Calculation of Parameters

Average rates of accumulation of reducing sugars (Q_{RS}), glucose (Q_{GLU}), cell biomass (Q_C), and PHA (Q_{PHA}) (mg DCW/L/h) were generally calculated in the same way: $Q_I = P/t$, where P is concentration (mg/L) of certain controlled parameter I (RS, GLU, CB, or PHA) in time of moment t (h).

Harvesting efficiency was calculated as the ratio of the difference between the initial and final concentrations of microalgae biomass in the medium to the initial concentration of biomass, expressed as a percentage.

The results performed are obtained as the mean values of at least three independent experiments ± standard deviation (±SD). SigmaPlot (ver. 12.5, Systat Software Inc., San Jose, CA, USA) was used for the statistical analysis.

5. Conclusions

In this work, for the first time, the possible effective biosorption of whole microalgae *C. vulgaris* cells on "a sorbent" in the form of immobilized mycelial fungal cells used in other processes and subject to further utilization was investigated. The production of mixed biomass and its mechanical–enzymatic processing allowed for obtaining a substrate that can be successfully used for the biosynthesis of PHA at a high rate. Such a process may be of interest not only to those who are looking for new substrates for their biocatalytic conversion into commercially significant products, solving the issues of biomass valorization, but also to those who develop new processes in solving environmental issues related to the need to utilize the spent biomass of mycelial fungi functioning as producers in various biotechnologies. The production of PHA is a well-known industrial process characterized by an increased cost, including the high cost of raw materials (food glucose obtained from starch or syngas) and the use of synthetic nutrient media. The approaches demonstrated in this work make it possible to significantly minimize the costs of raw materials and, at the same time, not only involve in processing but also rationally mix these wastes for their joint successful treatment. With the application of the results of this study, the modernization of current industrial systems for the production of PHA can be discussed based on scaling results in order to reduce the cost of polymer production, especially near those enterprises that use *Chlorella* biomass to clean their wastewater, including in the composition of active aerobic sludge.

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

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