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Original Research

Fungal Production of Fumaric Acid From Crude Glycerol: Different Approaches to the Process Including Intermediate Accumulation of Microalgal Biomass

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Abstract

Background: Interest in processing high-tonnage biodiesel production waste (crude glycerol and microalgal biomass after lipid extraction) into commercial products is steadily growing. Moreover, it is attractive to utilize these wastes for the microbiological production of fumaric acid (FA) using immobilized microorganisms, as it combines chemical and biological processes. **Methods**: The authors developed original approaches to FA production, which immobilize microalgal *Chlorella vulgaris* cells and the mycelium of the fungus *Rhyzopus oryzae*. Traditional techniques were employed for cultivating microorganisms, extracting lipids from accumulated microalgal biomass, and producing biomass hydrolysates through thermolysis under acidic conditions. The biochemical composition of *C. vulgaris* biomass obtained under different growth conditions (autotrophic, heterotrophic, and mixotrophic) was analyzed using standard methods. **Results**: The maximum accumulation of microalgal biomass was achieved under mixotrophic conditions using an inoculum immobilized in polyvinyl alcohol (PVA) cryogel in Tamiya medium supplemented with crude glycerol and bubbled with air containing 1% vol. CO_2. A total of 4.8 $\pm$ 0.2 g DW/L of free *C. vulgaris* biomass was accumulated in five batch cycles. Two types of *C. vulgaris* hydrolysates were obtained: hydrolysates from whole cell *C. vulgaris* biomass (ChVH1) and hydrolysates from *C. vulgaris* biomass waste after lipid extraction (ChVH2), respectively. In combination with crude glycerol (10–50 g/L), microalgal hydrolysates were transformed to FA using fungi *R. oryzae* immobilized in PVA cryogel. The highest concentration and yield of FA from the converted substrate were obtained when 20 g/L of crude glycerol, in combination with *C. vulgaris* hydrolysates, was administered alongside the fungi for 40 h. Multiple uses (five cycles, 200 h total) of immobilized fungi added to the medium with 20 g/L of crude glycerol and ChVH2 produced 186 g of FA. The average FA yield was 309 $\pm$ 12 mg/g substrate. **Conclusion**: A combined approach to producing FA by bioconversion of crude glycerol and hydrolysates of lipid-free microalgae biomass waste is of practical importance. These results expand the potential of bioscience in utilizing microorganisms in an immobilized form, contributing to the integration of environmental biotechnology into bioenergy production.

Keywords: *Chlorella vulgaris*; *Rhizopus oryzae*; polyvinyl alcohol; immobilized cells; hydrolysates; thermolysis; bioconversion; waste valorization; waste management

1. Introduction

Biochemical processes play a crucial role in the development of applied bioscience, biotechnology, and microbiology, enabling the production of valuable commercial products, such as proteins, antibiotics, enzymes, alcohols, and polysaccharides, and facilitating waste valorization in accordance with the principles of sustainable economic development [1,2,3]. The development of new approaches to realizing environmentally friendly chemical, biocatalytic, and hybrid processes is relevant and in demand [4,5,6].

Despite numerous promising studies in the field of biotechnological production of valuable products and waste processing [7,8], the development of integrated approaches combining renewable raw materials, waste, and resource-saving chemical and biological processes remains a topical area of research. Biotechnological and chemical processes related to biomass processing are inevitably accompanied by the formation of certain waste products that must be subsequently processed. Wastes from some processes can represent a promising source of raw materials for other processes in the complex. Therefore, the search for and implementation of such solutions can significantly expand the boundaries of modern applied biotechnology and chemical technologies.

Crude glycerol is the main multi-tonnage byproduct of biodiesel production from various types of biomass, including microalgae [9,10,11]. The accumulation of microalgal biomass is associated with photosynthetic CO_2 conversion, which does not require the use of territories with fertile soils, where crops of plant cultures with high nutritional value can be obtained instead. The biomass of microalgae is characterized by a high lipid content, which makes microalgae suitable for conversion into biodiesel [12,13]. The basic conditions for microalgal cultivation (maintaining temperatures above +18 °C, providing the necessary lighting,

and access to CO_2) can be easily met. Therefore, the organization of such a nature-like process seems attractive for implementation [14]. However, the greatest potential lies in the accumulation of microalgal biomass in various agro-industrial wastewaters [15] during their parallel treatment. This makes the processes involving microalgae especially attractive in terms of their environmental and economic impact.

The production of biodiesel from lipids extracted from microalgal biomass results in the accumulation of crude glycerol and lipid-free cell waste. Currently, scientific and technical tasks related to the subsequent processing of these wastes remain relevant [16]. The content of crude glycerol depends on the feedstock and the technology of biodiesel production (alkaline or enzymatic transesterification) [17]. Moreover, glycerol holds potential as a resource for the biosynthesis of essential cellular metabolites in various microorganisms [18]. Numerous studies have demonstrated that glycerol can be converted into high-value-added products (methane, hydrogen, ethanol, 1,3-propanediol, 2-phenylethanol, succinic acid, citric acid, propionic acid, lactic acid, lipids, and others) with or without minimal purification costs using whole-cell biocatalysts [10,19]. There has been an increase in research papers over the past 15 years that address the challenges of biodiesel production, the utilization of crude glycerol, and lipid-free microalgal wastes (Fig. 1).

Among the products that can be obtained from crude glycerol, fumaric acid (FA), a four-carbon dicarboxylic acid (trans-1,2-ethylenedicarboxylic acid), is of special interest. FA has a global market size of USD 660.9 million and is expected to grow at a rate of 5.5% annually from 2021 to 2026 [20]. FA is synthesized and secreted by some filamentous fungi, which can be used for processing various waste [21,22,23]. Moreover, up to 40.3 g/L of FA has been demonstrated to accumulate in a medium with *Rhizopus oryzae* under nitrogen-limited conditions, when the glucose concentration in the medium containing the fungus was 80 g/L [24]. Notably, to our knowledge, the introduction of complex mixed substrates, which are waste products from specific industrial processes, into the biocatalytic production of FA technology, as well as the integration of waste into closed production cycles, has not yet been studied.

Between 2010 and 2025, the annual number of papers on topics such as “Crude glycerol”, “Fumaric acid”, and “Microalgae” has increased by a factor of 4.5 (Fig. 1). The studies on “Crude glycerol” form the highest number of studies. However, a growth rate of only 2.6% was observed over a 10-year research period. Meanwhile, the number of papers on “Microalgae biorefinery” and “Lipid-free microalgae cell waste” increased by factors of 33 and 17, respectively, over the same period. This suggests that these topics remain highly relevant today, and many problems in this field remain unresolved.

The number of papers on FA production is increasing annually, while the total number of studies combining the topics “crude glycerol” and “FA” remains limited (Fig. 1). This is because FA production is currently based on chemical synthesis with conversion of butane from hydrocarbon raw materials into maleic anhydride and further through maleic acid [25].

Biotransformation of crude glycerol in combination with microalgae biomass hydrolysates (before and after lipid extraction) to produce FA has yet to be studied. However, this represents a promising area of research, and one that was chosen for this study. Crude glycerol was selected as the main object in this research because, on the one hand, crude glycerol can be a waste product of biodiesel manufacture from microalgal lipids [9] and, thus, be available in large quantities. Additionally, crude glycerol can be used as a raw material for other biotechnological processes, such as the accumulation of microalgal biomass and production of FA by the filamentous fungus *R. oryzae*.

This work focuses on developing the techniques for producing FA using crude glycerol and microalgal biomass of *Chlorella vulgaris* (whole cells and lipid-free cells) as the main substrates. Three different approaches were studied in the production of FA using immobilized microbial cells. The first (I) approach involved a single-stage bioconversion of crude glycerol using immobilized fungal mycelium of *R. oryzae*. The second (II) and third (III) approaches combined the bioconversion of crude glycerol with hydrolysates of microalgal biomass. The experimental scheme implemented in this work is shown in Fig. 2.

This work used the inoculum of microalgal cells and the fungal mycelium of *R. oryzae* in an immobilized form within polyvinyl alcohol (PVA) cryogel. Numerous studies have previously demonstrated the advantages of using PVA cryogel-immobilized live cells for bioconversion purposes compared to free cells [23,26,27,28]. PVA cryogel is a promising cellular carrier that can be successfully used to immobilize cells of various microorganisms [29]. Immobilization enables the capture of high concentrations of living cells in the carrier matrix, allowing for the reuse of cells without a loss in their stable target activity, easy separation from the spent medium, and transfer to a fresh reaction medium. Therefore, this study used the immobilized form of both the microalgal cells and the fungal mycelium of *R. oryzae*.

2. Materials and Methods

2.1 Materials

Green microalgal *Chlorella vulgaris* C-2 cells were obtained from the IPPAS Collection of Microalgae and Cyanobacteria of Institute of Plant Physiology RAS (Moscow, Russia, http://cellreg.org/catalog/, date of access — 01.06.2025). The Russian National Collection of Industrial Microorganisms (Moscow, Russia, http://eng.genetika

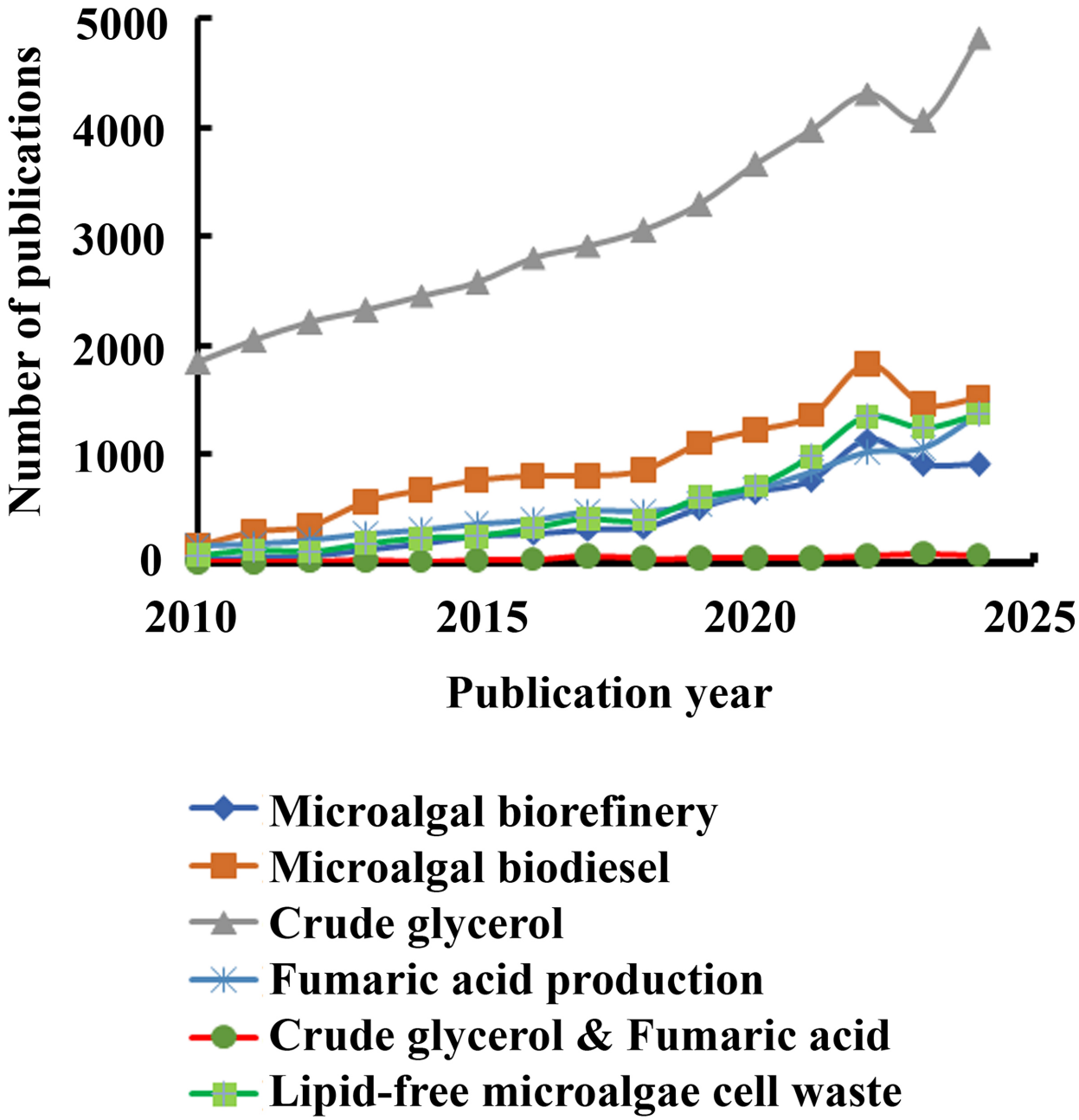

Fig. 1. Dynamics which is observed in publication activity of scientists according to Science Direct (www.sciencedirect.com, data of access — 02.07.2025). Publications corresponding to certain research areas developed in the period 2010–2025 are highlighted in different colors.

.ru/service-offer/vkpm/, date of access — 01.06.2025) supplied fungal *Rhizopus oryzae* F-1127 cells as a FA producer.

Crude glycerol was obtained as a byproduct of biodiesel production from the lipids of *C. vulgaris* biomass and was used in all the experiments. Crude glycerol was prepared as described earlier [30]. A solution of KOH in CH_3OH was used for lipid transesterification, and the reaction was performed at 60 °C for 10 minutes. The product was cooled in an ice bath and separated from the reaction medium by centrifugation at 5000 rpm for 5 minutes. Crude glycerol feedstock contained 81.9% glycerol, 6.5% ash, 0.6% methanol, and 11% water. Ash content was determined using a Nabertherm LT3/11 muffle furnace (Lilienthal, Germany) at 590 ± 10 °C for 24 h. Ash is expressed as a percentage of the weight difference before and after ashing.

PVA (type 16/1, 84 kDa) was purchased from Sinopec Corp. (Beijing, China).

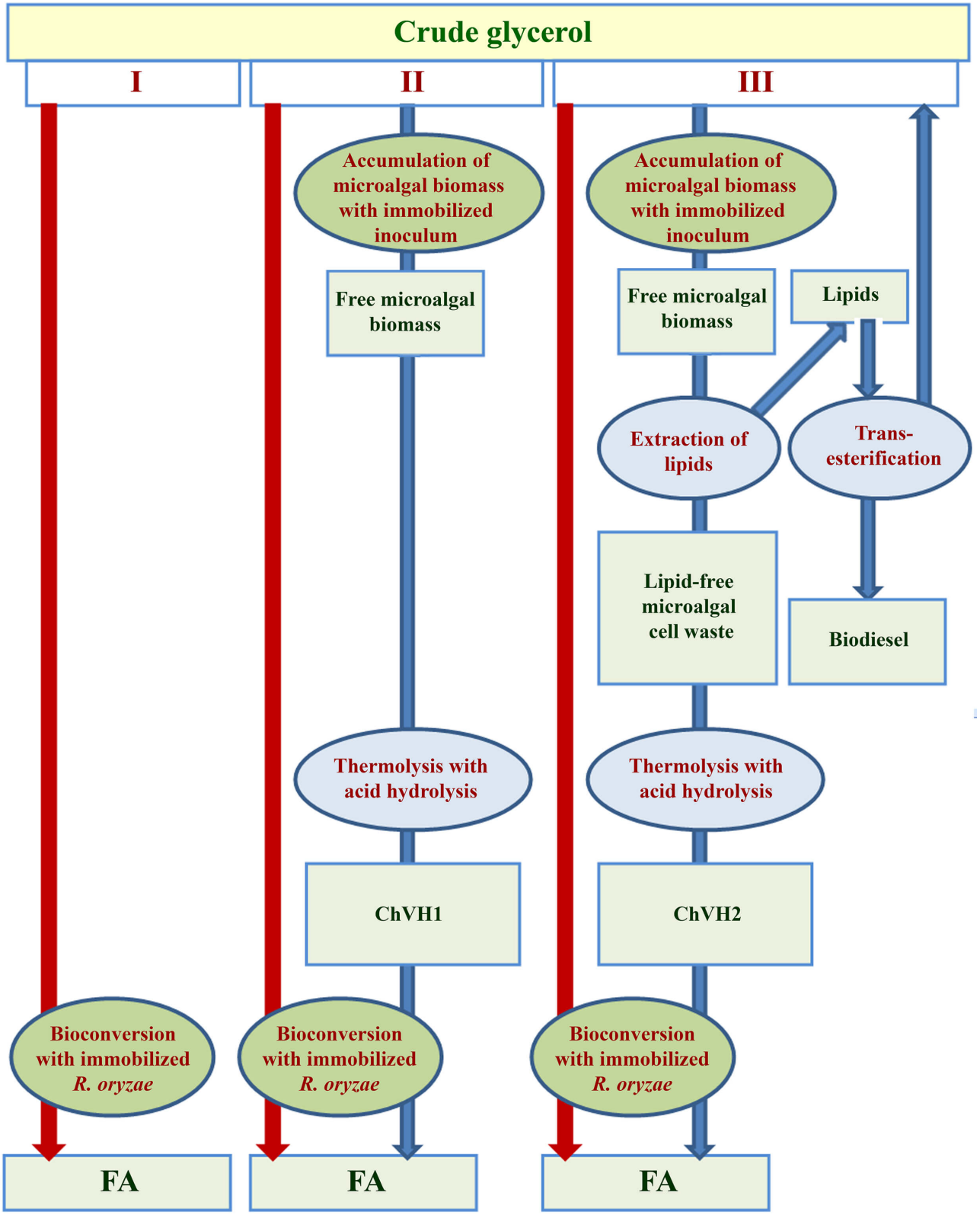

Fig. 2. The approaches to biosynthesis of FA by immobilized fungi *R. oryzae* from crude glycerol with or without hydrolysates of free microalgal biomass obtained from the use of immobilized *C. vulgaris* inoculum. Direct bioconversion of crude glycerol (I); bioconversion of crude glycerol with addition of hydrolysates from whole cell *C. vulgaris* biomass (II) or hydrolysate of the same *C. vulgaris* biomass waste after lipid extraction for biodiesel production (III). FA, fumaric acid; ChVH1, hydrolysates from whole cell *C. vulgaris* biomass; ChVH2, hydrolysates from *C. vulgaris* biomass waste after lipid extraction.

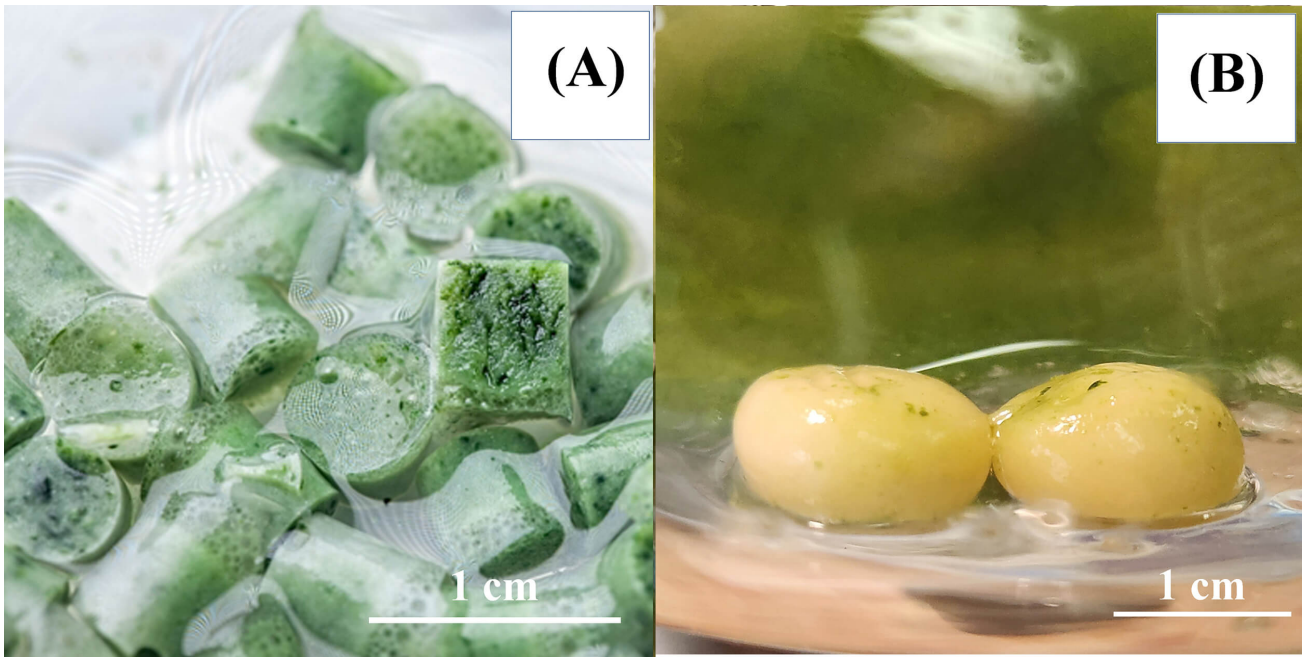

Fig. 3. Immobilized microorganisms. PVA cryogel granules with the immobilized *C. vulgaris* cells, which were used as inoculum for free microalgal biomass accumulation (A), and the mycelium of *R. oryzae* immobilized in the PVA cryogel granules in hydrolysate of free *C. vulgaris* biomass with the addition of crude glycerol for FA production (B). Scale bar = 1 cm. PVA, polyvinyl alcohol.

2.2 Immobilization of Microorganisms and Biomass Accumulation

The immobilization of cells into the PVA cryogel was achieved by incorporating microalgal biomass or fungal spores into the macroporous polymer matrix during its cryoformation, in accordance with a well-established technique [23,26,28].

The immobilized microalgal cells were used as inoculum for the accumulation of free cell biomass (Fig. 3A), and the immobilized fungal spores were cultivated for the growth of immobilized mycelium in and over the polymer matrix before their use in the FA production (Fig. 3B).

To obtain the immobilized cells, the microalgal biomass or fungal spores were thoroughly mixed with an aqueous PVA solution and pipetted into 96-well microplates. These plates were then placed in a freezer at –20 °C for 24 h, after which the thawing procedure was performed. A concentration of 4% was observed for the microalgae dry weight (DW) biomass in the immobilized samples [26]. Prior to freezing, the PVA solution contained a concentration of 10^7 spores/mL of fungal spores [31].

Immobilized microalgal cells were cultivated in a photobioreactor (Labfors 4 Lux, Infors AG, Bottmingen, Switzerland) with illumination (16 h-day/8 h-night mode) provided by Osram Fluora 77 luminescent lamps (30 W, Munich, Germany) to accumulate biomass at 24 ± 2 °C under autotrophic, heterotrophic and mixotrophic conditions.

Tamiya medium (g/L) was used as the basic mineral growth medium in all the studied variants: KNO_3, 5.00; $MgSO_4 \cdot 7H_2O$, 2.50; KH_2PO_4, 1.20; $FeSO_4 \cdot 7H_2O$, 0.03; 1 mL of solution (mg/L): H_3BO_3, 2.80; $MnCl_2 \cdot 4H_2O$, 1.80; $ZnSO_4$, 0.20 g; 1 mL of solution B (mg/L): MoO_3, 17.6; NH_4VO_3, 22.9, pH 7.0.

Air with 1%vol. CO_2 was constantly bubbled through a suspension of grown cells cultivated under autotrophic and mixotrophic conditions in the photobioreactor. In experiments conducted under heterotrophic and mixotrophic conditions, 5 g/L glycerol was added into the medium. The accumulation of free cell biomass was controlled by measuring the optical density of the cell suspensions at 540 nm (OD_{540}) using an Agilent UV-4853 spectrophotometer, Agilent Technologies, Waldbronn, Germany. *C. vulgaris* cells were harvested after cultivation for 72 h using centrifugation (centrifuge Avanti J 25 Beckman Coulter, Brea, CA, USA) at 8000 rpm for 10 min.

2.3 Preparation of Hydrolysates

Extraction of lipids from *C. vulgaris* biomass was conducted with a 2:1 chloroform–methanol mixture using a traditionally employed method [32]. The mass of extracted lipids was determined gravimetrically. For this purpose, the extractants were brought to a constant weight in a drying chamber at 50 °C.

Both whole cell and lipid-free *C. vulgaris* biomass samples were suspended in distilled water with H_2SO_4 (10^{-4} M) to achieve a concentration of 100 g DW/L. These suspensions were subjected to thermal treatment (thermolysis) at 121 °C for 0.5 h. Two types of *C. vulgaris* hydrolysates were obtained: hydrolysates from whole cell *C. vulgaris* biomass (ChVH1) and hydrolysates from *C.*

vulgaris biomass waste after lipid extraction (ChVH2). ChVH1 and ChVH2 were used as the bases of the culture media for fungal production of FA.

To obtain the immobilized fungal mycelium, granules of PVA cryogel with immobilized spores of *R. oryzae* were cultured in a liquid medium at 33 ± 1 °C under aerobic conditions for 50 h: Agitation rate of 150 rpm (thermostatically controlled Adolf Kuhner AG shaker, Basel, Switzerland) and the ratio of the liquid and gas phases in the bioreactor was 1:2. FA was further obtained under the same conditions due to the functioning of the produced immobilized mycelium (15 g DW/L). The duration of one cycle was 40 h based on the knowledge about the production of organic acids by a similar fungal culture [23].

The composition of the liquid medium for the growth of immobilized mycelium capable of producing FA was as follows (g/L): glycerol, 5; glucose, 50; yeast extract, 5; $(NH_4)_2SO_4$, 2.36; $MgSO_4 \cdot 7H_2O$, 0.2; $ZnSO_4 \cdot 7H_2O$, 0.07; $K_2HPO_4 \cdot 3H_2O$, 1.0.

To produce FA, a medium with the same salts but with an addition of glycerol (10–50 g/L) was implemented. For processes II and III (Fig. 3), the glycerol was added to the corresponding sample of *C. vulgaris* hydrolysates to produce FA using the immobilized fungal mycelium.

For the repeated use of immobilized microalgal and fungal cells in a periodic regime, granules with immobilized cells of each type (microalgae and fungi) were washed with a sterile 0.9% NaCl solution after each working cycle, and a new portion of the appropriate medium was loaded into the bioreactor.

2.4 Analytical Methods

The DW of the cells was determined using a standard gravimetric method, where the samples were dried at 105 °C to a constant weight.

The Shomody–Nelson method was applied to determine the concentration of the reducing sugars (RSs). The glucose concentration was determined using the standard impact reagent kit (Impact, Moscow, Russia). The concentration of FA was determined using a standard (colorimetric) assay kit (Abcam, Cambridge, UK).

Chemical oxygen demand (COD) was measured using a standardized method [33]. Potassium dichromate was used as the oxidizing agent, and glucose was used as the control oxidizable substrate in the spectrophotometric determination of COD (600 nm, Agilent UV-4853 spectrophotometer, Agilent Technologies, Waldbronn, Germany).

The concentration of glycerol was determined spectrophotometrically at 410 nm using a standard method with calibration curves [34].

To monitor the cell viability, a bioluminescent luciferin–luciferase method was used. This method enables the estimation of adenosine triphosphate (ATP) concentration using a standard ATP reagent (Biochimka, Moscow, Russia) based on recombinant firefly luciferase. Immobilized *R. oryzae* (0.10–0.15 g) was transferred to dimethyl sulfoxide (1 mL) and incubated at 25 °C for 1 h to extract intracellular ATP. Then, each sample was diluted with distilled water (1:10), and 50 µL of the resulting solution was placed in the cuvette of the 3560 microluminometer (New Horizons Diagnostics Co., Columbia, MD, USA) [3], containing 50 µL of the luciferin–luciferase reagent for registration of luminescence intensity. The ATP concentration was determined using the calibration curve plotted for standard ATP solutions (10^{-13}/10^{-7} M).

The biochemical composition of the *C. vulgaris* cell biomass (content of lipids, proteins, carbohydrates) was analyzed using the standardized methods described previously [35].

2.5 Calculations of Main Characteristics and Statistical Treatments of Results

The main characteristics of the investigated processes I, II, and III were calculated as follows (Fig. 2):

For (I): FA yield (mg/g substrate) = 1000 • FA (g/L)/glycerol (g/L);

For (II) or (III): FA yield (mg/g substrate) = 1000 • FA (g/L)/(glycerol (g/L) + 100 g/L), where 100g/L is *C. vulgaris* initial biomass concentration or lipid-free biomass concentration for *C. vulgaris* hydrolysates production.

The average yield of FA for five cycles (mg/g substrate) = $\sum_{i=1}^{5} FA_i$ yield for each cycle (mg/g substrate)/5.

Volumetric productivity (mg/(L • h)) = 1000 • FA (g/L)/40 h, where the duration of one cycle is 40 h.

Total amount of FA produced for five cycles (g) = $\sum_{i=1}^{5} (FA_i(\text{g/L}) \cdot 1\ \text{L})$, where 1 L is the volume of the medium used for one cycle.

To determine the standard deviation (±SD) of the results, data were obtained in at least three independent experiments. Statistical analysis was conducted using SigmaPlot, ver. 12.5 (Systat Software Inc., San Jose, CA, USA). A one-way analysis of variance (one-way ANOVA) was performed. All pairwise multiple comparison procedures were conducted using the Holm–Sidak method, with an overall significance level of 0.05. A p-value (<0.05) was considered to indicate statistical significance.

3. Results

3.1 Accumulation of Chlorella vulgaris Biomass With the Use of Immobilized Inoculum

The accumulation of microalgal biomass was conducted in a periodic regime. The experiment was performed using various concentrations of *Chlorella* cell inoculum immobilized in PVA cryogel under different cultivation conditions (autotrophic, heterotrophic, and mixotrophic) (Fig. 4). The process duration was 72 hours. These experiments controlled the total accumulation of biomass and estimated its daily accumulation (Fig. 4; Table 1).

The introduction of glycerol to the Tamiya medium was shown to have a positive effect on the rate of accumula-

Table 1. The results of free *C. vulgaris* biomass accumulation after multiple uses of the immobilized inoculum under mixotrophic conditions with 5 g/L crude glycerol in the medium (the duration of one batch cycle was 72 h)*.

Batch cycle number	Biomass concentration, mg DW/L	Biochemical composition of microalgal biomass, % from DW		
		Proteins	Lipids	Carbohydrates
1	993 ± 30	7.8 ± 0.3	23.5 ± 0.8	51.7 ± 1.8
2	1008 ± 41	7.9 ± 0.2	24.1 ± 0.7	50.9 ± 1.6
3	975 ± 36	7.4 ± 0.4	23.4 ± 0.8	51.9 ± 1.9
4	924 ± 33	7.7 ± 0.3	23.5 ± 0.6	51.8 ± 1.9
5	942 ± 31	8 ± 0.3	23.1 ± 0.7	52.2 ± 1.7

*Results are shown as the mean ± SD (n = 3). SD, standard deviation; DW, dry weight.

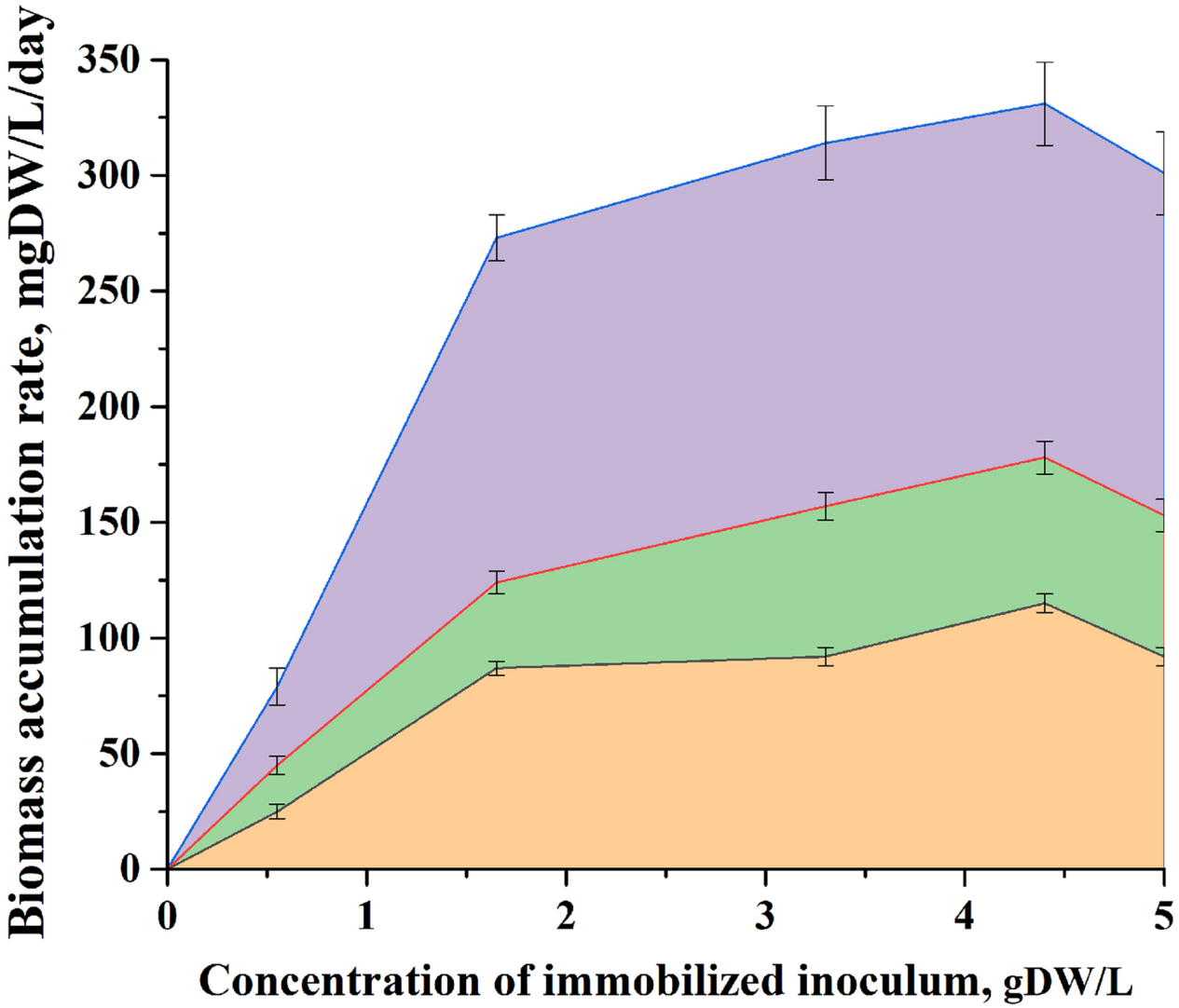

Fig. 4. Microalgal biomass accumulation with the immobilized *C. vulgaris* inoculum. Influence of the initial concentration of the immobilized *C. vulgaris* cells on the rate of free biomass accumulation under autotrophic (■), heterotrophic (■), and mixotrophic (■) conditions. Results are presented as the mean ± SD (n = 3). SD, standard deviation.

tion of suspended microalgal biomass. Under heterotrophic conditions, using glycerol (5 g/L) as the primary substrate resulted in a 42% increase in the rate of biomass accumulation compared to growth under autotrophic conditions

(without glycerol). In the mixotrophic process, provided by a combination of inorganic (CO_2 from the air) and organic (glycerol) carbon sources, the rate of microalgal biomass accumulation was maximum (Fig. 4).

The accumulation of *Chlorella* biomass improved for all the used substrates, with an increase observed following the initial concentration of the inoculum loaded to the media until the maximum at 4.4 g DW/L (Fig. 4). The maximum amount of biomass (993 ± 30 mg DW/L) was accumulated in the process conducted under mixotrophic conditions with the mentioned optimum concentration of immobilized inoculum. A further increase in the concentration of the inoculum in the form of granules with immobilized microalgae was impractical, as it was accompanied by a decrease in the rate of biomass accumulation (Fig. 4).

The dependence of the biomass accumulation under autotrophic conditions on the concentration of inoculum in the medium proved to be the strongest. Indeed, 4.6 times more biomass was obtained under autotrophic conditions (25 ± 0.7 mg DW/L at 1 g DW/L and 115 ± 5 mg DW/L at 4.4 g DW/L, respectively) with an 8-fold increase in the inoculum concentration over 72 hours. In contrast, these indicators were 4.0 and 4.2 times higher, respectively, under heterotrophic and mixotrophic conditions. Under mixotrophic conditions, the productivity (in terms of accumulated biomass amount) of the cryoimmobilized inoculum decreased by no more than 7% after five sequential batch cycles (Table 1). Moreover, the biomass of *C. vulgaris* obtained by cultivating an immobilized inoculum had a stable biochemical composition after each cycle (Table 1).

A total of 4.8 ± 0.2 g DW/L of free *C. vulgaris* cell biomass was obtained during five cycles (15 days) of application of the immobilized inoculum (4.4 g DW/L) under mixotrophic conditions. There was a decrease of at least 82% in the COD at the end of each cycle.

The lipids were extracted from half of the accumulated *C. vulgaris* biomass. Further, the subsequent thermolysis combined with weak acid hydrolysis of the whole *C. vulgaris* cell biomass and lipid-free microalgal waste were used, and two kinds of hydrolysates were obtained, ChVH1 and ChVH2, respectively. ChVH2, obtained from lipid-free microalgal waste, contained more RSs and glucose than ChVH1, which was obtained from whole cell microalgal biomass (50 ± 2.1 g RS/L; 28.2 ± 0.7 g glucose/L; 94.2 ± 5.7 g COD/L; and 40 ± 1.6 g RS/L; 34.8 ± 0.9 g glucose/L; 124.2 ± 5.7 g COD/L; respectively).

3.2 Biotransformation of Crude Glycerol and Hydrolysates of C. vulgaris Biomass in FA

Immobilized *R. oryzae* cells were applied for the production of FA from various substrates according to the three types of process (I, II, and III; Figs. 1,5). The use of crude glycerol as a monosubstrate for 40 h ensured the accumulation of minimal concentrations of FA in the medium (no more than 5 g/L) (Fig. 5A). The joint bioconversion of microalgal biomass hydrolysates and crude glycerol led to a 5–7-fold increase in the concentration of accumulated FA (Fig. 5; Table 2; Ref. [23,36,37]). The highest concentrations of FA during the 40 h-batch cycle were recorded in a medium with glycerol (20–40 g/L) and ChVH2 (Fig. 5A). The latter was characterized by 25% higher concentration of RS compared to ChVH1 obtained from the whole biomass of microalgae (Fig. 5A).

The maximum yield of FA (mg/g substrate) was obtained in a medium with 15–30 g/L glycerol and 50 g RS/L from ChVH2 (Fig. 5B).

The immobilized mycelium was repeatedly used to obtain FA in a batch process using 20 g/L glycerol and a joint bioconversion of *C. vulgaris* hydrolysates with 20 g/L glycerol for five cycles (Fig. 6). Meanwhile, it was noted that, in total, the productivity of the immobilized fungal mycelium was virtually stable for 200 h (Fig. 6A).

An increase in the concentration of FA was observed at the end of the second cycle using immobilized fungal cells, compared to the first cycle. The concentration was 24% higher in the medium with glycerin, while the concentration was 18% and 6% higher in the media with ChVH1 and ChVH2, respectively.

The maximum concentration of FA accumulated in the medium at the end of one working cycle did not exceed 39 g/L.

The maximum amount of FA after five cycles was obtained by combining glycerol and ChVH2 (Fig. 6B). Considering the results obtained in five cycles, the average FA yield (mg/g substrate) was the highest in an environment with crude glycerol. Meanwhile, the decrease in this indicator when crude glycerol was combined with ChVH1 did not exceed 14%, whereas this indicator varied within the margin of error in the medium with ChVH2 (Fig. 6C).

For immobilized *R. oryzae* cells, the intracellular ATP level was stable enough for the whole period of investigation of FA production and varied in the range of 0.58–0.73 μmol/mg. This finding testifies to the stable state of cell metabolism and the viability of fungi. These results align with the published data on the properties of immobilized cells [29,31].

4. Discussion

The results obtained on the accelerated (in comparison with the autotrophic regime) accumulation of microalgal biomass with high lipid content in a medium with added glycerol are consistent with the published data (Fig. 3). For example, the addition of glycerol, as a byproduct of biodiesel production, at a concentration of 3 g/L to synthetic wastewater with *Chlorella pyrenoidosa* (inoculum concentration was 10% v/v), contributed to an increased accumulation of lipids in the microalgal biomass to 30.76 ± 0.93% of cell DW compared to the control without glycerol (13.16 ± 0.25% of cell DW) [38].

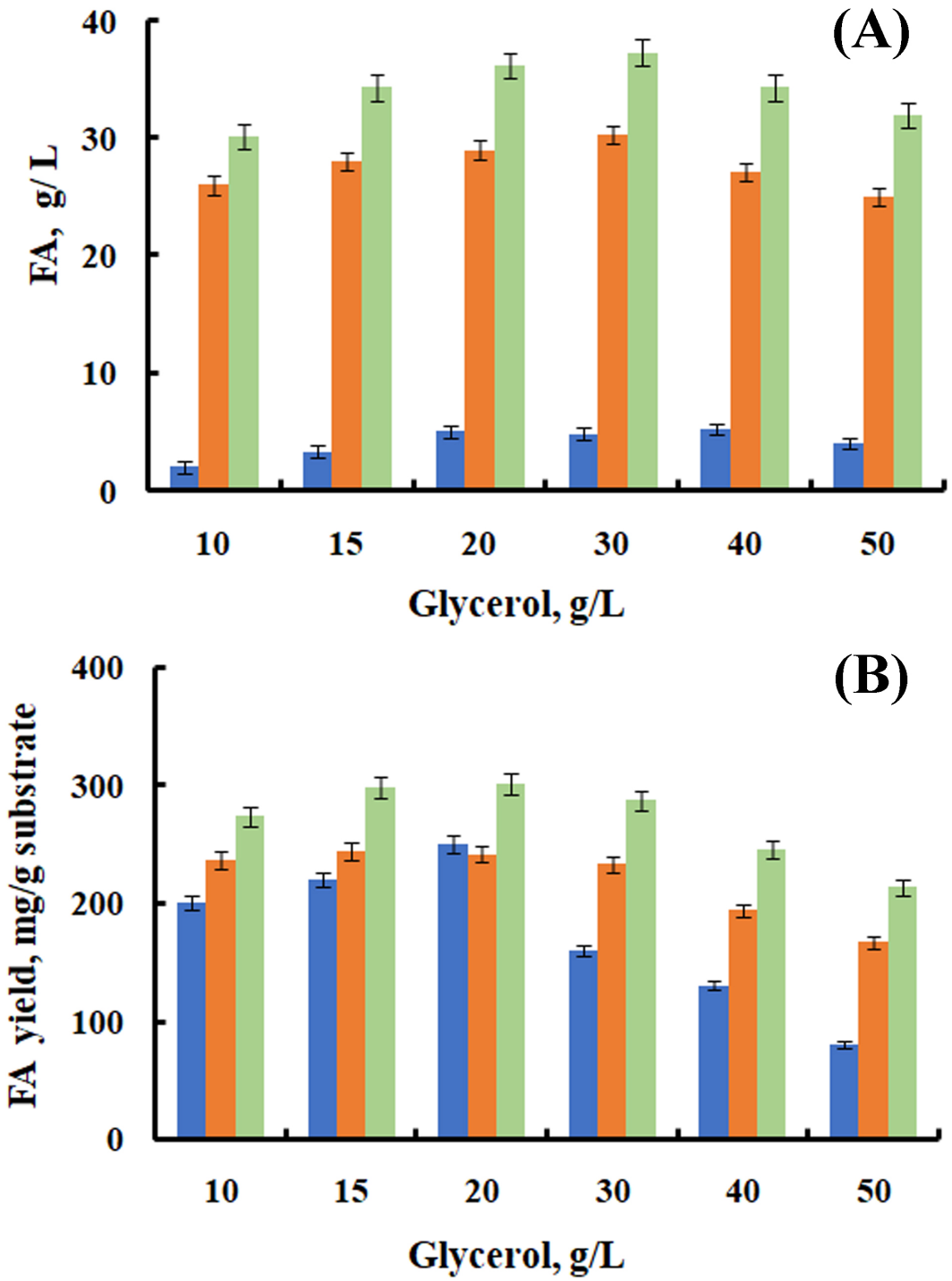

Fig. 5. FA production after one cycle with immobilized *R. oryzae* cells. The concentrations of FA (A) and the specific FA yield (B) estimated after the bioconversion of substrates to FA for 40 h within the three process types: I, crude glycerol (■); II, crude glycerol + ChVH1 (■); III, crude glycerol + ChVH2 (■). Results are presented as the mean ± SD (n = 3).

A positive effect on the accumulation of lipids in the biomass of *C. vulgaris* was also noted in another study (maximum lipid accumulation was 15.11% of the cell DW) when glycerol (5 g/L) was added to wastewater from sewage treatment plants [39]. In this study, the use of an inoculum immobilized in a PVA cryogel and the accumulation of *C. vulgaris* biomass under mixotrophic conditions with 5 g/L crude glycerol also produced a high accumulation of lipids in the biomass (up to 24% of cell DW) (Table 1).

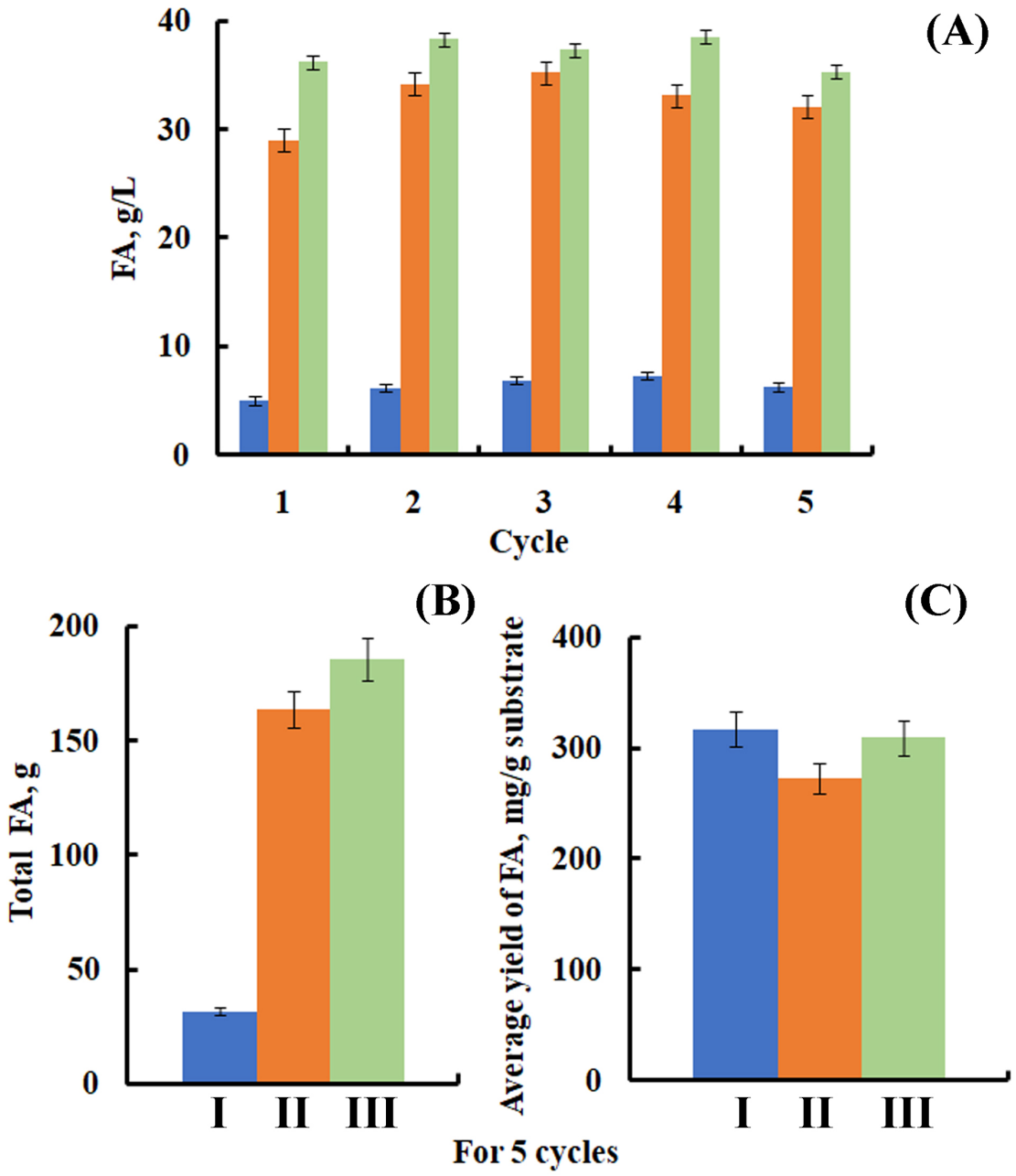

Fig. 6. Multiple uses of the immobilized *R. oryzae* cells for FA production. The FA concentrations after each 40 h-cycle (A), total amount of FA produced for five cycles (B), and the average yield of FA for five cycles (C) during multiple uses of immobilized *R. oryzae* in the bioconversion of substrates to FA within the three process types: I, crude glycerol (■); II, crude glycerol + ChVH1 (■); III, crude glycerol + ChVH2 (■). Results are shown as the mean ± SD (n = 3).

The application of cryoimmobilized *Chlorella vulgaris* cells as an inoculum enables a high concentration of microalgal cells to be loaded into the media, facilitating free biomass accumulation and increasing the process rate [26]. With the addition of 2 g/L of glycerol, the maximum biomass productivity (56 mg/(L•day) was achieved in a mixotrophic culture of *C. vulgaris* for 12 days with the introduction of free cells into the medium as an inoculum (10% v/v of 7-day-old microalgal culture) [39]. After 15 days of periodic cultivation, the highest concentration of *Chlorella pyrenoidosa* biomass (1.43 ± 0.02 g/L) was obtained in a medium with 3 g/L of glycerol [38].

The introduction of an immobilized inoculum (4.4 ± 0.05 g CDW/L) and 5 g/L of glycerol into the mineral medium in our study promoted a 5.9-times faster accumulation of microalgal biomass (331 mg/(L/day) compared to known literature data [38,39]. Meanwhile, a high rate of COD utilization was observed: the COD index of the

Table 2. Results of FA production by bioconversion of various substrates with free or immobilized *R. oryzae* cells.

Substrate*	Approach to FA production	FA, g/L	Volumetric productivity, mg/(L/h)	FA yield, mg/g substrate	Reference
Glycerol (20 g/L) + xylose (40 g/L)	-	28	233	467	[36]
Glycerol (40 g/L) + glucose (40 g/L)	-	16	111	200	[37]
Glycerol (50 g/L) + glucose (30 g/L)	-	28	194	350	[37]
Glucose (80 g/L)	-	24	167	300	[37]
$ChVH_f$ (44 g RS/L)	-	21	525	477	[23]**
Glycerol (20 g/L)	I	5	125	250	
Glycerol (20 g/L) + ChVH1 (40 g RS/L)	II	29	725	242	
Glycerol (30 g/L) + ChVH1 (40 g RS/L)	II	30	758	233	
Glycerol (40 g/L) + ChVH1 (40 g RS/L)	II	27	678	194	This work**
Glycerol (20 g/L) + ChVH2 (50 g RS/L)	III	36	905	302	
Glycerol (30 g/L) + ChVH2 (50 g RS/L)	III	37	933	287	
Glycerol (50 g/L) + ChVH2 (50 g RS/L)	III	32	800	213	

*ChVH is a *C. vulgaris* hydrolysate obtained from 100 g DW biomass; $ChVH_f$ is a *C. vulgaris* hydrolysate obtained after enzymatic treatment of biomass; ChVH1 and ChVH2 are hydrolysates of *C. vulgaris* biomass obtained after thermolysis with acid hydrolysis; ** result obtained with *R. oryzae* cells immobilized into PVA cryogel. For this work, the standard deviation (± SD) did not exceed 5% for each value (n = 3). FA, fumaric acid; RS, reducing sugars.

medium decreased every 72 hours from 5846 ± 175 mg/L to 1084 ± 33 mg/L.

When comparing the approaches implemented in this study to the pretreatment of microalgal biomass accumulated in the mixotrophic regime of *Chlorella* cultivation (obtaining hydrolysates from native biomass or from biomass waste after extracting lipids from it), it becomes obvious that pre-extraction of lipids is advisable in the case of joint biotransformation of *C. vulgaris* hydrolysates with crude glycerol for FA production. The ChVH2 hydrolysate of lipid-free microalgal biomass was characterized by a higher RS content (Table 1) and provided improved FA accumulation rates during its co-transformation with crude glycerol. The results obtained are generally consistent with the published data (Table 2), confirming accelerated and increased accumulation of FA in the medium when glycerol undergoes biotransformation in combination with a co-substrate containing RSs. The advantage of the proposed solution for obtaining FA compared to previously known processes is the utilization of waste in the form of a mixed (complex) substrate and its integration into a biodiesel production cycle.

The influence of glycerol in the medium on the FA productivity of fungi is related to the composition and permeability of the fungal cell membrane. In particular, the membrane of *Rhizopus arrhizus* cells in the FA co-fermentation process exhibited improved characteristics related to permeability and fatty acid composition, resulting in enhanced FA emission from fungal cells and leading to the stimulation of glucose biotransformation [37].

To our knowledge, this study established for the first time that during the bioconversion of *C. vulgaris* biomass hydrolysates (40–50 g RS/L) and glycerol (15–30 g/L), immobilized cells of the fungus *Rhizopus oryzae* are capable of successful simultaneous biotransformation of both substrates. Subsequently, up to 39 g/L of FA can be accumulated in the medium, which exceeds the previously known levels of parameters in processes related to the bioconversion of glycerol, microalgal biomass hydrolysates, and glucose, used as substrates individually or in combinations (Table 2).

Thus, in comparison with data obtained by other authors [36,37] at glycerol concentrations of 20 g/L and 40 g/L with the addition of 40 g/L glucose or xylose, the current results, which used 20 or 30 g/L glycerol and the addition of ChVH1, containing 40 g RS/L, accumulated comparable concentrations of FA (27–29 g/L). However, the immobilized mycelium exhibited 3–4 times higher volumetric productivity levels (Table 2). This confirms that, in the processes of glycerol and *C. vulgaris* hydrolysates bioconversion, the use of immobilized fungal mycelium ensures the accumulation of higher FA concentrations compared to using free cells [23,36,37,40]. Multiple uses (five cycles, 200 h total) of immobilized fungi added to the medium with 20 g/L of crude glycerol and ChVH2 produced 186 g of FA. The average FA yield was 309 ± 12 mg/g substrate.

The FA yield in the bioconversion of ChVH2 (50 g RS/L) with 20 g/L glycerol by immobilized *Rhizopus oryzae* cells was comparable to the results obtained during biotransformation of 80 g/L glucose by free mycelium. The FA volumetric productivity of immobilized *Rhizopus oryzae* was five times higher with immobilized cells, and it was possible to accumulate FA concentrations up to 50% higher (Table 2). The accumulation of high concentrations

of FA (more than 30 g/L) in the media containing immobilized cells, compared with the results obtained using free cells, confirms the advantage of using an immobilized form of mycelium.

The possibility of repeatedly using the same biomass of immobilized mycelium to produce FA is expected to reduce the cost of regular cultivation of the fungi as a producer, which increases the practical importance of using cell immobilization (Fig. 6). The noted increase in FA concentration at the end of the second cycle compared with the first one (Fig. 6A), which manifested most significantly in a medium that contained glycerol as a substrate and ChVH1, is most likely due to the adaptation of the cellular biochemical apparatus to the biotransformation of crude glycerol and lipids in the hydrolysates of the native biomass of microalgae.

At the end of five cycles, the average FA yield (mg/g substrate) was at its maximum precisely in the medium with crude glycerol (Fig. 6C), indicating that the cells had successfully adapted to the biotransformation of this medium. Comparing the results on the accumulation of FA in the media with different compositions revealed that it is possible to achieve the highest mycelium productivity in terms of FA yield in a medium with *C. vulgaris* hydrolysates and crude glycerol (Fig. 6A,B).

To scale the proposed process, it is advisable to use a mixed substrate, as this solution has been shown to be more effective in the synthesis and secretion of FA by the *R. oryzae* cells compared to other options (Table 2). Apparently, the "carbon to nitrogen" ratio in the culture medium plays an important role in the synthesis and secretion. Carbohydrates and proteins from *C. vulgaris* biomass were used as carbon and nitrogen sources for fungal cells. FA is synthesized through three metabolic pathways: the reductive or the oxidative branches of the tricarboxylic acid (TCA) cycle, and the glyoxylate route [22]. The reductive branch in the TCA cycle is the main metabolic pathway in the production of FA by *R. oryzae* [24]. The carbohydrates in the microalgae biomass include glucan and galactan [41]. The final products from their hydrolysis are predominantly glucose and galactose. RSs from *C. vulgaris* biomass enter the TCA cycle by converting pyruvate and ethyl-CoA. Glycerol is introduced to TCA through acetyl-CoA, which is formed following oxidation. Limiting the nitrogen content is known to increase the activity of cytosolic fumarase, one of the main enzymes in the TCA cycle and the FA production pathway. However, the presence of nitrogen sources in the medium is necessary for the synthesis of key cellular enzymes. The addition of crude glycerol to the medium with microalgae hydrolysates promotes a shift in the Carbon to Nitrogen (C/N) ratio towards carbon predominance, while preserving the possibility for fungal cells to synthesize their own enzymes.

Apparently, crude glycerol, in addition to its function as a C-substrate, can also perform another function: crude glycerol had a positive effect on the membrane structure and the removal of FA from cells. Thus, increasing the efficiency of the FA production process by combining monosaccharides with glycerol corresponds to a prior study, when the production of FA by *R. oryzae* was increased in the dual presence of glycerol and monosaccharides (fructose, galactose, and mannose) [36].

A comparison of the investigated processes (I and III) found that option III was the most attractive for obtaining FA in terms of waste management, efficiency, and principles of circular economy, because this process assumes the complete use of the initially available resources and ensures the production of FA as a result of the simultaneous transformation of two wastes.

When assessing waste management efficiency, we can conclude that crude glycerol and acidic microalgae hydrolysates (biodiesel production waste) should be converted specifically into FA, rather than, for example, into bioethanol. For example, the hydrolysate from *C. vulgaris* was fermented using the yeast *Brettanomyces custersii* H1-603, and a bioethanol yield of 370 mg/g was obtained from microalgal sugars [41]. The yield of bioethanol is comparable to that of FA; however, the efficiency of waste valorization (creating added value) is higher in the latter case, since the market value of FA is higher (FOB price: for FA USD 1280–1700 per ton) [42], as compared to bioethanol (USD 775–870 per ton) [43]. Currently, compared to the case of bioethanol, the biotechnological process of obtaining FA is more attractive for investment due to the almost complete lack of competition in this market segment.

When assessing environmental and energy efficiency, it is evident that incorporating microalgae waste into the FA production process is more suitable than, for example, utilizing plant waste. The buildup of microalgae biomass for biodiesel production can be performed simultaneously with wastewater treatment [44]. Microalgae can efficiently use sunlight and CO_2 through photosynthesis, achieving a much higher growth rate and a 10–50 times higher carbon fixation capacity than terrestrial plants [45]. New solutions are being actively developed to enhance the energy efficiency of processes associated with microalgae growth. Notably, more than 37 kWh/m^2 of electrical energy was generated from the semi-transparent photovoltaic-photobioreactor during microalgae cultivation, which was enough to provide the electric power required to operate the lights [44].

5. Conclusions

To our knowledge, this work successfully demonstrated an effective approach to producing FA through the biotransformation of crude glycerol in a medium of microalgal biomass hydrolysates for the first time. Glycerol, as a substrate, was gradually transformed into FA. Moreover, combining glycerol with different variants of microalgal biomass hydrolysates promoted the faster production

of high FA concentrations (up to 39 g/L over 40 hours). For the accumulation of microalgal biomass and the subsequent production of hydrolysates from the biomass to synthesize FA, we recommend using mixotrophic conditions for the cultivation of *Chlorella* cells with the addition of crude glycerol to the medium. When obtaining microalgal biomass hydrolysates, it is advisable to pre-extract lipids from the hydrolysates, which can then be used to produce biodiesel and glycerol. Due to the use of microbial cells, microalgal inoculum, and fungal mycelium in the immobilized form, it was possible to obtain high yields of microalgal biomass and FA quickly. This work also confirmed the possibility of repeatedly using both types of immobilized cells in the corresponding processes without reducing their target activity. The approaches to waste valorization investigated in this study for the joint conversion of crude glycerol and microalgal biomass hydrolysates to produce FA are of potential high practical importance. The results obtained generally indicate the investment attractiveness of the proposed solution for FA production. Studying the process under scaling conditions and selecting techniques for the easy recovery of FA from the fermented broth will enable us to evaluate the economic and energy efficiency of the process at a specific stage, simplify the assessment of potential limitations of the technology, and assess the prospects for its industrial implementation.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

OM and OS performed the Investigation, Methodology and Writing of the original draft. EE performed the Conceptualization, Supervision, Review and Editing of the original draft. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all the aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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