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Research Article

Application of a Microalgal Peptide-Enriched Extract as Media Component in *E. coli* Culture

José Antonio Callejo-López, Martín Ramírez,* José Joaquín González-Cortés, Antonio Valle, Domingo Cantero, and Jorge Bolívar

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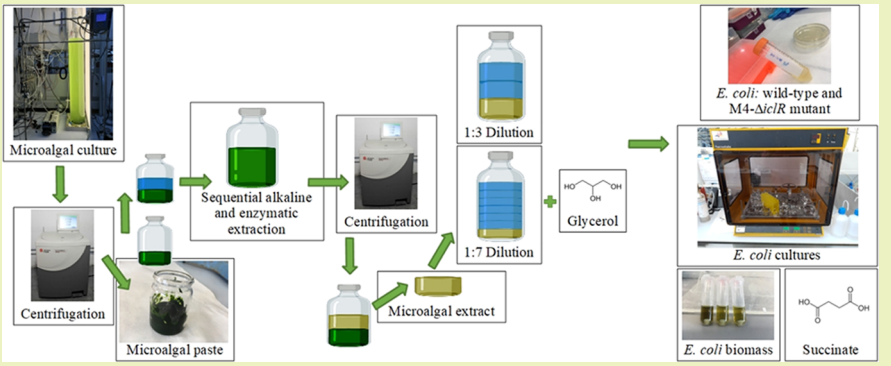

ABSTRACT: This study evaluated the feasibility of applying a microalgal peptide-enriched extract, obtained through a sequential alkaline and enzymatic method, as a fundamental media component in *Escherichia coli* culture. The results demonstrated that the extract is viable as the primary nutrient source for *E. coli* cultures (wild type), even as a complete substitute for the widely used Luria−Bertani (LB) medium, using an extract dilution of 1:3. Its application in a proportion carbon limiting (dilution 1:7), with glycerol supplementation at the early stationary phase, improved the nutrient use efficiency and controlled the undesirable acetate production. Furthermore, this last strategy applied in an *E. coli* mutant (M4-Δ*iclR*) enhanced the production of succinate by 67.3% compared with the M9 medium. The innovative approach proposed in this study opens a wide range of applications and studies related to the production of bio-compounds through diverse microorganisms.

KEYWORDS: *microalgae, peptide-enriched extract, culture medium, E. coli, succinate*

INTRODUCTION

In recent years, the interest in the protein fraction of microalgae has increased notably as the main bio-product target or as a co-product obtained in integrated processes or biorefineries due to its broad range of potential uses.[1] Among the potential uses, a promising one could be its application as a substitute for the nitrogen fraction of microorganisms' culture media.[2] Indeed, the successful implementation of this approach would open a wide variety of possibilities for the production of multiple bio-compounds through different microorganisms in the scope of the bio-economy. This approach would be supported by the high potential existing for the production of protein-enriched biomass through the use of microalgal technologies for the depuration of residual streams derived from human activities,[3] using them as a source of nutrients (nitrogen, phosphorus, CO_2, etc.), in the perspective of a circular economy. Using residual streams for the production of the raw material—microalgae—allows the development of efficient processes from the economic and environmental points of view, considering all of their life cycles. This perspective could facilitate the expansion of the use of microalgae and other microorganisms in the production of bio-products of interest.

The use of microalgal extracts as a source of nutrients for microorganism culture is not a new idea. However, practically the totality of the studies related to this application has focused on the carbohydrate fraction exploring the extraction of the polysaccharides and their hydrolysis to simple sugars to be used as carbon sources.[4,5] To our knowledge, only a study has addressed the viability of using a peptide-based extract obtained from microalgae for this purpose, although in that

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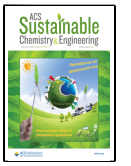

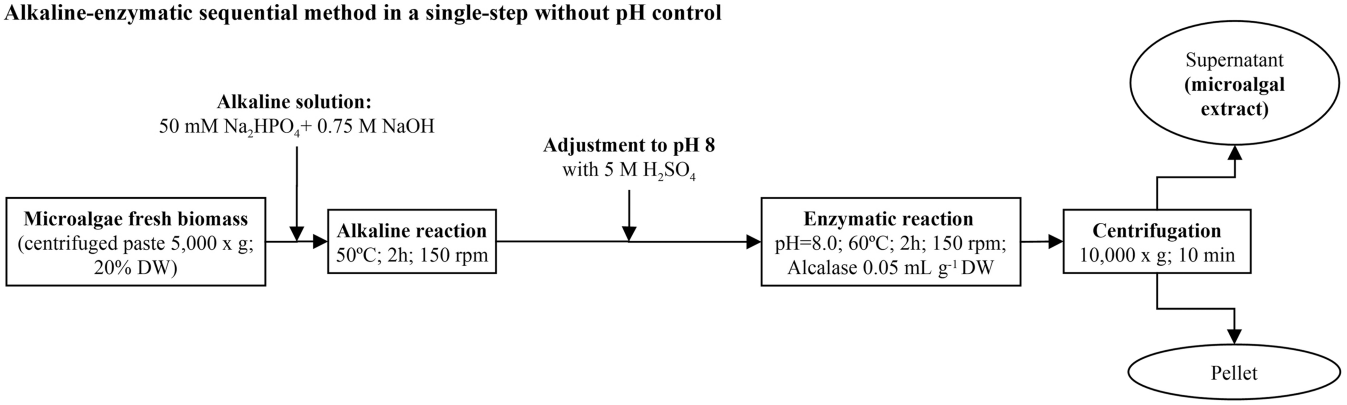

Figure 1. Diagram of the method used to obtain the microalgal extract enriched with hydrolyzed peptides directly from fresh biomass of recalcitrant microalgae (adapted with permission from ref 2. Copyright 2020 Elsevier).

case, as a partial substitute, i.e., for the yeast extract component of the medium.[6] Indirectly, through the evaluation of the production of biogas, as a product of anaerobic biological processes, this matter has also been addressed although using the complete biomass after treatment, not a soluble extract.[7]

Numerous technological alternatives exist for cell disruption and extracting proteins from microalgae.[8] The selection of the more appropriate technique requires considering several factors, such as economic and environmental aspects, microalgal species used, etc., apart from those related to the specific application. In a previous study, we developed a high-yielding method for extracting proteinaceous material from microalgae, rich in hydrolyzed peptides and amino acids, specifically designed for this application, although also suitable for other potential uses.[2] This method was effective over different recalcitrant microalgal species belonging to various genera and using fresh biomass.

Among the possible target microorganisms for this specific application, *Escherichia coli* is one of the most suitable models to be addressed, taking into account its consideration as the "workhorse" cell factory for producing chemicals.[9] This microorganism is widely used for the production of recombinant proteins and has great potential to be used for the production of different fine chemicals and building blocks, such as dicarboxylic acids (succinate and malate), biohydrogen, bioethanol, and biopolymers, among others.

Besides the interest in using a peptide-based medium as the exclusive source of nutrients for *E. coli* culture, the strategy of adding carbohydrates or other alternatives such as glycerol as supplementary carbon sources could also offer other essential aspects. On the one hand, this could improve the efficiency of using the nutrients contained in the microalgal extracts, especially the nitrogen constituent. On the other hand, its addition is necessary for producing specific bio-products obtained through the genetic manipulation of the tricarboxylic acids cycle (TCA), such as the previously mentioned succinate, malate, ethanol, etc. Among them, glycerol is an ideal candidate given its character as a byproduct from the biodiesel industry, whose notable development in the last years has led to an increasing surplus of this compound in the global market, with a consequent decreasing price.[10] Glycerol has been successfully used in previous studies as a carbon source for its biotransformation in those bio-products.[11,12]

In this study, we attempt to demonstrate if it is possible to use a microalgal peptide-enriched extract obtained through a sequential high-alkaline and protease-assisted method as a peptide-based culture medium component for *E. coli* growth, or even as a complete substitute for the widely used LB medium. Furthermore, we try to prove if it is feasible to combine this microalgal extract with glycerol—a residue of biodiesel industry—as a supplementary carbon source. In this case, we try to elucidate if the moment of the addition of glycerol can affect the microorganism metabolism. Finally, we test the feasibility of using this combination in succinate production through an *E. coli* mutant strain as an example of application for obtaining a bio-product of interest.

EXPERIMENTAL SECTION

Microalgae Identification and Culture. A recalcitrant nonaxenic microalgae culture, isolated previously from a landfill leachate by our research group,[13] was used to obtain the raw material for the production of the peptide-enriched extract. To characterize this nonaxenic microalgae culture, identification of operational taxonomic units (OTUs) was performed by next-generation sequencing (NGS). For this, a 1.4 mL sample from a batch culture was centrifuged at 8000*g* for 10 min and the biomass pellet was recovered. The genomic DNA (gDNA) was extracted from the pellet using DNeasy Plant Mini Kit (Qiagen, Germany) and was used as input material for NGS. Previously, gDNA was checked for quality control for library construction with 5′ and 3′ adapter ligation and after that, sequencing the 18S RNA V4 sequence (~400 bp) using Illumina SBS technology. The sequencer generated raw images utilizing sequencing control software for system control and base calling through an integrated primary analysis software called RTA (real-time analysis). The BCL (base calls) binary was converted into FASTQ utilizing the Illumina package bcl2fastq. Adapters were not trimmed away from the reads. These reads were subsequently organized and assigned into OTUs using the basic local alignment search tool (BLAST). The NGS, including bioinformatic analysis, was performed in Macrogen, Inc. (Seoul, Korea).

The microalgal cultures were accomplished as described by Callejo-López et al.[14] In brief, batch cultures were carried out in 5 L glass bottles, bubbled with air mixed with CO_2 (5−10% v/v) at a flow rate of 0.1 vvm, at room temperature (23−26 °C). LED light was supplied to the cultures at an intensity of 170 μmol photons m^{-2} s^{-1} and using a 12/12 h photoperiod. Combo formulation, enriched with an excess of nitrogen (10 mM) and phosphorus (0.5 mM), was used as a culture medium. The resulting biomass was harvested after 7 days of culture at a final biomass concentration of 0.7−0.8 g L^{-1}. At this moment, cultures were within the exponential phase according to previous evaluations.[14] This phase was selected due to microalgae achieving the maximum protein content.[15] The microalgal biomass was concentrated by centrifugation at 5000*g* at 4 °C for 10 min (Beckman Avanti JXN-26, JLA-8 rotor). The centrifuged paste was washed two times with distilled water and subsequent centrifugations.

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The resulting centrifuged paste, hereafter referred to as fresh biomass, was immediately processed for the production of the microalgal peptide-enriched extract as described subsequently. The total protein content of the microalgal biomass was 39.5 ± 0.27% (w/w of dry weight (DW)), as previously reported.[2]

Microalgal Peptide-Enriched Extract Preparation. The hydrolyzed peptide- and amino acid-enriched extract (hereafter referred to as peptide-enriched extract) was obtained through the sequential alkaline-enzymatic method developed in our previous research, particularly, using the called single-step process without pH control (Figure 1).[2] In short, for the alkaline reaction, the microalgal centrifuged paste (fresh biomass) was resuspended and diluted with a highly alkaline phosphate-buffered solution (prepared previously with 50 mM Na_2HPO_4 and 0.75 M NaOH; pH 13.4), up to a final biomass concentration of 100 g L^{-1} of microalgal suspension (DW), in 50 mL centrifuge tubes, which were subsequently subjected to 50 °C for 2 h in an orbital incubator at 150 rpm. After the alkaline reaction, H_2SO_4 (5 M) was added to the suspension to adjust the pH to 8. Immediately after, the enzymatic reaction was performed with the protease Alcalase 2.5L (Novozymes Spain, S.A., Spain) at a dosage of 0.05 mL g^{-1} microalgal biomass (DW) at 60 °C for 2 h and 150 rpm. Finally, the suspension was centrifuged at 10 000*g* for 10 min (Beckman Avanti JXN-26, JLA-8 rotor), and the supernatant (microalgal extract) was recovered and stored at −80 °C. The microalgal extract content in total dry solids, N, free amino nitrogen (FAN), and proteinaceous material was 78.8 ± 0.3, 6.7 ± 0.01, 1.6 ± 0.12, and 30.7 ± 1.4 g L^{-1}, respectively, as previously reported.[2] Before applying to *E. coli* cultures, the microalgal extract was centrifuged at 10 000*g* for 15 min and filtrated through 0.45 μm nylon filters, for further solid suspension removal, and sterilized by autoclaving at 120 °C for 20 min as the rest medium components. The sterilization by autoclaving was performed following the typical procedures applied in *E. coli* cultures for the conventional medium LB, also rich in hydrolyzed peptides, and used in this study for comparison.

***E. coli* Strains and Culture.** Two *E. coli* strains were used as a function of the group of experiments. The wild-type strain BW25113, purchased from CGSC (Coli Genetic Stock Center, Yale University), was used to evaluate the effect of the microalgal extract concentration and its supplementation with glycerol. The quintuple M4-Δ*iclR* mutant strain (BW25113 Δ*sdhA*::Δ*ack-pta*::Δ*pox*::FRTΔ*iclR*::kan; defective in succinate dehydrogenase subunit A acetate kinase, phosphate acetyltransferase, pyruvate oxidase. and DNA-binding transcriptional repressor IclR), obtained in previous work,[12] was used for succinate production experiments. These *E. coli* strains were initially picked up from 15% glycerol stocks at −80 °C and streaked on Luria−Bertani (LB) agar plates (peptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and NaCl, 10 g L^{-1}; containing 1.5% agar). The inoculums for the experiments were prepared by transferring a colony from the agar plates to 5 mL of LB medium and incubating overnight (12 h) at 37 °C and 200 rpm.

Culture assays were performed in mini-reactors consisting of 5 mL Eppendorf tubes (wild-type strain) or 15 mL centrifuge tubes (M4-Δ*iclR* mutant strain) containing 0.9 or 3.0 mL of the different tested media, respectively, allowing a free volume of 5 times the volume of the liquid that assured the aerobic conditions (volumes after the first sample aliquot extraction at t = 0 h). The inoculums were added to the cultures to reach an initial concentration of 0.06 ± 0.02 and 0.22 ± 0.02 g DW L^{-1}, for wild-type and M4-Δ *iclR* mutant, respectively. These values were selected according to previous studies[11,12] and preliminary experiments. The cultures were performed at 37 °C in an orbital incubator (Laboshake THO 500/1, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) at 200 rpm for the times indicated below.

For the determination of glycerol, organics acids, and ammonia, the sample aliquots were centrifuged at 10 000*g* for 10 min and the supernatants were recovered and stored at −20 °C until analysis.

The different media tested were prepared using concentrated stocks of the nutrients prepared previously. All of the concentrations of the media indicated here are expressed as the final concentration in the medium tested.

Study of the Effect of the Microalgal Extract Concentration and the Supplementation with Glycerol on Wild-Type *E. coli* Culture. First, the effect of the microalgal peptide-enriched extract's concentration, as the culture medium's primary nutritional component, on wild-type *E. coli* culture was evaluated by testing decreasing proportions of the extract in the culture medium. For this, different media were prepared by diluting the microalgal extract with the other medium components according to the following proportions (hereinafter, namely, microalgal extract concentration levels MECLs): 1:1, 1:2, 1:3, 1:5, 1:7, and 1:9. The other medium components were phosphate buffer (KH_2PO_4, 7.19 g L^{-1}; Na_2HPO_4, 1.97 g L^{-1}; pH 6.3) and NaCl (up to 1.5 g L^{-1} from an initial NaCl content in the extract before the dilution of 4.33 g L^{-1}). NaCl was not added in MECLs 1:1 and 1:2 since they overcame 1.5 g L^{-1} after the dilution. An experiment with Luria−Bertani (LB) medium (tryptone, 10 g L^{-1}; yeast extract, 5 g L^{-1}; and NaCl, 10 g L^{-1}) was also performed in the same conditions for comparison. The *E. coli* biomass growth (g DW L^{-1}), the final *E. coli* biomass yield (mg of biomass produced per g^{-1} of microalgal extract consumed (on a DW basis considering a total dry solids content in the extract of 78.75 g L^{-1})), and the ammonia concentration (g L^{-1}) were evaluated for 24 h cultures.

Subsequently, an assay by refreshing the media with new microalgal extract was performed for the selected reference MECL 1:7 at the early stationary phase (6 h) to confirm potential nutrient exhaustion. The amount of extract added to refresh the media was the same initially used for this condition, i.e., for producing a final proportion equal to 1:7. Furthermore, an experiment supplementing with 5 g L^{-1} of glycerol at the same culture moment was performed for the same medium reference MECL 1:7 to assess if carbon could be the limiting element in the potential nutrient exhaustion.

Finally, the effect of the moment of the addition of glycerol (5 g L^{-1}) as a supplementary carbon source for the selected MECL 1:7 medium was evaluated at two different time points: initial moment (0 h) and early stationary (6 h). The final *E. coli* apparent biomass concentration (g DW L^{-1}), the *E. coli* biomass productivity in the first 2 and 6 h (g DW L^{-1} h^{-1}), and the final concentration of degradation products (acetate and ammonia; g L^{-1}) at the end of the experiments (24 h) were evaluated. In this case, the final apparent biomass concentration, instead of the final concentration, was considered to avoid the biomass decrease caused by the dilution effect produced when the glycerol concentrate stock was added at 6 h. This variable was calculated by summing to the final biomass concentration, the difference of the biomass concentration measured just after the glycerol addition with respect to that obtained by the baseline reference (MECL 1:7 without glycerol) measured at this same moment, as expressed in the following equation

$$B_{FA}\ (\text{g DW L}^{-1}) = B_F + (B_R - B_{AD})\ (\text{g DW L}^{-1}) \quad (1)$$

where B_{FA} (g DW L^{-1}) is the final *E. coli* apparent biomass concentration (DW) per liter of culture, and B_F, B_R, and B_{AD} (g DW L^{-1}) are the *E. coli* biomass concentrations in the culture final, in the reference without glycerol, and after the glycerol addition, respectively.

Study of the Effect of the Microalgal Peptide-Enriched Extract on the Production of Succinate with the M4-Δ*iclR* Mutant. Experiments with the *E. coli* quintuple M4-Δ*iclR* mutant were performed to evaluate the succinate production using a medium prepared with the microalgal peptide-enriched extract at the proportion 1:7 (MECL 1:7) and glycerol (10 g L^{-1}, added at t = 8 h). The medium was completed with phosphate buffer (KH_2PO_4, 3 g L^{-1}; Na_2HPO_4, 9.97 g L^{-1}; pH 7.2) and NaCl (0.88 g L^{-1}). Besides, it was supplemented with 2 g L^{-1} of $NaHCO_3$,[12] increasing the medium pH to 7.8. Another experiment with a lower initial pH was performed, varying only the salts proportions of the buffer (KH_2PO_4, 7.19 g L^{-1}; Na_2HPO_4, 1.97 g L^{-1}; pH 6.3), which achieved a final pH of 6.9 after the supplementation with 2 g L^{-1} of $NaHCO_3$. The *E. coli* biomass growth (g DW L^{-1}), the glycerol consumption (g L^{-1}), and the acetate and succinate production (g L^{-1}) were evaluated. Succinate

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molar yield was also assessed as mol of succinate produced per mol of glycerol consumed.

For its comparison, an assay was performed under the same culture conditions using minimal medium M9 and 10 g L^{-1} of glycerol, whose composition is detailed in a previous study performed with this same M4-Δ*iclR* mutant.[12] The medium was also supplemented with 2 g L^{-1} of $NaHCO_3$ as in the reference study, increasing the final pH of the medium up to 7.8.

Analytical Methods. *E. coli* biomass growth was estimated by measuring OD_{570}[12] with a Multiskan FC microplate photometer (Thermo Scientific, CA), using 100 μL sample aliquots.

Glycerol and organics acids (acetate and succinate) were analyzed by high-performance liquid chromatography (HPLC), as described by Cofré et al.[11] In short, the samples were filtered through 0.22 μm nylon filters and quantified using the LaChrom Elite VWR-Hitachi equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA). 5 mM H_2SO_4 was used as the mobile phase, fluxed at 0.6 mL min^{-1}, and a temperature of 50 °C was applied for the column.

Ammonia was determined by the Nessler reactive method,[16] measuring the absorbance at 405 nm with the Multiskan FC microplate photometer (Thermo Scientific, CA).

Statistical Data Analysis. Data were analyzed through a one-way analysis of variance (ANOVA) or Student's *t*-test, where appropriate, with a significance level of $p = 0.05$. Mean values were analyzed by Tukey's multiple comparison tests or Student's *t*-test. Statgraphics Centurion 19 (Statgraphics Technologies, Inc.) was used for the statistical analysis. Experiments were performed in triplicate, and the means and standard deviations are reported.

RESULTS AND DISCUSSION

Microalgae Identification. A nonaxenic microalgae culture was isolated from landfill leachate by our research group in a previous study,[13] which was not precisely characterized. To determine the taxonomic and abundance composition of the culture, a genetic analysis was addressed through NGS technology in the present work. The gDNA isolated from a batch culture yielded 47 ng μL^{-1} and was analyzed as previously described in the Experimental section. A total of 242 582 raw sequence reads were generated which is consistent with the expected output (around 100 000 sequence reads). 94.57% obtained a Phred quality score of over 20, indicating a base call accuracy of 99%. After denoising, a total of 108 528 joined paired-end sequences were obtained and 5 unique features of OTUs were taxonomically identified. The most abundant OTU was the unidentified species of *Chlorella* sp. with a relative abundance of 99.45%. The remaining 0.35% was composed of the genera *Acanthamoeba* and *Vermamoeba*, free-living amoebae widely distributed in the environment as well as less than 0.2% are contaminated or not identified sequences whose influence on the peptide extract composition could be considered negligible (data not shown).

The microalgal strain is a factor that can influence the application proposed in this study in various ways, considering that it is the raw material for the production of the microalgal peptide-enriched extract. First, the protein content, amino acid composition, and cell wall resistance, a crucial factor in bio-compound extraction from microalgae, vary between species. Protein content and cell wall resistance affect the extraction yield of the proteinaceous material, and thus its concentration in the extract, affecting the nutrient availability for the *E. coli* growth if used directly without a drying step. The protein content of the microalgae culture used in this study (39.5%), composed of *Chlorella* sp., is within the range of those reported for different microalgal species,[17] and the extraction yield of the proteinaceous material using the method applied in this study was similar to other recalcitrant species.[2] Regarding the amino acid composition, the microalgae culture showed a profile similar to those reported from other microalgal species,[15,17] with some variations between amino acids which cannot be related to a phylogenetic group.[17] Furthermore, certain metabolites or substances associated with some microalgal genera or species can also influence *E. coli* growth since they have shown active functionalities such as antimicrobial.[18] The nonselective extraction method applied in this study and the absence of subsequent purification steps can produce the co-extraction of these potential active bio-products and thus could negatively affect *E. coli* growth. However, the satisfactory results obtained in this study with the peptide extract obtained from *Chlorella* sp. using the proposed method suggest that this extract did not contain these substances (at least not in a sufficient concentration to inhibit bacterial growth) or that the extraction process inactivated them, as discussed below.

Effect of the Microalgal Extract Concentration and the Supplementation with Glycerol on Wild-Type *E. coli* Culture. First, the effect of the concentration of the microalgal peptide-enriched extract, as the primary nutritional component of the culture medium, was evaluated on the culture of wild-type *E. coli*. Figure 2a shows the biomass growth of wild-type *E.*

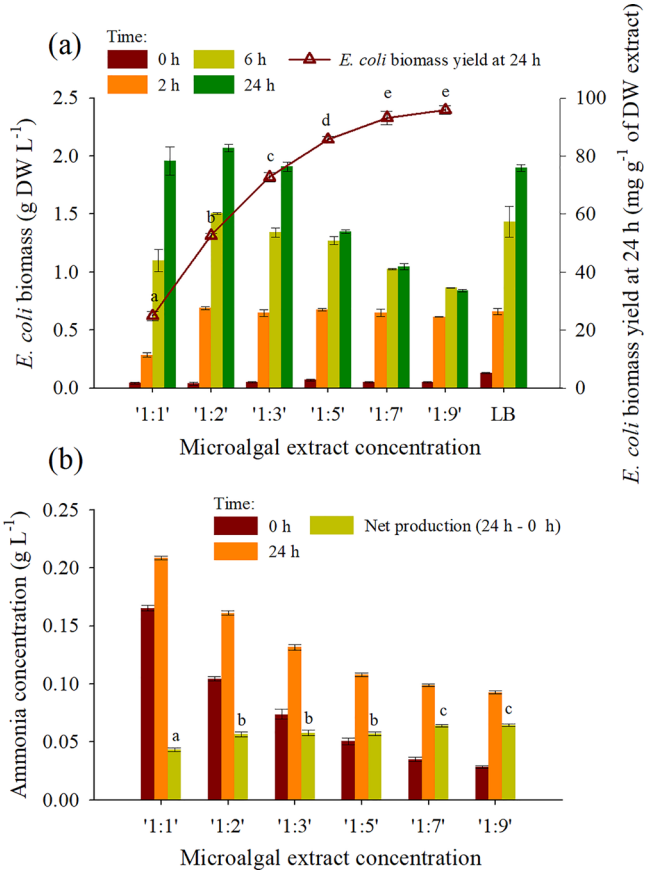

Figure 2. Effect of the concentration of the microalgal peptide-enriched extract, as the primary nutritional component of the culture medium, on wild-type *E. coli* culture. (a) *E. coli* biomass growth and final *E. coli* biomass yield and (b) ammonia concentration at $t = 0$ and 24 h and ammonia net production (NH_4 at $t = 24$ h − NH_4 at $t = 0$ h). Different letters represent statistically significant differences among the means according to Tukey's test ($p < 0.05$).

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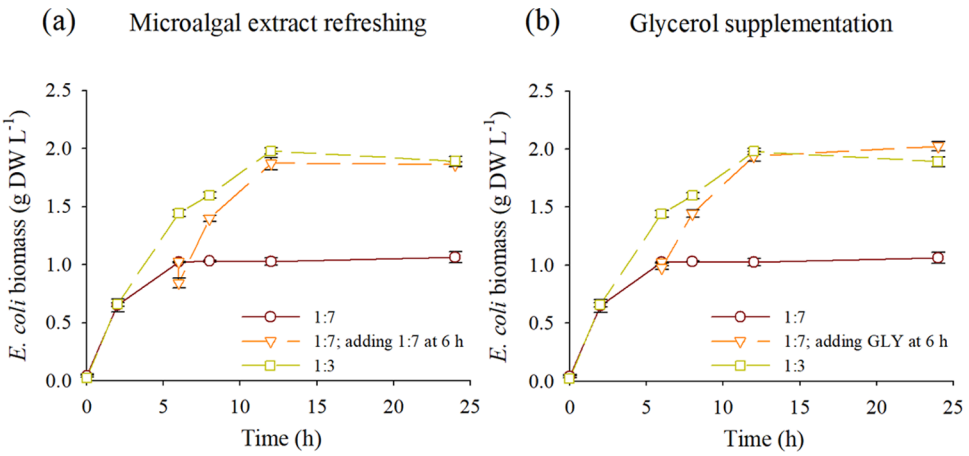

Figure 3. Effect of the addition of (a) new microalgal peptide-enriched extract or (b) glycerol at the early stationary phase on the biomass production of *E. coli* (wild-type strain) cultivated in a microalgal peptide-based medium at the proportion of 1:7.

coli obtained with the different proportions tested of the microalgal extract in the medium composition, called microalgal extract concentration levels (MECLs). As exposed, in the first 2 h, *E. coli* achieved a similar biomass concentration in all of the tested MECLs and the LB reference, except in the highest MECL 1:1 ($p < 0.001$), in which *E. coli* evidenced difficulties for its adaptation in the initial growth phase of the culture. Nevertheless, from this time, the *E. coli* biomass concentration began to show notable differences among the different conditions, dropping markedly with the decrease of the microalgal extract concentration level, a fact that seemed to be related with the nutrient availability and its exhaustion. Indeed, at 6 h or before, *E. coli* already achieved the stationary growth phase for the less concentrated media of 1:5, 1:7, and 1:9, evidencing their lower nutrient availability. At this time, the differences among the conditions were statistically significant in multiple comparisons according to Tukey's test ($p < 0.001$).

At the culture end ($t = 24$ h), *E. coli* biomass achieved the maximum differences among the different conditions ($p < 0.001$). At this time, the second most concentrated medium (MECL 1:2) achieved the maximum final *E. coli* biomass concentration of 2.07 ± 0.03 g L^{-1}, followed by the highest concentrated one (MECL 1:1), which reached a statistically similar final biomass concentration (1.96 ± 0.12 g L^{-1}). Despite the adaptation difficulties exhibited by *E. coli* in this last case at the previous time points, its biomass growth improved substantially during the next culture hours showing a progressive adaptation, up to achieving this similar value to the MECL 1:2 at the culture end. This difficulty found in the *E. coli* adaptation in the MECL 1:1 at the initial culture phases could be due to the high salts content or the presence of certain compounds in the extract with inhibitory effects from a threshold concentration in the culture medium.[18] On the other hand, the fact that *E. coli* grew in all cases confirms that the microalgal proteins extracted were functionally inactivated. The material in the extract consisted of hydrolyzed peptides and amino acids produced as a consequence of the extraction method applied—a sequential high-alkaline and protease-assisted procedure. Indeed, nonhydrolyzed proteins obtained from microalgae have shown potential antimicrobial properties in previous studies.[19]

As shown in Figure 2a, the final *E. coli* biomass yield, as the ratio of the biomass production at 24 h per mass of microalgal extract (DW) used, increased significantly at decreasing microalgal extract concentration levels, especially in the three conditions that achieved the highest final biomass concentrations (MECLs 1:1, 1:2, and 1:3). This fact suggests higher efficiency in the use of the nutrients at lower extract concentrations where they were early limiting, reflecting potential better assimilation and biotransformation to biomass. In particular, the condition MECL 1:3, with a biomass yield of 72.7 ± 1.6 mg g^{-1} of microalgal extract (DW) used, achieved a final biomass concentration of 1.91 ± 0.04 g L^{-1}, which was only 7.8% lower than the highest value achieved by MECL 1:2, but using 33.3% less of extract. In addition, note that the *E. coli* cultured with this MECL 1:3 grew similarly to that grown in LB used as a reference, achieving statistically similar *E. coli* biomass concentrations in all of the time points according to Tukey's test. These two media were comparable regarding their content in total solids (25 and 26.3 g L^{-1} for LB and MECL 1:3, respectively) and total nitrogen (1.8 and 2.2 g L^{-1} for LB and MECL 1:3, respectively). Therefore, this condition could be considered the most interesting for *E. coli* biomass production, according to the final biomass concentration achieved and yield regarding the consumption of microalgal extract, constituting a complete substitute for the traditional LB medium.

The concentration of ammonia was analyzed at the initial and final times of the cultures. As shown in Figure 2b, the final ammonia concentration increased notably compared with the initial moment in all of the concentration levels revealing the catabolization of the proteinaceous material of the microalgal extract and its usage as a carbon source. Higher MECLs led to higher final ammonia concentrations because the microalgal extract contained significant initial ammonia content, taking into account that the net production (NH_4 at $t = 24$ h − NH_4 at $t = 0$ h) increased slightly at decreasing MECLs (Figure 2b). These high final ammonia concentrations at increasing MECLs, and the consequent pH increment, did not have an inhibitory effect on the *E. coli* growth, given the higher final biomass concentrations obtained in these cases. Using a low initial pH (6.3) contributed to having enough margin of pH increase within the tolerable range for *E. coli*. For instance, the pH of the media increased from the initial buffered pH of 6.3 to 7.4−7.6 for the MECLs with the highest final biomass concentrations at the culture ends (1:1, 1:2, and 1:3). As mentioned before, the ammonia net production (Figure 2b)

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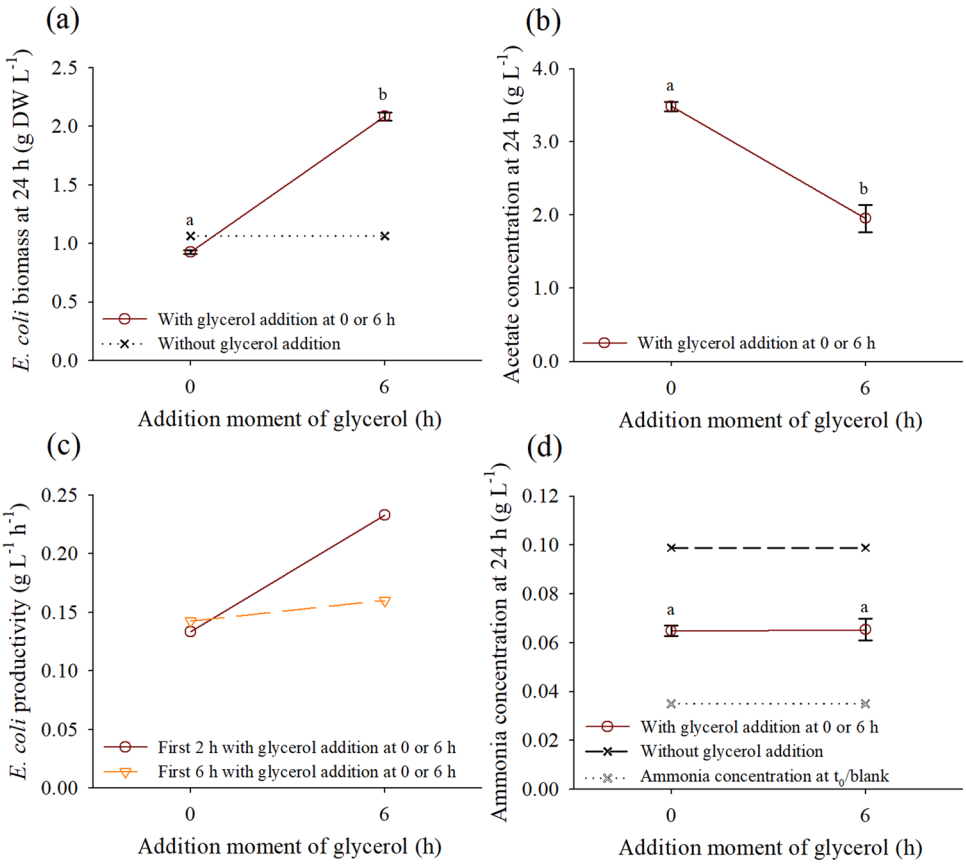

Figure 4. Effect of the moment of the addition of glycerol at different culture time points (0 and 6 h) on *E. coli* (wild-type strain) cultivated in a microalgal peptide-enriched extract at the proportion of 1:7 during 24 h. (a) Final *E. coli* apparent biomass concentration; (b) final acetate concentration; (c) *E. coli* biomass productivity in the first 2 and 6 h in the presence of glycerol; and (d) final ammonia concentration. Different letters represent statistically significant differences among the means according to Student's *t*-test ($p < 0.05$).

increased slightly at decreasing concentrations of the microalgal extract, with differences statistically significant ($p < 0.001$), supporting the suggestion mentioned previously for the biomass yield of possible higher efficiency in the catabolism of the proteinaceous material at lower extract concentrations where this carbon and nitrogen source was early limiting. These results suggested that the proteinaceous material of the extract was practically the exclusive carbon source used by *E. coli* that supported its biomass growth. This result is in accordance with the study of Sezonov et al.[20] using LB, which concluded that catabolizable amino acids are the exclusive carbon sources for *E. coli* grown in that complex culture medium also rich in hydrolyzed peptides and amino acids.

To confirm a nutrient exhaustion, experiments were performed supplementing MECL 1:7 cultures after the apparent exhaustion (t = 6 h), through two procedures: refreshing with new microalgal extract or adding glycerol. Cultures MECL 1:7 and 1:3 without supplementation were also carried out under the same conditions for comparison. As shown in Figure 3a, the MECL 1:7 without supplementation achieved the stationary phase before 6 h, with an *E. coli* biomass concentration of 1.02 ± 0.01 g L^{-1}, whereas the same condition refreshed with new microalgal extract at this time experimented a noticeable renovate biomass growth just after the refreshing moment, achieving a final biomass concentration of 1.86 ± 0.02 g L^{-1} (at 24 h) and 1.73 ± 0.03 g L^{-1} (at 48 h), respectively, close to that obtained for the upper reference MECL 1:3. Considering the net production, after the 6 first hours of the refreshing phase, the biomass increment was similar to that obtained in its analogue period of the initial growth (of around 1 g L^{-1}), which was consistent with the nutrient amount refreshed. Likewise, the supplementation with glycerol at 6 h (Figure 3b) produced a renovate and remarkable biomass growth just after this moment, almost doubling it, reaching up to 2 g L^{-1}. Indeed, the behavior of the biomass growth, in this case, was similar to that exhibited by refreshing with the new microalgal extract described previously. To contrast these results, these same experiments were replied using glucose and for MECL 1:3, obtaining a similar behavior (data not shown). According to the results obtained, it can be concluded that the use of the proteinaceous material of the extract for catabolization by *E. coli* exhausted the carbon source and was the cause of the growth stopping in the different MECLs and the drop of the biomass production at decreasing microalgal extract concentrations. Similarly, Sezonov et al.[20] found that the stop of the *E. coli* growth cultured in LB medium is due to the exhaustion of the carbon source, which in this medium is provided by catabolizable amino acids and not by carbohydrates.

The effect of the supplementation with glycerol, media prepared with a carbon limiting condition of the microalgal peptide-enriched extract (MECL 1:7), was further evaluated given its interest as a strategy to maximize the use of the nutrients contained in the microalgal extract. Two addition moments (initial and early stationary) were evaluated for a glycerol concentration of 5 g L^{-1}—higher than the total

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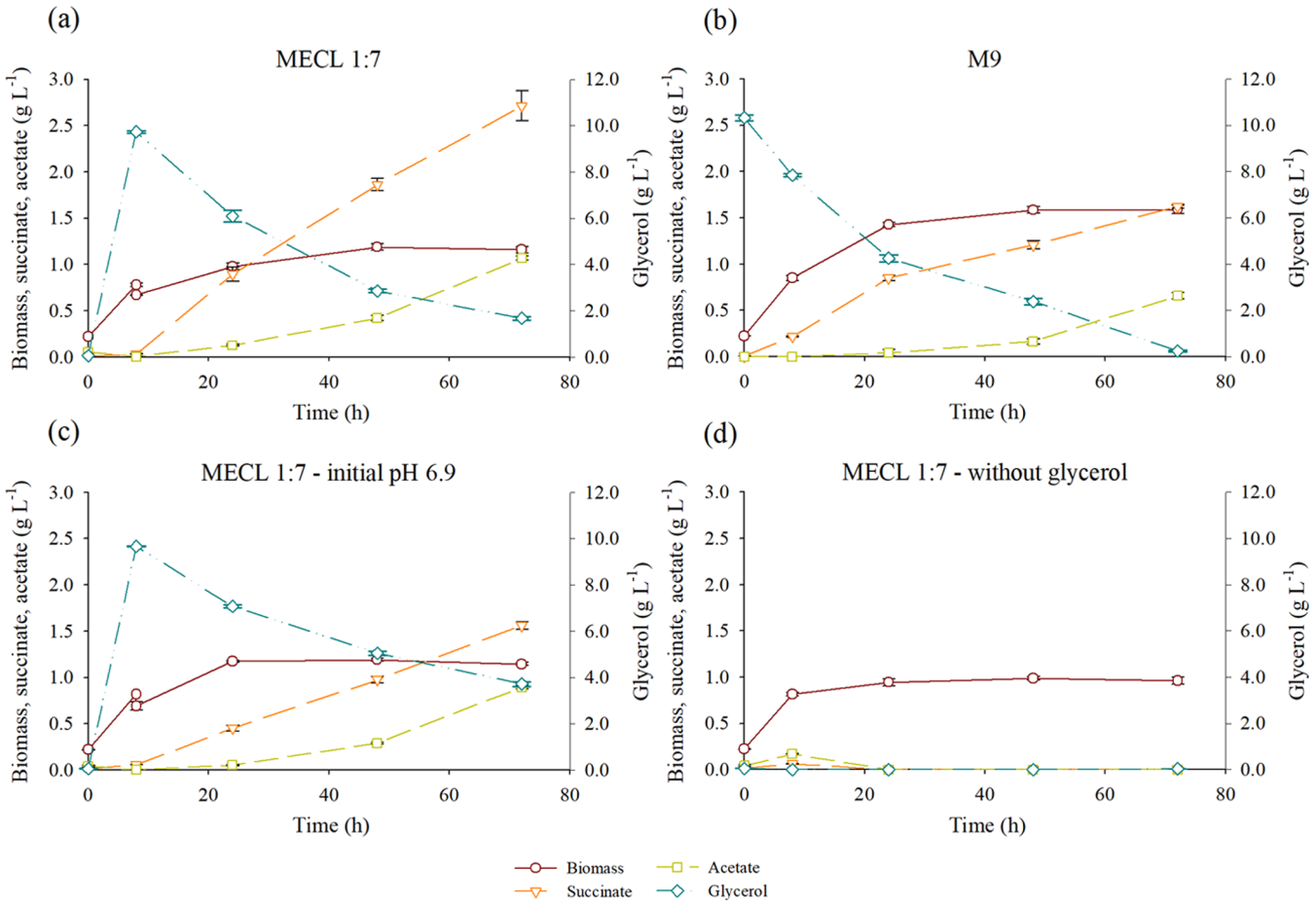

Figure 5. Effect of the microalgal peptide-enriched extract in succinate production with the M4-Δ*iclR* mutant. Production of *E. coli* biomass, succinate, and acetate, and glycerol consumption for the conditions (a) MECL 1:7 and glycerol added a t = 8 h; (b) M9 and glycerol added a t = 0 h; (c) MECL 1:7 at an initial buffered pH of 6.9 and glycerol added a t = 8 h; and (d) control—MECL 1:7 without glycerol.

glycerol consumed in the time-lapse considered of 24 h, and thus consisting in a nonlimiting nutrient condition. This same assay was replicated with glucose, an easily assimilable carbon source for *E. coli*, obtaining a similar behavior as this described subsequently (data not shown).

As shown in Figure 4a, the culture supplemented at the initial moment achieved a final *E. coli* apparent biomass concentration (0.93 ± 0.01 g L^{-1}) substantially lower (p < 0.001) than that supplemented at the early stationary phase (2.09 ± 0.03 g L^{-1}), with a value less of the half. Indeed, in this case, the result was close to that obtained by the reference without glycerol supplementation (1.06 ± 0.04 g L^{-1}). This substantial lower final apparent biomass concentration obtained when the glycerol was added at the initial moment was due to the metabolic response of *E. coli* as a function of its availability in the culture beginning, revealed by the acetate production (Figure 4b), which was not generated without glycerol addition. Acetate is a fermentation product in anaerobic conditions; however, it is also typically produced and accumulated in *E. coli* aerobic cultures when they grow at high rates.[21] When the glycerol was provided at the initial moment, the *E. coli* biomass growth was supported by this compound from the culture beginning, with an earlier progressive medium acidification due to the acetate production, up to achieving a final pH below 6. This very low pH would have hindered further growth based on the catabolism of the proteinaceous material to use it as a carbon source once glycerol was exhausted, leading to a low final biomass concentration. In contrast, when the glycerol was added in an advanced culture moment (t = 6 h), *E. coli* grew in two different phases separated by the moment of this addition, as shown above (Figure 3b). In the first phase, the biomass growth was supported by the carbon obtained from the proteinaceous material of the extract, and in the second phase, by the carbon obtained from the glycerol added. In the first phase, the pH increased due to the ammonia production (as described above), and in the second one decreased due to the acetate generation, up to reach a final pH around 6. Therefore, under this last strategy, higher potential of the carbon component of the microalgal extract was used, leading to substantially higher final biomass production.

Another significant effect of the moment of the glycerol addition was the different patterns experimented in the generation of acetate. In this case, the effect was contrary (Figure 4b); when glycerol was added at the initial moment, the acetate production was substantially higher (3.48 ± 0.06 g L^{-1}) than that obtained when it was added at 6 h (1.95 ± 0.19 g L^{-1}), with a value 1.8 times superior. The cause would be in the growth state of the *E. coli* culture at the moment of this addition. Previous studies have shown that adding carbohydrates through fed pulses during the culture can reduce acetate production.[21,22] This effect would support the strategy of adding glycerol at an advanced culture moment for reducing this harmful compound in highly dense *E. coli* cultures, apart

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from as a means for improving the biomass growth as shown before. Furthermore, the productivity in the first 2 or 6 h of the growth in the presence of glycerol was higher when it was added at 6 h (Figure 4c). Consequently, using fed pulses during the culture could represent an advantage in developing more efficient processes using the approach proposed in this work for producing high-density *E. coli* cultures.

Concerning ammonia (Figure 4d), this compound was produced with a final concentration statistically similar in the two addition times, of around 0.065 g L^{-1} (p = 0.893). It is worth to note that that ammonia was produced even when the glycerol was added at t = 0 h (0.065 ± 0.002 g L^{-1}), where it was used as the carbon source instead of the proteinaceous material, as observed when comparing with the blank (0.035 g L^{-1}). This fact would reveal the use of the proteinaceous material of the extract as the nitrogen source, probably in the form of amino acids. The ammonia would be produced by the hydrolysis and degradation of this proteinaceous material for this purpose. Furthermore, the final ammonia concentration for the addition time of 6 h was significantly lower (0.065 ± 0.004 g L^{-1}) than that obtained by the reference without glycerol addition (0.099 g L^{-1}). This lower amount would be due to its consumption as the nitrogen source in the second growth phase after glycerol addition. Therefore, in this case, higher potential of the nitrogen component of the microalgal extract was used in ammonia form, a degradation product of the proteinaceous material assimilated as the carbon source in the first growth phase. This fact would prove the suitability and interest of the strategy of supplementing with glycerol at an advanced phase of the microorganism culture for improving the efficiency of the overall nitrogen balance.

Effect of the Microalgal Peptide-Enriched Extract on the Production of Succinate with the M4-Δ*iclR* Mutant. Experiments with the *E. coli* quintuple M4-Δ*iclR* mutant strain were performed to test succinate production as an example of a particular application to obtain a bio-product of industrial interest through a genetically modified microorganism. This strain is the base for other mutants capable of co-producing other interesting bio-products such as malate.[12] Figure 5a shows the results obtained with the medium prepared with the microalgal extract concentration of 1:7 (MECL 1:7) and adding glycerol at the culture time of 8 h, and Figure 5b with the minimal medium M9 and glycerol. As shown in these figures, the culture with the microalgal extract achieved a final succinate production of 2.71 ± 0.16 g L^{-1}, which was noticeably higher (67.3%) than that obtained using the M9 medium of 1.62 ± 0.02 g L^{-1}. Likewise, the molar yield (mol of succinate/mol of glycerol consumed) was also significantly higher in the culture with MECL 1:7 (0.26) compared with that obtained with M9 (0.16). The opposite occurred with the biomass production, which was remarkably higher in the case of M9 (1.59 ± 0.04 g L^{-1}) than MECL 1:7 (1.16 ± 0.03 g L^{-1}).

A possible cause of this higher succinate production obtained with the culture MECL 1:7 compared with M9 could be the slightly better pH conditions displayed for this case during the culture. The M9 cultures started at a pH of 7.8, dropping progressively from an earlier moment with the glycerol consumption and the formation of organic acids, up to reaching a pH of 5.8 at the end of the culture after 72 h. The MECL 1:7 culture also started with a pH of 7.8, but this increased to 8 at the time of 8 h just before the glycerol addition due to the ammonia generation from the catabolism of the proteinaceous material of the extract. After this, the pH began its progressive drop, a cause of the organic acids generation, up to end in 6 at the culture final. This fact is supported by the study of Soto-Varela et al.,[12] which suggested that an initial higher pH, obtained by adding increasing carbonate amounts, improved succinate production. Additionally, certain compounds existing in the microalgal extract with buffering properties, such as hydrolyzed proteins, phosphates, etc., could have slowed down the pH drop, promoting better environmental conditions for a further glycerol metabolism.

Another experiment using a buffer with a lower initial pH of 6.9 was performed to verify this point. As expected, the succinate production was noticeably lower in this case than in the condition starting at a higher pH (Figure 5c), achieving a value of 1.56 ± 0.04 g L^{-1}, with a conversion yield of 0.20 mol of succinate mol^{-1} glycerol consumed. This lower succinate production was associated with a lower glycerol consumption, which reached around 60% of total glycerol against the 80% starting at pH 7.8. In addition, in this case, another organic acid (acetate) was also produced in a similar amount—around 1 g L^{-1}—and thus achieved a higher proportion (50%) of the total organic acids found in the culture medium. Despite this, the succinate production was close to that produced by the M9 culture, and the conversion yield was even higher. This fact suggested that there would be other causes for the better results of succinate production obtained in the case of using the microalgal extract apart from the pH. The main cause would be that the M9 is a minimal medium, in which the microorganism has to produce all of the metabolites de novo, and thus the energy and carbon obtained from the glycerol were necessarily used for the biomass growth, producing succinate as a degradation product. In contrast, in the microalgal extract peptide-rich medium, the first part of the growth was supported by the peptide catabolism without succinate production. Adding the glycerol near the stationary phase (t = 8 h) produced an excess of carbon that the microorganism canalized to succinate production. Indeed, this mutant has two pathways of acetate production suppressed, so the pyruvate produced in the energy generation from the glycerol catabolism is removed as succinate instead of acetate. Therefore, succinate is produced while glycerol is available and used as an energy source, even in the stationary phase, as shown in Figure 5. This fact would support the strategy of using two sequential growth phases, peptide and glycerol-based, to improve the production of this type of bio-products, apart from enhancing the efficiency of the use of nutrients. In addition, certain compounds existing in the microalgal extract, as a complex medium ingredient, with nutritive characteristics, such as trace elements, co-factors, vitamins, and other metabolites, could have had positive effects on the microorganism development.[11,23]

As a reference, Soto-Varela et al.[12] studied this same M4-Δ*iclR* strain, among others, in similar medium conditions, i.e., using M9 and 10 g L^{-1} of glycerol, although in other culture conditions (250 mL Erlenmeyer flask). In that study, M4-Δ*iclR* achieved the stationary phase a little after that in our culture conditions, at around 30 h, and reached a higher biomass concentration (around 2.75 g L^{-1}). As in our case, succinate was produced during the stationary phase while glycerol was available, up to a maximum close to 2 g L^{-1} at 48 h when the glycerol was completely exhausted, which was slightly higher than that obtained in our study with M9 (1.62 g L^{-1}). In our case, the maximum succinate production and the total glycerol

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consumption were achieved at 72 h, showing a slower production rate that could be associated with the culture conditions used. Therefore, taking into account the similar behavior, it can be inferred that the application of that culture conditions in cultures grown with the microalgal extract could improve even more the succinate production obtained in our study.

CONCLUSIONS

In this study, we have demonstrated the feasibility of applying a microalgal peptide-enriched extract as the primary medium component for *E. coli* culture, even as a complete substitute for the widely used LB medium at an extract dilution of 1:3. We have also proved that its application in a proportion carbon limiting (dilution 1:7), with glycerol supplementation at the early stationary phase, improved the nutrient use efficiency and controlled the undesirable acetate production. Besides, this strategy applied in an *E. coli* mutant enhanced the production of succinate by 67.3% compared with the M9 medium.

AUTHOR INFORMATION

Corresponding Author

Martín Ramírez − *Department of Chemical Engineering and Food Technology, Institute of Viticulture and Agri-Food Research (IVAGRO), University of Cádiz, Puerto Real 11510, Spain;* orcid.org/0000-0002-5929-8783; Phone: +34956016286; Email: martin.ramirez@uca.es; Fax: +34956016411

Authors

José Antonio Callejo-López − *Department of Chemical Engineering and Food Technology, Institute of Viticulture and Agri-Food Research (IVAGRO), University of Cádiz, Puerto Real 11510, Spain;* orcid.org/0000-0002-9856-5821

José Joaquín González-Cortés − *Department of Chemical Engineering and Food Technology, Institute of Viticulture and Agri-Food Research (IVAGRO), University of Cádiz, Puerto Real 11510, Spain;* orcid.org/0000-0003-0244-9074

Antonio Valle − *Department of Biomedicine, Biotechnology and Public Health-Biochemistry and Molecular Biology, Institute of Biomolecules (INBIO), University of Cádiz, Puerto Real 11510, Spain;* orcid.org/0000-0002-4459-4627

Domingo Cantero − *Department of Chemical Engineering and Food Technology, Institute of Viticulture and Agri-Food Research (IVAGRO), University of Cádiz, Puerto Real 11510, Spain*

Jorge Bolívar − *Department of Biomedicine, Biotechnology and Public Health-Biochemistry and Molecular Biology, Institute of Biomolecules (INBIO), University of Cádiz, Puerto Real 11510, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.2c07765

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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