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Mimicking Marine Conditions to Improve Prodigiosin Yields in Bioreactor

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Abstract: Prodigiosin is a red bacterial pigment with great potential as a natural dye and drug precursor, while presenting several pharmacological properties, including antimicrobial and anticancer activities. Its commercialization for biomedical applications, however, remains scarce. The major limitations are related to the lack of efficient bioprocesses and scaling up from laboratory to production. In the present work, the upstream process for prodigiosin production was developed using a marine *Serratia rubidaea* isolated from a sample collected near a shallow-water hydrothermal vent. The yield of product per biomass was found to be influenced by the cell concentration in the inoculum. The system was scaled up to 2 L stirred tank reactors with two different vessel geometries. It was shown that the vessel geometry and a cascade control mode for regulating the dissolved oxygen concentration influenced the volumetric oxygen mass transfer coefficient (k_La) and thus prodigiosin production. To improve product yields, strategies to mimic the aeration conditions found at the sampling site were tested. When the inoculum was grown for 5 h at 200 rpm and for 19 h at 25 rpm, which significantly decreased the oxygen available, the cells produced 588.2 $mg_{product}/g_{biomass}$, corresponding to a production of 1066.2 mg of prodigiosin in 24 h and a productivity of 36.1 $mg_{product}/(L.h)$. This is a 3.7-fold increase in prodigiosin yield and a 4.5-fold increase in productivity in relation to when no particular strategy was promoted. Additionally, it was shown that lipid analysis and flow cytometry may be used as reliable at-line analytical tools, allowing the monitoring of cell condition and prodigiosin production during fermentation.

Keywords: *Serratia rubidaea*; bioreactors; marine bioprocess; prodigiosin; lipidomics; flow cytometry

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Citation: Pereira, R.F.S.; de Carvalho, C.C.C.R. Mimicking Marine Conditions to Improve Prodigiosin Yields in Bioreactor. *Processes* **2024**, *12*, 1794. https://doi.org/10.3390/pr12091794

Academic Editor: Clayton Jeffryes

Received: 21 June 2024
Revised: 10 August 2024
Accepted: 19 August 2024
Published: 23 August 2024

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1. Introduction

With the increasing demand for more eco-driven processes, synthetic products are gradually being substituted by natural compounds. These natural compounds are presented as safer products in comparison with their synthetic counterparts, which are often hazardous to the environment, thus affecting human and animal health [1,2]. Amongst these natural products, the red pigment prodigiosin possesses a great potential not only as a natural dye but also as a drug precursor, with wide pharmacological properties ranging from antimicrobial to anticancer activity [1–3]. Although the beneficial properties of prodigiosin have been shown repeatedly, its industrial production and commercialization are still far from being a reality.

One of the many limitations to prodigiosin application is related to the lack of systematic production of prodigiosin at laboratory scale and its appropriate scale-up [1]. Marine bacteria may be good candidates to address this issue, together with the optimization of medium and fermentation conditions. The conditions found in the marine environment favor the adaptability of marine bacteria to rapid environmental changes, to a broad range of ecological niches, and to survive under environmental conditions found to be extreme by other bacteria [4]. This evolutionary pressure gave marine bacteria properties such as high

genetic plasticity [4], and unique cellular properties as high salt tolerance, piezophilicity, hyperthermo-stability, and cold adaptability [5–7]. However, regarding marine bioprocess development, research data on bioreactor engineering and fermentation protocol design is still scarce. The available strategies, used for the production of commercially interesting biochemicals, are mostly carried out in shaken flasks with low prospects for successful scale-up [8,9].

In this work, a bioprocess was developed using a marine *Serratia rubidaea* isolated from a sample collected from a shallow-water hydrothermal vent [10]. The advantages of using this marine *S. rubidaea* strain are related to its high adaptability to changing biotic and abiotic conditions. In a previous study, we showed that by improving medium composition and conditions, a final concentration of 293.3 mg/L of prodigiosin could be produced in 2 L bioreactors in 24 h [11]. The adaptability of this *S. rubidaea*, which is able to grow and produce prodigiosin at least between 15–62 °C, 19–80 g/L NaCl, pH 6.2–8.6 [10,11], makes it a good candidate for industrial application since gradients of such parameters are found in large-scale bioreactors due to poor mixing.

In the present study, stirred tank reactors were used in batch mode, as they still remain the backbone for screening conditions and process optimization. Thus, at minimum costs, maximum biomass and product yields may be achieved [12–14]. Stirred tank reactors provide efficient mixing, oxygen transfer, temperature, and pH control while allowing the simulation, to some extent, of the natural conditions found at isolation sites with good control over the environmental parameters [8]. The strategy used in this work was a simulation of the conditions of the sampling site from which the bacterium was isolated. The medium contained nutrients that allowed its proliferation in the ecological niche, and the conditions in the laboratory mimicked the abiotic conditions present during high and low tides, meaning periods of starvation and oxygen depletion during fermentation. Besides bioprocess development, the use of lipidomics and flow cytometry as analytical tools to monitor cell condition and prodigiosin production were also demonstrated. The ultimate goal of the study was to increase product yield, i.e., to increase the amount of prodigiosin produced per *S. rubidaea* cell, so that productivity in the bioreactor could also be increased.

2. Materials and Methods

2.1. Bacterial Strain

A *S. rubidaea*, isolated from a shallow-water hydrothermal vent as previously reported [10], was used. The strain is kept at −80 °C in 20% glycerol at iBB, Lisbon, Portugal.

2.2. Cell Growth

A cryopreserved aliquot of a *S. rubidaea* cell culture was grown on a Marine Agar plate (MA; Condalab, Madrid, Spain), and colonies were used to inoculate 100 mL Erlenmeyer flasks containing 40 mL of Marine Broth (MB; Condalab, Madrid, Spain). MB was supplemented to enhance both cell concentration and prodigiosin, as described previously [11]. Briefly, MB was supplemented with 5 g/L of meat peptone, 5 g/L of sodium glutamate (both from Sigma-Aldrich, St. Louis, MO, USA), and 0.2 g/L of $Fe_2(SO_4)_3$ (Riedel-de Haën, Sleeze, Germany). This medium is designated as supplemented MB (MB + S) in the text. The flasks were incubated overnight (approx. 16 h–18 h) in an orbital shaker (Aralab, Almada, Portugal), at 30 °C, and 200 rpm.

2.3. Effect of Inoculum Concentration

For the experiment assessing the effect of cell concentration in the inoculum, isolated colonies of *S. rubidaea* were used to inoculate 40 mL of MB + S, and the cultures were grown for 20 h at 30 °C and 200 rpm. A 24-well OxoDish® plate (from PreSens Precision Sensing GmbH, Regensburg, Germany) containing 1.35 mL MB + S per well was used to monitor cell growth, which was started by adding 150 µL per well of inoculum. Different initial cell concentrations of inoculum were tested, namely, from 0.01 g/L to 0.21 g/L of dried

cell weight (DCW). The plate was closed with a Breathe-Easy® sealing membrane (from Diversified Biotech, Dedham, MA, USA) to prevent medium evaporation. Cell growth was monitored online for 24 h by the dissolved oxygen concentration. The Oxodish® microtiter plates, according to the manufacturer, have a resolution of ±0.4% O_2 and a precision of ±1% O_2 at 20.9% O_2, and a drift < 0.2% O_2 within one week. The dissolved oxygen concentration data were acquired with the SDR_v37 software (also from PreSens). The plate was incubated at 30 °C and 200 rpm. All assays were performed at least in duplicate, with the DCW and product concentration measured at the end.

2.4. Bioreactors

A total of four 2 L bioreactors were used: two Minifors (BI; from Infors HT, Basel, Switzerland) and two Fermac 360 (BE; from Electrolab, Gloucesteshire, UK), with 1.2 L and 1.5 L of working volume, respectively. Both models are stirred tank reactors.

For the inocula for the bioreactors, 250 mL Kitasato flasks containing a volume of medium equivalent to 10% (v/v) of the final working volume of each bioreactor were used: 120 and 150 mL for the BI and BE bioreactors, respectively. The use of Kitasato flasks allows for an easier inoculation of the bioreactors under sterile conditions than Erlenmeyer flasks. The incubation conditions used were identical to those previously described, unless stated otherwise. The inoculum cell concentration was measured before the inoculation and diluted appropriately with sterile MB + S, when necessary, to reach a concentration of 0.04 g/L.

2.4.1. k_La Determination

The k_La in the bioreactors was determined by the static gassing-out method, using a polarographic probe (Mettler Toledo, Greifensee, Switzerland) to measure the dissolved oxygen. A total of four 2 L bioreactors were used, two BI and two BE, filled with 1.2 L and 1.5 L of a 3% NaCl solution, respectively. Nitrogen was used in the deoxygenated step, and compressed air was used in the reoxygenation step. The air flow into the bioreactors and temperature were maintained constant at 1 vvm and 30 °C, respectively. The stirring speed range was established between 100 and 800 rpm. All measurements were performed at least in duplicate.

2.4.2. Bacterial Culture

After inoculation with 10% of the working volume of the bioreactor with inocula grown on MB + S, *S. rubidaea* cells were grown in the four 2 L bioreactors previously mentioned. The BE contained 1.65 L of culture medium, and the BI contained 1.32 L. All experiments were performed in duplicate, in batch mode, for 24 h to 48 h under the following conditions: 30 °C; pH 7.2 ± 0.2 adjusted with the addition of 3 N H_2SO_4 and 2 N NaOH; air supplied at 1 to 2 vvm.

In the initial experiments to assess the effect of stirring speed on cell and product production, stirring rates were kept constant from 100 rpm to 400 rpm, and no value for dissolved oxygen concentration (DO) was defined as a set point (although this parameter was monitored). In the following experiments, the DO set point was changed between 30%, 40%, and 50% of air saturation, and the cascade mode was activated in all bioreactors. With the activation of the cascade mode, the stirring rates were maintained in a range between 200 rpm and 600 rpm, depending on the strategy employed. In the BE fermenters, which have an additional control loop on the cascade mode, it was possible to maintain the aeration between 0 and 2 vvm to reach the desired set point. Biomass, prodigiosin production, and the lipid profile of the cells were determined by following the protocols described previously [11].

To mimic the marine conditions found at the isolation site, from which the bacterium was isolated, six strategies were developed by interchanging low oxygen conditions with aerobic phases, and all were tested in the BI. The amount of dissolved oxygen is largely affected by wave movement and water temperature, since oxygen solubility decreases with

temperature. The last strategy consisted in applying the conditions that best increased prodigiosin productivity in the previous strategies in an in-series bioreactor approach.

S1—Phase with low oxygen followed by an aerated phase applied in the bioreactor

The bioreactors were filled with MB + S, taking into consideration the volume of inoculum to be added. The inoculum was grown as described in Section 2.4. After inoculation, cellular growth was monitored for 48 h. During the first 24 h, agitation was kept at 100 rpm, and no forced aeration of the medium was promoted (low oxygen conditions). During the following 24 h, aeration was kept at 1 vvm, with a defined set point of 40% air saturation, with the agitation speed being maintained between 450–600 rpm (aerobic conditions).

S2—Phase with low oxygen concentration between two aerated phases in the bioreactor

As in S1, the inoculum was grown as previously described and added to the bioreactor at 10% (v/v) of the final volume. The batch process was carried out for 48 h, divided in the following manner: (i) during the first 5 h, aerobic conditions were applied; (ii) during the following 19 h, low oxygen conditions were applied; (iii) and, in the remaining 24 h, the same conditions as in the first 5 h were promoted.

S3—Aerobic conditions in the bioreactor and addition of fresh medium

The inoculum was grown for 19 h with 10% of the final volume of the bioreactor at 30 °C and 200 rpm in a 1 L Kitasato flask. Afterwards, 600 mL of fresh medium was added to the inoculum, and the cells were allowed to grow for another 5 h. The bioreactor, which had 600 mL of fresh medium, was then inoculated with the 720 mL of inoculum, and growth proceeded for the next 24 h under the aerobic conditions defined in S1.

S4—Phase with low oxygen concentration between two aerobic phases in the inoculum

With this strategy, the transition between phases promoted in S2 in the bioreactor was applied to the inoculum flask. The inoculum was grown for 24 h, and during the first and last 5 h, a stirring speed of 200 rpm was implemented. During the 14 h between these two periods, an agitation speed of 25 rpm was set, and thus oxygen was depleted in the broth. After inoculation, cellular growth in the bioreactor was carried out for 24 h under the aerobic conditions defined in S1.

S5—Low oxygen concentration in the inoculum, aerobic conditions in the bioreactor

The inoculum for the bioreactor was grown as described for S4, but the aerobic conditions were applied only during the first 5 h. During the remaining 19 h, agitation was kept at 25 rpm to limit oxygen concentration. The conditions applied to the bioreactor were similar to those in S4.

S6—In-series bioreactors

After determining the best conditions for prodigiosin production, a system using in-series bioreactors was tested. The first bioreactor was inoculated with an inoculum grown under the conditions described in Section 2.4, and operated at 30 °C, pH 7.2 ± 0.2, and agitated between 450 and 600 rpm depending on the strategy phase sequence. After 24 h, the second bioreactor was inoculated with the cells grown in the first one. The second bioreactor was filled with half of its working volume with fresh medium and inoculated with 60% of the working volume of the first bioreactor, the final volume being 1.32 L. Cellular growth in the second bioreactor was monitored for the following 24 h.

2.5. Analytical Methods

2.5.1. Off-Line Quantification of Biomass (X) and Prodigiosin (P)

For biomass and product quantification, 1 mL of sample was harvested. Cellular growth of *S. rubidaea* cells was monitored by measuring the optical density at 600 nm (OD_{600}) and converting its value into DCW according to a calibration curve of DCW vs. OD_{600}. This calibration curve was periodically checked by measuring the OD_{600} of samples and weighting. After centrifugation at 12,500× g for 5 min (in a centrifuge from HERMLE

Labortechnik GmbH, Wehingen, Germany), the respective cell pellets were dried at 65 °C for 24 h.

For product quantification, the off-line procedure consisted in its extraction from cell pellets (obtained by centrifugation of 1 mL of cell culture) with 1 mL of a 3:1 ethyl acetate–acetic acid solution (both from Sigma-Aldrich, St. Louis, MO, USA), as previously reported [11]. The mixture was analyzed between 200 and 800 nm, and the conversion of the 535 nm peak value into the concentration of the product was performed using a calibration curve. DCW and prodigiosin quantification were both determined in 96-well microtitter plates (MTP), with product quantification being performed in a glass-coated MTP (Plate+™, Greiner Bio-One GmbH, Kremsmünster, Austria). Measures were carried out by a Multiskan Go spectrophotometer microplate and cuvette reader from Thermo Fisher Scientific (Whaltham, MA, USA).

2.5.2. Lipid Analysis

The fatty acid profile of the cells was performed after their extraction and methylation to fatty acid methyl esters (FAMEs), as previously described [15]. Briefly, 1 mL samples were taken every hour during the fermentation process. Cells were harvested by centrifugation at 12,500× *g* for 5 min and washed with milli-Q water. The extraction and methylation of fatty acids were performed simultaneously using the Instant FAME method from MIDI (MIDI, Inc., Newark, DE, USA) [16]. FAMEs were analyzed by gas chromatography on an Agilent Technologies 6890N gas chromatograph, with a flame detector and a 7683 B series injector using a 25 m Agilent J&W Ultra 2 capillary column. The FAME profile was obtained from the Sherlock® software package (version 6.2) using the PLFAD1 method. The FAMEs were identified by the MIDI software and also by using calibration standards.

2.5.3. At-Line Monitoring of Cell Viability and Product Production

From the inoculum for the bioreactor, two 1 mL samples were taken, and the pellet was washed with milli-Q water. After centrifugation at room temperature at 12,500× *g* for 5 min, the pellet was resuspended in Tris-HCl buffer pH 7.0 (Eurobio Scientific, Les Ulis, France) and further diluted to approximately 3.0×10^8 cells/mL (corresponding to 1.0 McFarland standard). To make the negative control, where only non-viable cells were present, 500 µL of a 25% glutaraldehyde solution (Acros Organics, Geel, Belgium) was added. The cells were stained using the LIVE/DEAD™ *Bac*Light™ Bacterial viability kit (Thermo Fisher Scientific), which contains Syto 9 and propidium iodide (PI), according to the specifications of the manufacturer [17]. From the bioreactor, two 2 mL samples were taken along the 24 h of growth and diluted to a concentration of 6.0×10^5 cells/mL. Two sets of diluted samples were prepared, with only one set being stained with the SYTO 9/PI fluorescence dye mixture and the other analyzed directly.

Both sets and controls were analyzed with a Cyflow Space flow cytometer (Sysmex Partec, Görlitz, Germany), and acquisition and data treatment were carried out with the FloMax® software (also from Sysmex Partec). The marked samples, diluted to 3.0×10^8 cells/mL, were also analyzed by fluorescent microscopy with an Olympus CX40 microscope (Olympus, Tokyo, Japan) equipped with an Olympus U-RFL-T burner and an U-MWB mirror cube unit. The images were captured by an Evolution™ MP5.1 CCD color camera using the software Image-Pro Plus 5.1, both from Media Cybernetics, Inc. (Rockville, MD, USA).

2.6. Statistics

A one-way ANOVA was applied to oxygen consumption rate and product from biomass yield data, with an unplanned comparison being made using Tukey's B test ($\alpha = 0.05$) using IBM SPSS Statistics 29.

3. Results and Discussion

3.1. Assessing the Best Initial Inoculum Concentration

A suitable inoculum is an essential part of the development of a new bioprocess based on whole cell cultivation. In order to have an active and proliferative cell population, the nutritional requirements of the strain must be taken into consideration when developing the culture medium. In this work, supplemented MB (MB + S) was used since it was shown to be the most suitable for *S. rubidaea* growth and prodigiosin production [11]. To assess the effect of inoculum concentration on biomass and prodigiosin yields, experiments were carried out in microtiter plates with real-time oxygen monitoring, with the inoculum concentration ranging from 0.01 to 0.21 g/L of DCW (Figure 1).

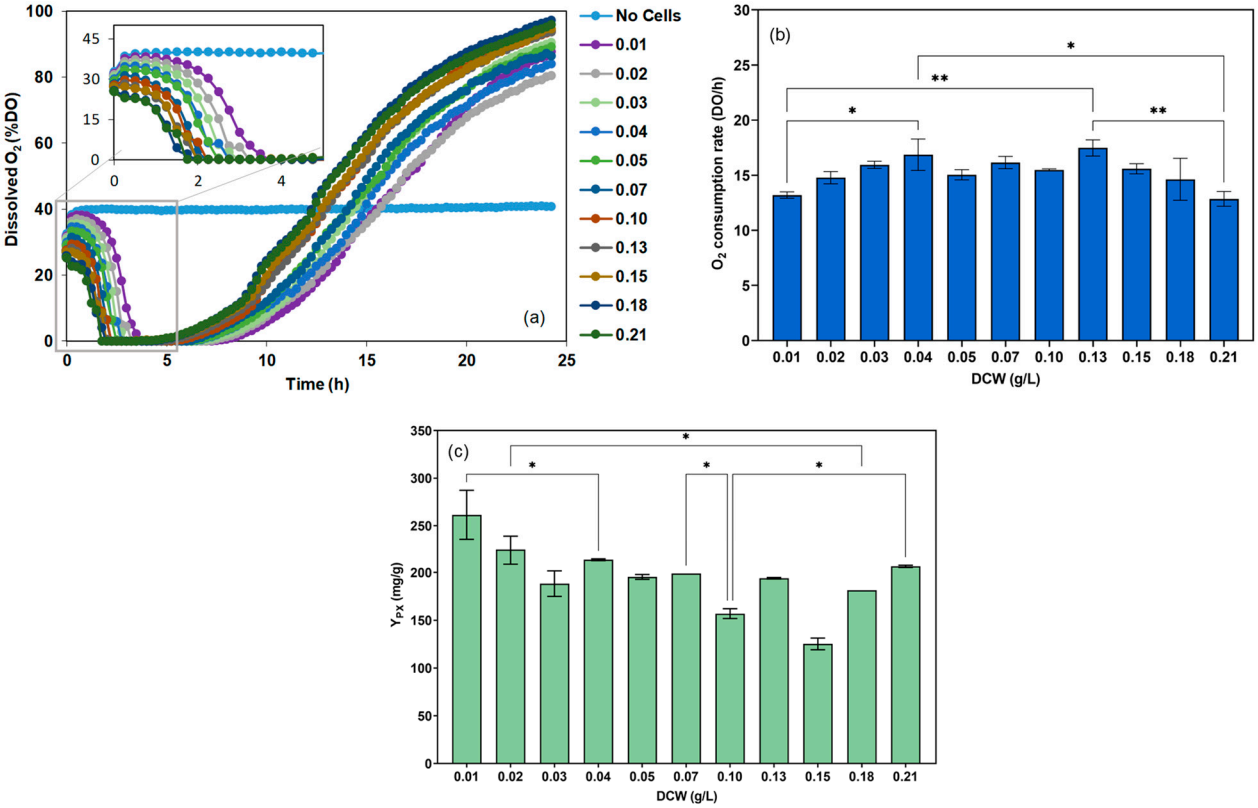

Figure 1. Effect of inoculum cell concentration on: oxygen profile measured in real time during growth (**a**); oxygen consumption rate (**b**); and product per biomass yield (**c**). DCW refers to the initial concentration in the well. The cells were grown for 24 h at 30 °C and 200 rpm in MB + S in microtiter plates with dissolved oxygen monitoring in real-time. With the exception of (**a**), the values are represented as the average ± standard deviation. Statistical significance was determined by one-way ANOVA (* $p < 0.05$; ** $p < 0.003$; only significant differences are indicated).

After inoculation with different cell concentrations, the dissolved oxygen concentration in each well was monitored in real time (Figure 1a) and used as a proxy for *S. rubidaea* growth [18]. The fast oxygen depletion, which corresponds to the exponential growth phase, increased with the inoculum's increasing cell concentration. Curiously, the growth curves of cultures inoculated with 0.13 and 0.15 g/L and with 0.18 and 0.21 g/L were identical. However, as expected, the growth obtained in the cultures inoculated with a higher cell density was faster. The specific oxygen consumption rate, up to a concentration of 0.04 g/L of DCW, increased 3.7% DO/h (Figure 1b). This increase in oxygen consumption suggests a relation between the initial low concentration of cells and a greater abundance of resources available per cell. Between the initial concentrations of 0.05 and 0.10 g/L of DCW, an oxygen consumption plateau was reached, indicating a possible equilibrium between the cell population and resources. From 0.13 to 0.21 g/L of DCW concentrations,

the oxygen consumption decreased 4.6% DO/h, indicating a corresponding lower growth rate. Regarding prodigiosin production, a decrease in the product per biomass yield (Y_{PX}) until 0.03 g/L of DCW concentration in the inoculum was observed (Figure 1c). For cell cultures inoculated with 0.04 g/L or higher cell concentrations, similar Y_{PX} values were obtained. To ensure the highest yield of Y_{PX} and growth rate, the inocula used in the following experiments were prepared so that their cell concentration was ca. 0.04 g/L of DCW. Higher inoculum concentrations could be used, but this allows for lower costs as cells may be transferred to the fermenter earlier.

3.2. Fermentation Conditions

3.2.1. Effect of Stirring Speed

The maintenance of the volumetric mass transfer coefficient, k_La, as a scale-up parameter has the advantage of allowing oxygen transfer rates to be transversal throughout scales and geometries. To determine the k_La values obtained in the bioreactors used in the present study, the static gassing-out method was applied.

The values of k_La increased with the stirring speed used in the bioreactors up to 800 rpm (Figure 2a). The variations in k_La values obtained between bioreactor types are mostly related to the geometric design of the vessels (Figure 2b) and mixing apparatus [19]. It was noticed that from 100 rpm to 300 rpm, the k_La values attained in the BE were, on average, 25% higher than the ones in the BI. However, from 400 rpm to 800 rpm, the BI allowed for better oxygen transfer, resulting, on average, in a 76% increase in k_La.

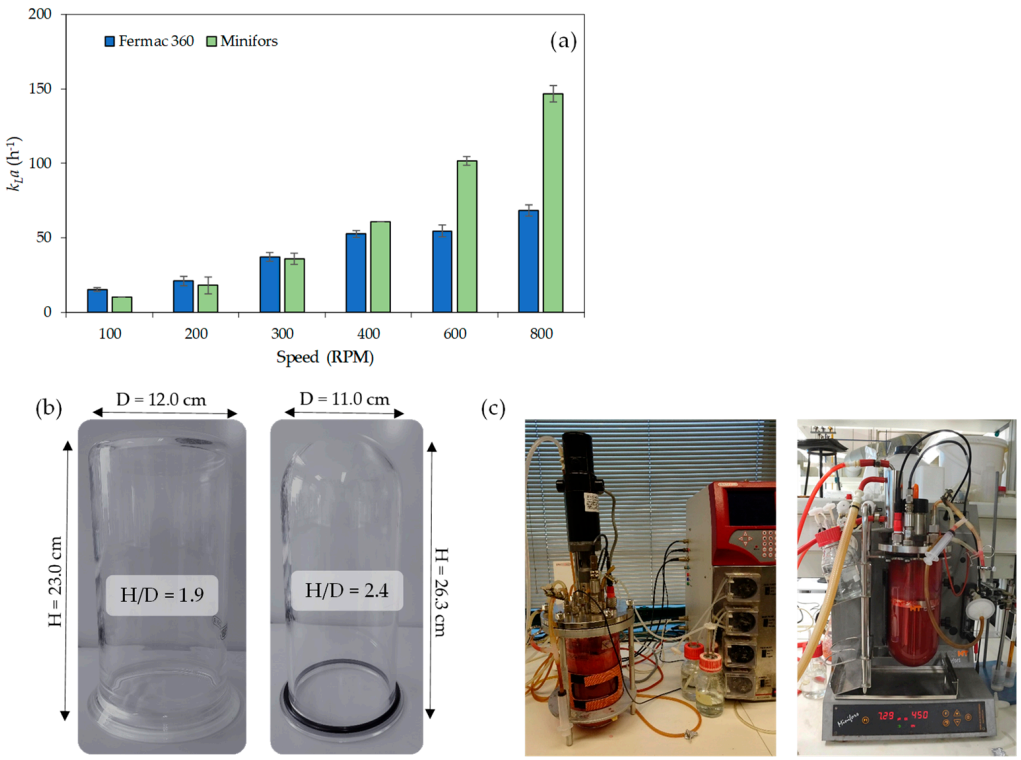

Figure 2. Effect of stirring speed on the volumetric mass transfer coefficient, k_La, on bioreactors with different vessel geometries: (**a**) variation of k_La with agitation speed in Fermac 360 (BE) and Minifors (BI) bioreactors, using a 30 g/L NaCl solution at 30 °C. The values are represented as the average ± standard deviation. (**b**) Vessel geometry: BE (left); BI (right). (**c**) Bioreactors during operation: BE (left); BI (right).

To assess the influence of stirring speed on biomass and prodigiosin yields while maintaining 1 vvm of aeration, 8 fermentations with 4 different stirring speeds were carried out (each condition tested in duplicate). The culture medium contained only MB, and stirring speeds between 100–400 rpm were tested (Figure 3). Other parameters, e.g., pH

and temperature, were kept constant at the values that allowed the best results in biomass and product production in shaken flask studies previously conducted [10,11]. Due to the similarity of k_La values obtained from 100 to 400 rpm in both bioreactor models, these experiments were only performed in the BE.

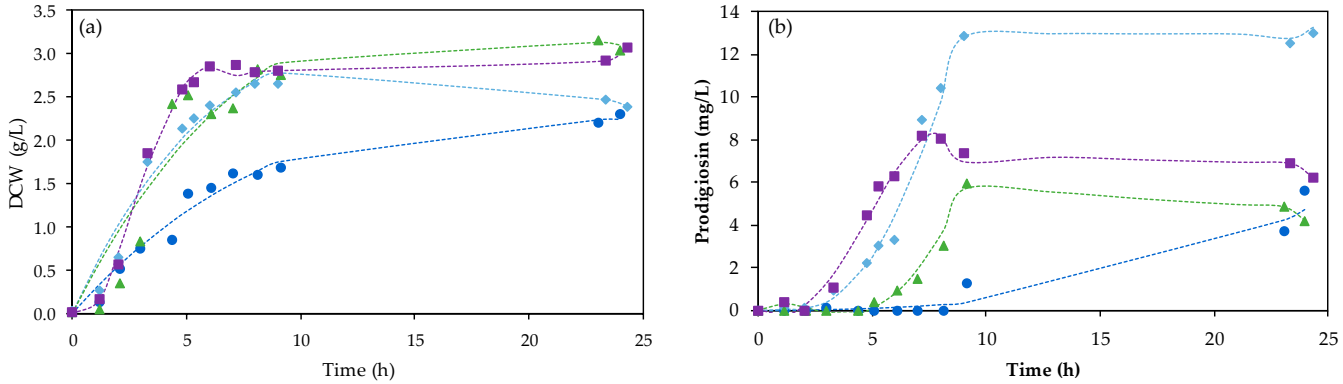

Figure 3. Influence of different stirring speeds on the BE fermenter on: (**a**) biomass concentration; and (**b**), prodigiosin production. Temperature was kept at 30 °C, pH at 7.2 and aeration at 1 vvm. The stirring speed was maintained at 100 rpm (●), 200 rpm (♦), 300 rpm (▲), or 400 rpm (■). The values are represented as the average ± standard deviation.

With the increase in agitation speed, a better aeration of the medium was obtained, resulting in a 33% increase in biomass (Figure 3a). Although prodigiosin production is known to be oxygen-dependent [20–22], the highest concentration was obtained under an agitation of 200 rpm, with similar concentrations being attained for the remaining stirring speeds (Figure 3b). On average, a 2.2-fold increase in prodigiosin concentration was achieved at 200 rpm. At 300 rpm and 400 rpm, the lower quantity of prodigiosin might be related to shear stress or metabolic changes in the cells. Higher biomass productivity was thus attained at 300 rpm, but higher prodigiosin productivity was achieved at 200 rpm (Table 1).

Table 1. Growth parameters and volumetric productivities achieved for *S. rubidaea* biomass and prodigiosin at different agitation speeds in MB. μ_{max}—maximum growth rate; t_d—duplication time; Pr^{DCW}—cell productivity; $Pr^{Product}$—product productivity.

Stirring Speed (rpm)	Measured DO (%)	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/(L.h))	$P_r^{Product}$ (mg/(L.h))
100	0–80	1.26 ± 0.22	0.55 ± 0.03	95.3	0.23
200	0–90	1.16 ± 0.21	0.60 ± 0.03	104.4	0.54
300	11–96	1.23 ± 0.22	0.56 ± 0.03	136.2	0.21
400	47–97	1.31 ± 0.24	0.53 ± 0.03	123.9	0.30

Interestingly, comparison of biomass and product curves (Figure 3) indicates that product formation may be classified as mixed-growth-associated because its presence is initially observed during the middle of the exponential phase, and its concentration increases during the stationary phase.

3.2.2. Applying Cascade Control to Maintain Oxygen Concentration

Due to the inherent complexity of a fermentation, the occurrence of disturbances, which are in most cases difficult to predict, may become the reason for batch-to-batch variability. Using process control, specifically "cascade" control, controlled elements that exhibit nonlinear behavior, e.g., dissolved oxygen (DO), are dealt quickly with low interference to the overall process [23]. By defining a percentage of DO that is near the optimum and above a critical value, the process oxygen requirements will be met [24].

With cascade control, the output from a master controller (DO) is compared with the established set-point, and any deviation from it is used to automatically adjust the desired value of a slave controller (e.g., agitation speed, air flow) so that the master reading has an insignificant deviation from the desired set-point [25]. This will assure the oxygen needs of the strain at every growth stage and may lead to the highest possible amount of product being produced.

According to Junker [26], in fermentations where cellular growth does not change considerably the overall viscosity of the medium, a DO set-point value in the range between 10–30% of air saturation in the media is sufficient to enable cellular growth and increase product production. However, if mycelial-forming strains are used or the goal is to achieve a high cell density culture in fermentation, DO set-point values transit to 30–70% of air saturation to allow a good oxygen distribution. In this work, the medium chosen has a viscosity similar to sea water, and a 40% DO saturation was initially defined. Using MB + S, the BE cascade mode was implemented with a 0 to 1 vvm of aeration and an agitation speed between 200 and 300 rpm, since 200 rpm improved the production of prodigiosin while 300 rpm increased biomass production. In order to maintain a similar k_La value in BI, the cascade mode was set as in BE but with a constant air flow at 1 vvm. Comparing the results shown in Table 2 with those obtained by Pereira et al. [11] in the bioreactor, the application of cascade mode increased, on average, the biomass productivity 7.9% and the prodigiosin productivity 12-fold.

Table 2. Growth parameters and productivities achieved for biomass and prodigiosin with 40% dissolved oxygen as the set point. * Data adapted with permission from Pereira et al. [11].

Bioreactor	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/(L.h))	$P_r^{Product}$ (mg/(L.h))	[X] (g)	[P] (mg)	Y_{px} (mg/g)
BE	0.63 ± 0.04	1.1 ± 0.06	117.8	15.03	4.02	541.3	134.5
BE *	0.29 ± 0.04	2.43	73.2	1.64	2.63	59.0	22.4
BI	0.28 ± 0.04	2.5 ± 0.35	50.9	8.02	1.83	288.6	157.6
BI *	0.24 ± 0.04	2.88	69.1	0.22	1.99	6.30	3.2

Even when maintaining the same k_La in both bioreactors, the productivity of both biomass and product in the BE was 2-fold higher than in the BI. However, oxygen limitation was observed in the experiments conducted in the BE at stirring speeds higher than 400 rpm, due to the vessel geometry. The following experiments were carried out to evaluate the effect of oxygen concentration on prodigiosin production in the BI.

3.3. Fermentation Strategies to Mimick Sampling Site Conditions

The fluctuating conditions found in marine environments and the variability in nutrient composition resulted in the selection of particular species, which, in order to survive and thrive, may produce specific substances such as quorum-sensing molecules [7,27]. This marine *S. rubidaea* strain belongs to one of these species that have adapted to an intertidal zone in which cycles of nutrient abundance and starvation (including of oxygen) are dependent on seawater movement. Additionally, since the sampling site is located near an intertidal shallow-water hydrothermal vent, the conditions vary significantly between low tide, when the temperature of the sampling site is ca. 60 °C, and high tide, when the sampling site is at the temperature of the Atlantic Ocean (ca. 15 °C in winter) [10,28]. This unique place located in S. Miguel Island, in the Azores, Portugal, is a semi-enclosed rock pool with a surface area of ca. 300 m^2. It is 3–4 m deep, with a south-facing aperture 3 m wide and 2 m deep that opens to the Atlantic Ocean. Its dimensions, low shore slope, and irregular profile flanks of basalt boulders shelter the sampling site from waves during low tide and calm seas [29]. These topological characteristics, combined with the Azores current propagating flow being usually weak with a dominantly southeast direction from December to April, which reduces wave formation energy and tidal range to a maximum of 1.8 m, even in winter when the stronger winds are formed, confer to the sampling site

gentler conditions and flatter waves than other parts of the Azorean coast [30,31]. On the day of sampling at the isolation site, the low and high tides corresponded to 0.6 and 1.5 m, respectively [32]. The developed strategies thus tried to mimic several possible conditions found in the sampling site, namely: submerged situation with nearly no wave movement (low aeration conditions); submerged situation with wave movement (aerated conditions); low amount of water in the pool (higher temperature due to a larger proportion of thermal water and thus lower dissolved oxygen concentration); and, a high amount of water in the pool (lower temperature due to a larger contribution of seawater; aeration conditions depending on wave movement).

To determine the conditions that would induce *S. rubidaea* cells to produce more prodigiosin, five strategies were developed to mimic the variability of the sampling site (Table 3). In S1, the cells were exposed to a 24 h cycle of oxygen starvation followed by 24 h of aeration to mimic conditions of low and high seawater movement, respectively. In S2, the goal was to mimic conditions of different amounts of water in the pool and with different wave energy: the cells were aerated for 5 h, followed by 19 h without aeration, and another 24 h aerated at 1 vvm. S3 mimicked not only oxygen starvation and aerated periods in the inoculum but also the addition of fresh medium to simulate the increased availability of nutrients to the marine microbes during seawater entrance in the natural pool. Strategies S4 and S5 are similar to S2, but oxygen starvation and abundancy periods were applied during inoculum growth. The difference between S4 and S5 is the application of the aerobic conditions during the last 5 h of the inoculum in S4.

Table 3. Fermentation conditions during the S1–S5 strategies for cell growth and production. MB + S*—note the different volume in S3.

Strategy	Inoculum (medium = 0.12 L, except *)			Reactor (medium = 1.32 L)		
S1	20 h, 200 rpm			24 h, 100 rpm, 0 vvm	24 h, 450–600 rpm, 1 vvm	
S2	20 h, 200 rpm			5 h, 450–600 rpm, 1 vvm	19 h, 100 rpm, 0 vvm	24 h, 450–600 rpm, 1 vvm
S3	19 h, 200 rpm, 0.12 L	5 h, 200 rpm, 0.12 L + 0.6 L MB + S*		24 h, 450–600 rpm, 1 vvm		
S4	5 h, 200 rpm	14 h, 25 rpm	5 h, 200 rpm	24 h, 450–600 rpm, 1 vvm		
S5	5 h, 200 rpm	19 h, 25 rpm		24 h, 450–600 rpm, 1 vvm		

The existence of an oxygen depletion period during *S. rubidaea* cell growth, followed by aerated conditions, considerably enhanced prodigiosin production (Table 4). However, more than 19 h of exposure of these cells to oxygen depleted conditions led to a fast cell proliferation rate but low amounts of product (S1). Intercalating a stage without forced aeration between two aerobic stages (S2) led to a 30% increase in prodigiosin concentration when compared to S1. A fed-batch strategy applied to the inoculum (S3) resulted in a 5% increase in prodigiosin production in comparison to S1. Comparison of S3 with other strategies showed that the amount of product was, on average, reduced in 74%. When the oxygen depleted condition was applied to the inoculum (S4), the amount of prodigiosin increased by 62% in relation to the previous strategies. In the case of S5, by removing the final 5 h under aerobic conditions during inoculum growth, the production of prodigiosin increased by 33% relative to S4. Of all the different strategies tested, S5 resulted in a 3.7-fold increase in both prodigiosin production and yield when compared to previous fermentations where no particular strategy was applied (Tables 2 and 4).

Table 4. Growth parameters, productivities, final amount of biomass and product in the bioreactor BI, and respective product from biomass yield following the application of the S1–S5 strategies.

Strategy	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/L.h)	$P_r^{Product}$ (mg/L.h)	[X] (g)	[P] (mg)	Y_{px} (mg/g)
S1	0.45 ± 0.02	1.6 ± 0.08	114.4	4.7	11.0	448.2	40.7
S2	0.36 ± 0.16	2.2 ± 0.83	43.7	10.6	2.4	583.7	242.1
S3	0.27 ± 0.04	2.6 ± 0.34	51.5	13.8	1.5	398.5	267.4
S4	0.40 ± 0.11	1.8 ± 0.53	73.6	28.5	2.3	802.0	350.5
S5	1.39 ± 0.03	0.5 ± 0.01	62.7	36.1	1.8	1066.2	588.2

Figure 4 shows how much productivity could be increased after implementing each strategy.

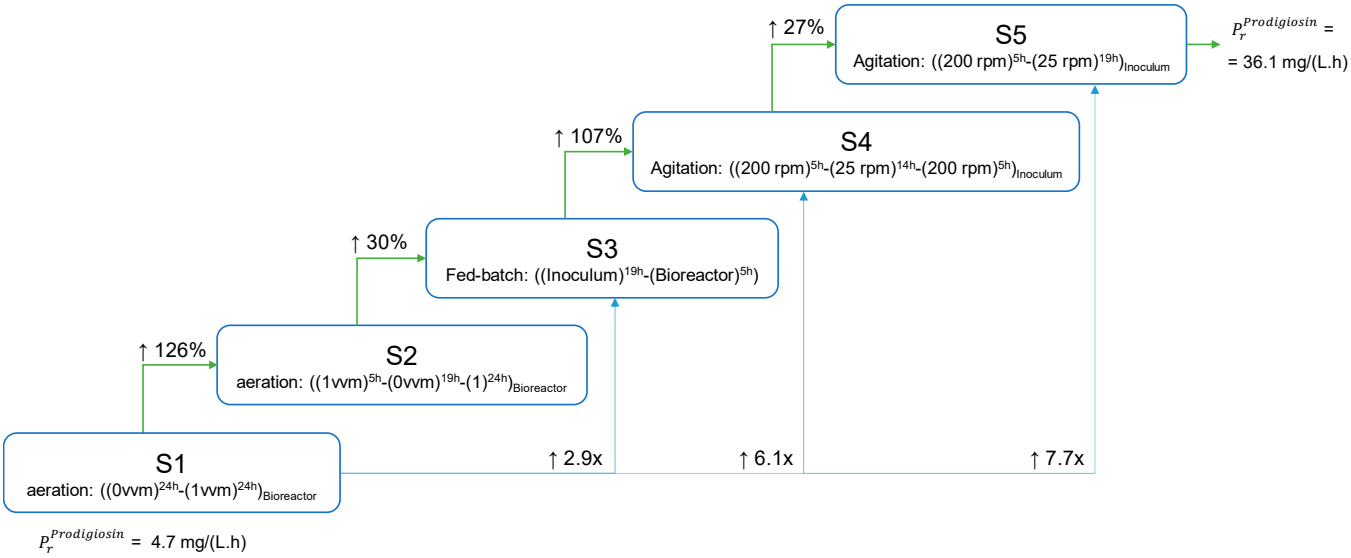

Figure 4. Conditions implemented in each strategy tested and the corresponding increase in productivity that was attained.

3.4. Assessing the Best Fermentation Conditions

To further optimize the fermentation process, the pH, aeration, and stirring conditions were further studied using S5 as the starting point and as a control for comparison of biomass and product yields. The first approach was to assess the effect of changing the DO set point between 30% and 50% saturation. The second approach carried out was to allow a larger range of stirring speeds to control DO inside the bioreactor by increasing the agitation range applied from 450–600 rpm to 200–600 rpm, as stirring speeds around 200–300 rpm led to an increase in prodigiosin productivity (Table 1). The third approach consisted in decreasing the pH setpoint from 7.2 ± 0.2 to 6.5 ± 1.5. As previously mentioned, we have shown that the *S. rubidaea* strain is able to produce prodigiosin under different temperature and pH values [10], which may be beneficial to endure the gradients of these parameters observed inside industrial-scale bioreactors.

With the first approach, when the DO value was changed from 40% to 30% and to 50%, the amount of biomass produced increased 2.3- and 3.0-fold, respectively (Tables 4 and 5). However, prodigiosin concentration decreased by 30% and 54%, respectively. These changes in production should have been related not only to the aeration conditions but also to the agitation profile, resulting from the cascade mode controlling DO in the bioreactor. As shown in Table 1, prodigiosin formation might be higher at lower stirring speeds. At 30% DO, the agitation speed might have limited mass transfer, leading to the formation of pH and oxygen gradients, as shown previously [33–36].

Table 5. Growth parameters and kinetics of *S. rubidaea* cells. Effect of aeration and pH set points on biomass and prodigiosin production, productivity, and yield.

Conditions	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/L.h)	$P_r^{Product}$ (mg/L.h)	[X] (g)	[P] (mg)	Y_{px} (mg/g)
DO 30% 450–600 rpm	0.34 ± 0.13	2.2 ± 0.87	144.3	11.2	4.2	323.8	77.8
DO 50% 450–600 rpm	0.64 ± 0.05	1.1 ± 0.09	186.4	19.9	5.4	577.9	107.2
DO 40% 450–600 rpm pH 5.0–8.0	0.56 ± 0.23	1.3 ± 0.55	139.0	9.5	4.00	273.7	68.3
DO 40% 200–600 rpm Aerated inoculum	0.55 ± 0.04	1.2 ± 0.08	135.0	20.3	3.91	595.3	150.3
DO 40% 200–600 rpm Inoculum as S5	0.32 ± 0.06	2.2 ± 0.46	166.0	16.2	4.79	468.1	97.7

In the second approach, the agitation range was increased to include lower stirring speeds, which resulted in a 2.4-fold increase in biomass but did not induce *S. rubidaea* cells to produce more prodigiosin; a 50% reduction in its concentration was observed in comparison with S5 (Tables 3 and 5). It was also noticed that under these conditions, it was more advantageous to grow the inoculum without an oxygen depleted stage than applying the conditions of S5.

The third approach used was based on our previous work [11], where we have shown the adaptability of this marine strain to different pH values and its capacity to produce prodigiosin at every value tested. It was also shown that, independently of the starting pH value and even in the presence of a buffer system added to MB + S, the by-products produced by this *S. rubidaea* change the medium pH value, reaching a value of 8.5 at the end of the fermentation, which was compensated in the bioreactor by the addition of acid (Figure 5). This is the common pH value of seawater. The lower control over the pH value of the fermentation broth, by allowing the pH set point to be 6.5 ± 1.5, led to more metabolic side reactions (other compounds could be detected by gas chromatography), reducing prodigiosin production (Table 5). As in the previous approaches, the amount of biomass increased 2.2-fold, but a 75% decrease in prodigiosin production was observed.

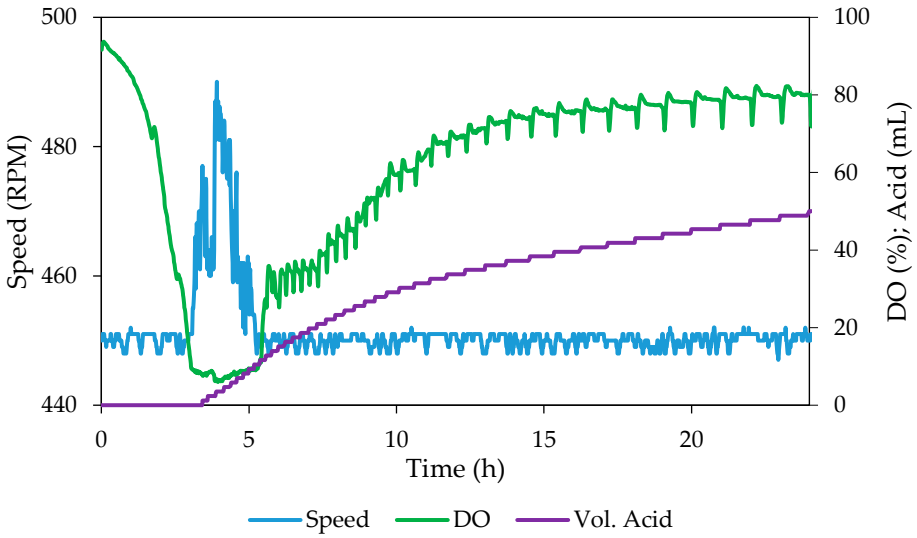

Figure 5. Influence of an imposed set-point of pH of 6.5 ± 1.5 on the stirring speed, dissolved oxygen concentration (DO), and volume of acid added to the fermenter.

3.5. In-Series Strategy to Enhance Prodigiosin Production (S6)

With strategy S6, the goal was to apply the operating conditions of strategies that led to the highest amount of prodigiosin (S4 and S5) in bioreactors placed in series. The typical flasks used to grow bacterial inocula do not possess systems to monitor and control pH and DO. To overcome this, a strategy to assess how the control of parameters during inoculum growth would influence prodigiosin production in *S. rubidaea* cells, during the main fermentation, was tested. In the first bioreactor, the cells were grown according to the "inoculum" conditions of S4 or S5 listed in Table 3 but updated to the following bioreactor operating conditions: the 25 rpm period was adjusted to 100 rpm at 0 vvm and the 200 rpm period was adjusted to 450–600 rpm and 1 vvm. After 24 h, 720 mL of the culture medium were transferred to the second reactor, which contained 600 mL of fresh MB + S. These strategies are designated as $S6_a$ (when S4 conditions were used in both reactors) and $S6_b$ (when S5 conditions were applied in both reactors). A third strategy involved similar conditions to $S6_b$, but the culture in the first bioreactor was allowed to grow for further 24 h, after inoculation of the second bioreactor, with an aeration set at 2 vvm and 450–600 rpm. This strategy is designated as $S6_c$. The values presented in Table 6 reflect the production in the combined bioreactors.

Table 6. Application of two in-series bioreactors for the growth of *S. rubidaea* cells: growth rates, productivities, and final amount of biomass and prodigiosin.

	Inoculum Bioreactor		**Production Bioreactor**		**System (Both Bioreactors)**				
Strategy	μ_{max} (h^{-1})	t_d (h)	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/L.h)	$P_r^{Product}$ (mg/L.h)	[X] (g)	[P] (mg)	Y_{px} (mg/g)
$S6_a$	0.46 ± 0.05	1.5 ± 0.06	0.36 ± 0.02	1.9 ± 0.23	104.8	32.0	3.76	802.4	305.1
$S6_b$	0.57 ± 0.17	1.2 ± 0.56	0.20 ± 0.09	3.4 ± 1.01	215.8	22.6	5.02	630.5	104.9
$S6_c$	0.57 ± 0.17	1.2 ± 0.56	0.02 ± 0.001	30.3 ± 1.51	204.6	30.5	5.92	879.5	149.2

$S6_a$ and $S6_c$ strategies gave similar results in terms of prodigiosin: $S6_c$ allowed a 58% higher concentration of biomass, but only ca. 10% higher prodigiosin concentration (Table 6). Based on these results, it is shown that higher control during "inoculum" growth (which could be obtained by growing it in bioreactors instead of Erlenmeyer flasks) results in higher production of biomass (Tables 4 and 6). However, this results in a lower amount of prodigiosin being produced and, especially, in lower product from biomass yields. It can be inferred that a larger variability of conditions improve prodigisin production in this *S. rubidaea* strain.

3.6. k_La Maintenance Conditions from BI to BE

Using the maintenance of k_La, S5 growth conditions were applied to the BE. To achieve k_La values in the BE similar to the BI, at 450–600 rpm, the aeration rate was increased to 2 vvm. By applying strategy S5 to a different bioreactor, it was possible to assess the influence of the geometry of the vessel, of aeration rate, and of the activation of a second control loop in cascade mode on biomass growth and prodigiosin production (Table 7). In the presence of a second loop in the cascade mode controlling aeration, the air entering the bioreactor is controlled to maintain the DO set point, contrary to when only a single loop in cascade mode is applied, during which only stirring speed is used to control the DO.

Using the conditions that induced the highest production of prodigiosin in the BI, the BE vessel geometry limited the production of prodigiosin, on average, 5.7-fold (Table 7). Comparing both conditions applied during fermentations in the BE, a higher aeration rate, without a cascade control over air flow, allowed for an increased production of prodigiosin of 26%. Nevertheless, biomass grew at a faster rate. As an overall comparison of the BE performance, it is possible to observe that the S5 strategy led to a decrease in biomass and prodigiosin yields, on average, of 1.4-fold and 2.9-fold, respectively, in relation to the results in Table 2.

Table 7. S5 strategy applied to BE: growth parameters, productivities, and final amount of biomass and prodigiosin as a result of doubling the aeration rate to 2 vvm and applying a second control loop over aeration.

Conditions	μ_{max} (h^{-1})	t_d (h)	P_r^{DCW} (mg/L.h)	$P_r^{Product}$ (mg/L.h)	[X] (g)	[P] (mg)	Y_{px} (mg/g)
cascade mode off	0.48 ± 0.02	1.4 ± 0.07	77.9	7.08	2.24	203.9	91.0
2 control loops in cascade mode	0.87 ± 0.04	0.8 ± 0.04	107.3	5.61	3.10	161.7	52.3

3.7. At-Line Analytical Techniques to Assess the Biological Condition of Cells and Prodigiosin Presence

Traditional methods of monitoring key bioprocess parameters are seldom sufficiently sensitive due to the dilution of the medium and the presence of non-culturable cells, such as in the case of optical density measurements and plate count methods [37]. These methods are unable to discriminate accurately between live and dead cells, debris and small-sized bacteria, and mixed subpopulations. So analytical methods allowing for the accurate monitoring of biological parameters in a fermentation are in great demand. The analytical methods should be able to give quick information about the processes, allowing rapid responses to optimize the overall yield [38]. Two examples are lipidomics and flow cytometry. Regarding lipidomics, when bacteria are under stressful environmental conditions, they change the fatty acid (FA) composition of their membranes as an adaptive mechanism with the intent of maintaining their integrity and fluidity level [39,40]. This adaptation is paramount to the several functions of the cellular membrane, which may include: permeability barrier to solutes; maintenance of turgor pressure and energy; quorum sensing; and other energy-transduction processes [40]. These adaptations to the environmental conditions and media composition influence the lipid content of the cells, and so lipidomics becomes an analytical tool to assess cell fitness and product production in a bioprocess [39]. Compared with traditional techniques, the use of flow cytometry for at-line monitoring is becoming common practice. Flow cytometry simultaneously offers individual cell level multiparametric discrimination like size and complexity, swift and statistically accurate measurements, and a better insight into a cell's viability, growth, and productivity in a myriad of different process microenvironments [38].

3.7.1. Analysis of Cell Lipids

Bacterial cells are able to modulate the fluidity of their cellular membranes by making changes in the fatty acid composition of their phospholipids as a response to, e.g., the carbon source, changes in temperature and salinity, and other environmental conditions [39,41]. We have recently shown how *S. rubidaea* cells are able to modify their lipid composition in response to the carbon and nitrogen sources used for cell growth, the growth temperature, the presence of metal ions, and prodigiosin [11].

In the present study, the FA profile of the cells was determined to study the influence of the imposed growth conditions, namely medium composition, 30 °C, pH 7.2, 40% DO, 200–300 rpm, and of the bioreactor vessel geometry, on *S. rubidaea* cells during a 24 h fermentation (Figure 6). The observed lipid profile of these cells followed the typical profile observed during cell aging throughout the stages of microbial growth [11,39]. However, it was possible to observe significant differences between the profiles of the cells grown in the two models of bioreactors (Figure 6). These differences were related to prodigiosin production and accumulation, which in the BE begins at the 5th h, whilst in the BI it was only observed after the 8th h of fermentation.

These alterations to the FA profile composition were studied to assess the possibility of using the FA profile of the cells as a tool to predict the amount of prodigiosin that would be attained at a certain time of fermentation. This was achieved by applying partial least squares (PLS) statistical analysis, where the lipid composition of the cell membrane was

used as the matrix variables, and the concentration of prodigiosin as the response array (Figure 7).

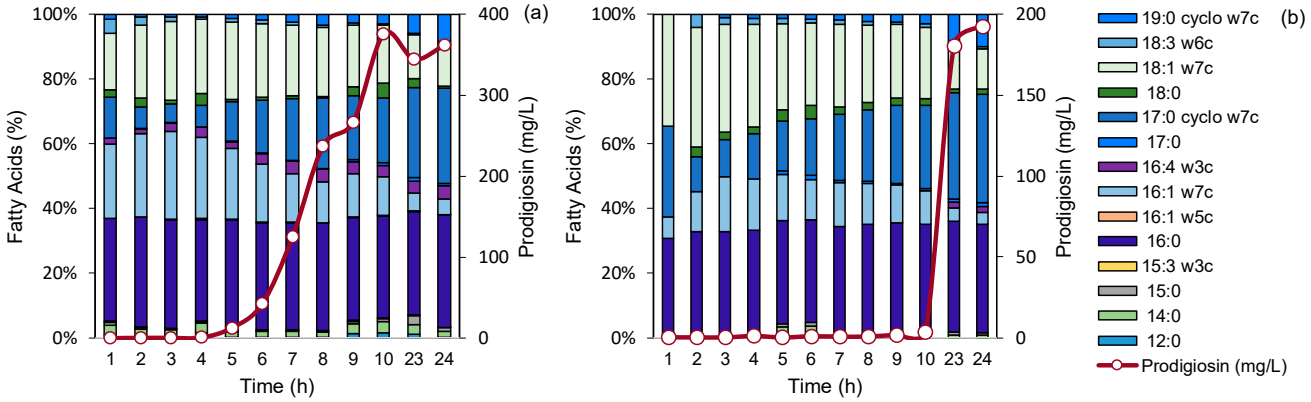

Figure 6. FA profile and prodigiosin production of *S. rubidaea* cells grown in geometrically different bioreactors for 24 h. (**a**) BE; (**b**) BI. The values represented are the average of data points obtained from at least two independent experiments.

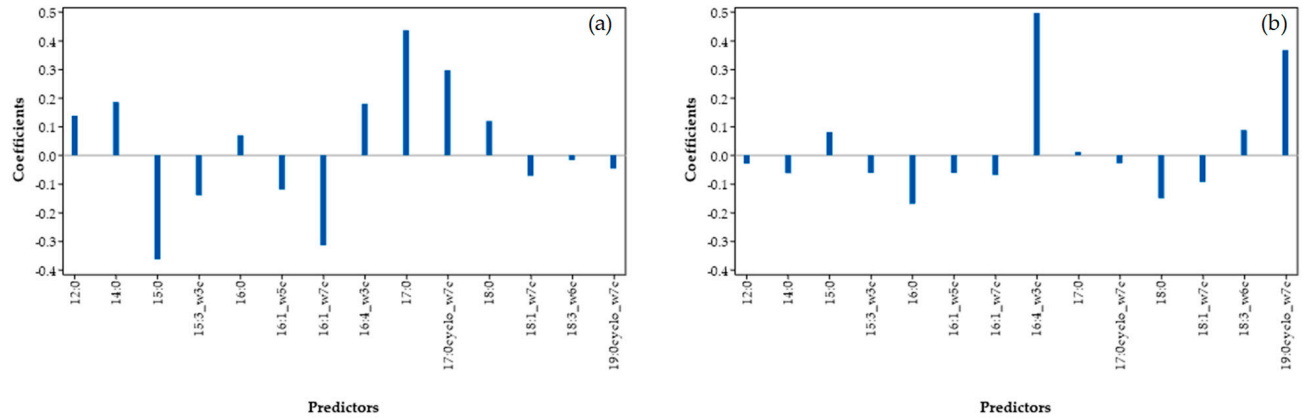

Figure 7. Coefficients of the PLS model relating the fatty acid composition of the cells with prodigiosin production by *S. rubidaea* cells in the bioreactors BE (**a**) and BI (**b**). Calculations performed with Minitab 14.1.

The increased concentration of prodigiosin was positively correlated to the production of the FAs 17:0 (0.44) and 17:0 cyclo w7c (0.30) FA and negatively correlated to the production of the FAs 15:0 (−0.37) and 16:1 w7c (−0.32) (Figure 7a). These FAs were used as potential biomarkers to assess the production of prodigiosin by *S. rubidaea* with time. The FA 19:0 cyclo w7c, although not significant as a biomarker in this analysis (its value is −0.05), was also considered since the production of these cyclo FAs demand a significant energy expenditure by the cells. In this case, the presence of cyclo FAs is related not only to cell aging, but also to the accommodation of prodigioisn in the membrane, since they promote membrane flexibility [39]. In Figure 7b, the most significant FAs were different from those selected for cells growing in the BE. The cells grown in the BI presented 16:4 w3c (0.50) and 19:0 cyclo w7c (0.37) as the most significant FAs. The latter was produced mainly during the last 2 h of growth (ca. 3 times more during this period than in the previous hours), when the concentration of prodigiosin was highest.

Since the concentration of prodigiosin was 50% higher under these conditions in the BE, the determined relation between FAs and prodigiosin was used for modeling. The established relation between FA composition and prodigiosin was as follows: to produce high amounts of product: (i) the amount of 15:0 should be below 1%; (ii) 16:1 w7c should decrease with time from 20% to 5%; (iii) the amount of 17:0 should remain

between 0.2% and 0.75%; and (iv) the proportion between the two predominant cyclo FA (17:0 cyclo/19:0 cyclo) should remain between 3.5 and 10 during the late exponential phase and beginning of the stationary phase. Using this analysis and applying it to the membrane FA profile of the cells in BI (Figure 6b), the following was noticed: (i) the amount of 15:0 did not increase beyond the 1%; and (ii) the amount of 16:1 w7c FA remained below 20% but never decreased beyond 10% in most samples, with the exception of the ones taken during the last 2 h, when its value was below 5%. Interestingly, when the values of these FA were outside the boundaries specified, the changes were correlated with specific events during bacterial growth, namely the beginning and/or arrest of prodigiosin production. Relatively to the remaining FAs taken into consideration in the FA/prodigiosin correlation established, the amount of the FA 17:0 and the proportion between the cyclo FAs remained within the specified range. This suggests that these FAs could be used to infer on prodigiosin production. The lipid profile of the cells can thus be used to study both the physiological condition of the cells and also their ability to produce prodigiosin.

3.7.2. Flow Cytometry

With flow cytometry and the LIVE/DEAD *Bac*Light kit, it was possible to monitor *S. rubidaea* growth using samples with a cell concentration of 6.0×10^5 cells/mL. The stained samples also allowed the monitoring of the non-viable cells. Although there are various issues involving the SYTO 9 dye, such as its permeability problems with Gram negative bacteria and the greater affinity of PI for DNA, the viability data given by this fluorescent dye for *S. rubidaea* cells was used. These issues have been presented as potential interference of the viability staining method, resulting in erroneous estimations of the number of viable cells in other studies [42–44], but it is a generally used dye to assess viability.

Due to prodigiosin's chromophoric nature, its fluorescence emission was detected in the FL3 flow channel (events presenting red fluorescence), which is also the one used to count the PI stained non-viable cells. Being both simultaneously quantified, in order to monitor the amount of product being produced, the number of non-viable cells in the stained samples was determined by the subtraction of events marked with SYTO 9 that were presenting fluorescence in the FL1 flow channel (events presenting green/blue fluorescence) and the sample total number of cells. The amount of prodigiosin present was determined based on the fluorescent intensity in the FL3 flow channel in the samples without any fluorescent staining. The total number of cells inside region R1, where the prodigiosin-producing cells were shown to be grouped (Figure 8), was also determined in the unstained samples. It was observed that there was some degree of aggregation between cells since the estimated concentration and the values obtained for stained and non-stained samples were different.

When production of prodigiosin started, during the middle of the exponential phase (ca. the 5th h of fermentation), a sudden decrease in the total number of cells was observed (Figure 9). This phenomenon was also observed when microbial growth was followed by optical density measurements (Figure 3a). To study this phenomenon, simultaneous analyses by flow cytometry and fluorescence microscopy were carried out (Figure 8). It was observed that when prodigiosin starts to be produced, as a response to the selective pressures imposed on the bioreactor, the cells change from reproduction and proliferation towards prodigiosin production. Once the metabolic route of prodigiosin was activated by the cells, and during the following hours until the culture reached the stationary phase (ca. the 24th h of fermentation), it was possible to observe multiple sub-populations of *S. rubidaea* cells (Figure 8). In stained samples, four sub-populations could be observed (Figure 8). At 24 h, the population present was homogeneous and producing prodigiosin.

Prodigiosin production followed cellular growth, and the number of cells containing prodigiosin inside remained between the total number of cells and the number of non-viable cells (Figures 8 and 9). This pattern confirms that the chromophoric nature of prodigiosin can be closely monitored using flow cytometry.

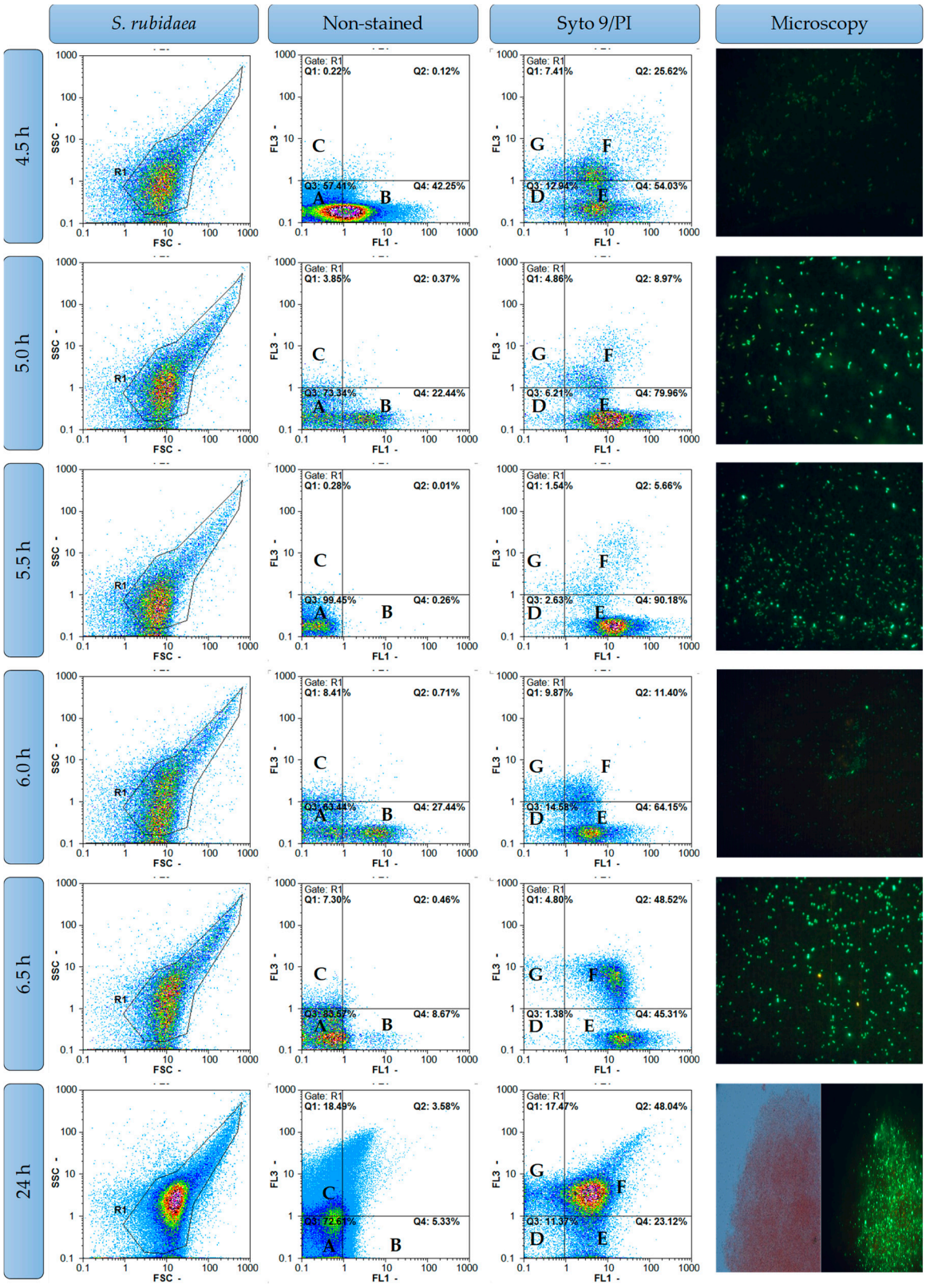

Figure 8. Use of flow cytometry and fluorescence microscopy as analytical tools for monitoring cell growth and production events. Non-stained samples: new cells (A), growing cells (B), and prodigiosin-producing cells (C). Syto 9/PI stained samples: cell debris (D), the viable cells stained with Syto 9 (E), viable cells stained with Syto 9/PI and producing prodigiosin (F), and the non-viable cells stained with PI (G). The magnification used in the microscope images was 1500×.

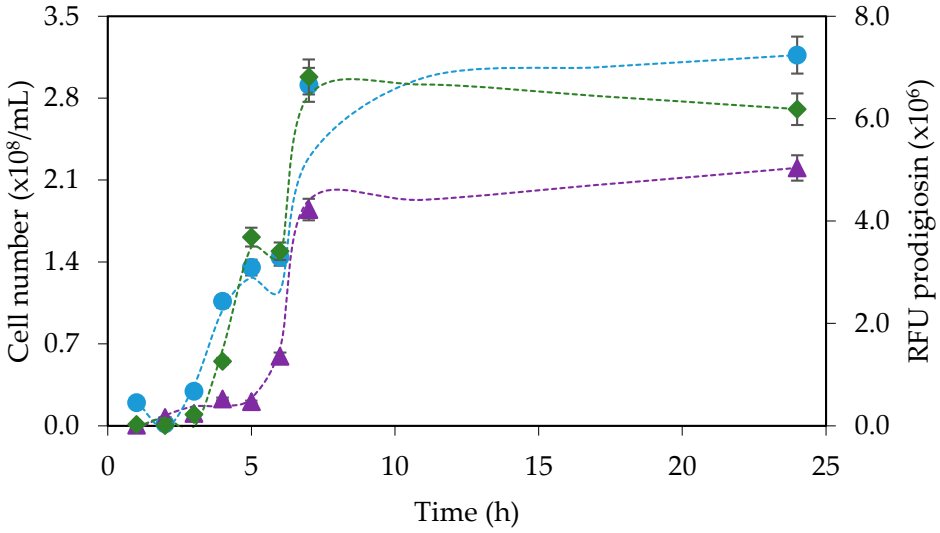

Figure 9. Growth and production of prodigiosin by *S. rubidaea* cells monitored by flow cytometry: total cells (●), non-viable cells (▲), and relative fluorescence intensity (RFU) of produced prodigiosin (♦). The data are represented as the average ± standard deviation.

4. Conclusions

Bioprocess development is not a straightforward process. Several parameters have to be studied in order to guarantee a successful scale-up from flask to bioreactor. In this work, cell concentration in the inoculum and bioreactor design were taken into consideration as possible variability factors. It was concluded that the inoculum should have a maximum concentration of 0.04 g/L of DCW, thereby reducing considerably the extent of the *lag* phase and allowing a greater amount of prodigiosin after 24 h.

The comparison between the two models of 2 L bioreactors used showed that the vessels' geometry is a major contributor to the fermentation result. The geometry can limit oxygen transfer (e.g., in the BE model), especially at stirring speeds above 400 rpm, when the oxygen transfer observed in the tested bioreactors resulted in high k_La differences and influenced the strategy used to increase product yields. Regarding the strategies developed for the BI, S5 was the one that led to the greatest amount of prodigiosin produced. To further optimize this strategy, each of the fermentation parameters of S5, namely DO, stirring speed range, and pH, were modified individually, but no increment in product yield or productivity was observed. When the S5 strategy was applied to BE, no significant improvement in prodigiosin production, relative to the first test made, was achieved in this bioreactor. In this study, the BI bioreactor showed better results for the production of prodigiosin, and its geometry should be maintained for the design of the fermenters in the scale-up process. However, the design of the BE vessel is the most commonly used for microbial growth at laboratory scale. A unique strategy for the BE bioreactors had to be developed to achieve similar values as in the BI.

This work also showed the applicability of using the fatty acid profile and flow cytometry for fermentation monitoring. With the lipid profile, it was possible, solely based on the pattern of the FAs of the cells, to infer the amount of prodigiosin present without the need for extracting and analyzing the product. Based on the identified FA that could be used as biomarkers, the results suggest that it is possible to infer how much prodigiosin will be produced by the cells, even when the environmental conditions and medium components change. Using multi-staining flow cytometry, it was possible to follow bacterial growth and obtain further insights into the formation of distinct bacterial sub-populations, as well as prodigiosin production, without disrupting the cells. It was shown that when the biochemical route for prodigiosin is triggered in this marine *S. rubidaea*, a decrease in the total cell number occurs. Multi-staining of the cells allowed us to determine that this phenomenon occurred not only because of cell death but also due to an arrest in cellular

growth that gave place to the production of prodigiosin, with two distinct populations observed: a producing population and a non-producing but viable population. During the stationary phase, both sub-populations became a single one where most cells were producing prodigiosin. Due to the chromophoric nature of prodigiosin, its production inside the cells could be monitored without the need for any additional staining compounds. Both tools allowed for time and resource savings.

This study thus indicates that by mimicking the conditions found at the isolation site during fermentation and by using analytical methods to assess cell physiological conditions, it is possible to significantly increase product yield.

Author Contributions: Conceptualization, R.F.S.P. and C.C.C.R.d.C.; methodology, R.F.S.P.; formal analysis, R.F.S.P. and C.C.C.R.d.C.; writing—original draft preparation, R.F.S.P.; writing—review and editing, C.C.C.R.d.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Union's Horizon 2020 Research and Innovation Programme, grant agreement no. 101000327 (Project FuturEnzyme). The research was also partially funded by national funds from Fundação para a Ciência e a Tecnologia (FCT, Portugal) in the scope of the projects UIDB/04565/2020 and UIDP/04565/2020 of the Research Unit iBB—Institute for Bioengineering and Biosciences, and of the project LA/P/0140/2020 of the i4HN—Associate Laboratory Institute for Health and Bioeconomy.

Data Availability Statement: Data will be made available on request.

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

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