

Scaling-Up of the Bioluminescent Strain *Aliivibrio* sp. (ref. 1485) from Blue Amazon: Implications for the Formulation of Sustainable Signaling Devices

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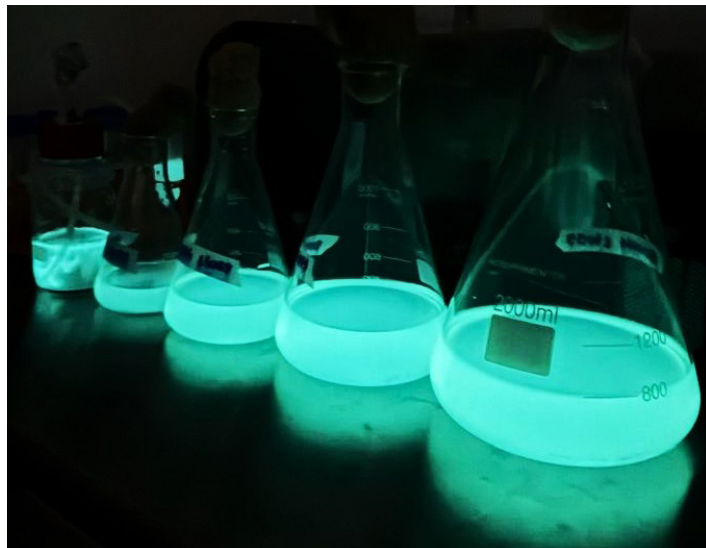
Abstract

This study detailed and validated the scaling-up protocol for the bioluminescent marine strain, *Aliivibrio* sp. (ref. 1485), sourced from the REGENERA BANK, using an INFORS HT Minifors 2 bioreactor. The primary goal was to establish optimal parameters for the light emission of the strain for use in sustainable signaling systems. The strain, collected in the coastal zone of Rio Grande do Sul, Brazil, was cultivated in batch mode at 28 °C with buffered pH at 6.50 in commercial Marine Broth (MB) medium. The results showed successful biomass production, with the culture reaching the stationary phase ($OD_{600nm} = 1.2$) at approximately 68 hours. However, the bioluminescence kinetics revealed a significant divergence from the canonical Quorum Sensing (QS) pattern. The maximum luminescent peak (276,000 photons) occurred just 3 hours after inoculation, demonstrating an inverse proportionality to cell growth. This suggests the presence of catabolite repression or inhibition by metabolic by products at high cell density, resulting in a “switching off” effect instead of the typical light induction in the stationary phase.

The protocol was validated as an efficient and reproducible method for mass production. Nevertheless, the commercial medium MB proved inadequate for long-term stability. The ideal harvesting window was confirmed to be immediately after inoculation (0-5 hours). The strategic solution involves replacing the commercial medium with optimized medium (BO1485 - under industrial secrecy) developed by REGENERA via Design of Experiments (DoE), which is designed to maintain bioluminescence viability for up to 100 days. Further optimization studies involving temperature and stirrer variations are recommended to maximize the initial light output and longevity of the strain.

Keywords

Minifors 2 bioreactor, eve bioprocess software, INFORS HT, marine bacteria, biotechnology, light emission



Objective

The objective of this study is to validate the scaling-up protocol for the bioluminescent *Aliivibrio* strain from the REGENERA BANK using a Minifors 2 bioreactor (INFORS HT), by determining the growth performance, bioluminescence stability and establishing the maximum luminescent emission and cell viability parameters necessary for developing sustainable signage systems.

Introduction

Marine bioluminescent bacteria represent a powerful resource at the intersection of biology and engineering. These microorganisms, primarily residing in symbiotic relationships or in the deep ocean, possess the remarkable ability to naturally emit light via the luciferase enzymatic reaction [1]. This intrinsic mechanism is not merely an ecological curiosity but a robust, non-invasive biological signaling system with profound technological implications across multiple sectors [2].

The stable and quantifiable light emission from these organisms makes them invaluable tools. They are currently being leveraged in fields such as biosensing for real-time toxicity testing, environmental monitoring, and the rapid detection of pollutants [3]. Furthermore, the potential of harnessing this natural illumination extends to novel applications, including the development of sustainable biological lighting systems [4]. A particularly promising and innovative area is the use of these bacteria for road and safety signage. By incorporating naturally glowing microorganisms, it is possible to design markers that are self-sustaining, energy-efficient, and responsive to environmental cues, potentially offering a revolutionary step away from traditional energy-intensive lighting methods [5].

From a production standpoint, marine microorganisms often present an advantage for bioreactor scaling compared to their terrestrial counterparts. These organisms are specifically adapted to aquatic environments with low nutrient availability and climate instability, distinguishing them for their nutrient plasticity and resilience. Therefore, they are suitable for large-scale controlled cultivation under laboratory conditions. These organisms are typically adapted to stable and nutrient aquatic environments, making them particularly amenable to large-scale, controlled cultivation under laboratory conditions [6]. Their relatively straightforward nutritional requirements and established tolerance to high cell densities facilitate the process of industrial upscaling and fermentation, ensuring the robust supply needed for technological development, especially in applications like light emission where consistent output is critical [7].

This technical document details the scaling-up protocol for a bioluminescent bacterial strain, a procedure of strategic interest to REGENERA. The strain was selected from the REGENERA BANK (BANCO REGENERA) — the first bank of marine and coastal microorganisms in the country legally available for bioprospection and technological development. Collected in the Blue Amazon, through a strategy previously directed by company studies, the bacterium belongs to the genus *Aliivibrio* and represents the Brazilian marine biodiversity, being notable for its high light emission capability.

The primary goal of this scaling-up is the development of sustainable signage systems. The scaling-up procedure is fundamental to verify the stability and bioluminescence characteristics of the strain following cryopreservation. Therefore, the central objective of this protocol is to ensure the quality, purity, and luminescent intensity of the sample, thus guaranteeing its successful introduction into subsequent production and research environments.

Methods and materials

The family of Vibrionaceae contains a large number of bacterial species, many of which are described from marine habitats [8]. *Aliivibrio* spp. (formerly *Vibrio* spp.) is a Gram-negative bacterium found globally in marine environments. The strain utilized for scaling-up was collected in the coastal zone of the state of Rio Grande do Sul (RS) from samples of seaweed and fouling invertebrates, thereby representing the Brazilian marine biodiversity. The bacterium had its genome sequenced and was identified as *Aliivibrio* sp. (ref. 1485), with the species designation being maintained under industrial secrecy.

Before cultivation in the Minifors 2 bioreactor, the scale-up process was carried out gradually, beginning with smaller volumes and increasing stepwise. The sequence followed was 15 mL (Falcon tube), 100 mL, 200 mL, 400 mL, and 800 mL (Erlenmeyer flasks), culminating in the 1.6 L Minifors 2 stage. This gradual increase in culture volume allowed the continuity of growth across each step prior to large-scale cultivation.

The scaling-up process was conducted in an 6 L Minifors 2 bioreactor under strictly controlled conditions. The eve® bioprocess platform software (2025 H1 R1 - 1.149.11.1) was used to monitor and control the parameters during the process. Inoculum preparation involved the re-activation of the *Aliivibrio* strain, which was sub-cultured onto 40 plates of solid Marine Broth (MB) medium (Difco BD) and incubated at 28 °C for 24 hours (Figure 1A). Following growth, the biomass was resuspended in a 0.9 % (w/v) saline solution (Figure 1B). The cultivation was carried out in a final working volume of 4 L of commercial Marine Broth medium (Difco BD). The initial conditions in the bioreactor were established with an Initial Optical Density (OD_{600nm}) of 0.05 and an Initial Luminescence of 55,000 photons.

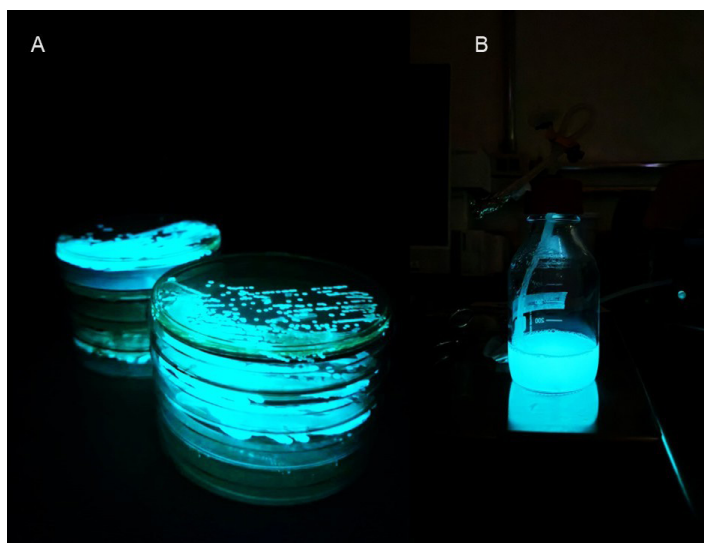


Figure 1. *Aliivibrio* sp. (ref. 1485) from REGENERA BANK. A. Growth bacteria in agar Marine medium. B: bioreactor inoculum in saline 0.9 %.

The culture was operated in Batch mode for a total duration of 92 hours. The operational parameters were maintained at the following setpoints: temperature at 28 °C, stirrer speed at 100 min⁻¹, GasMix at 21 % O₂, and Total Flow at 0.5 L min⁻¹. The pH was buffered and controlled at 6.50 throughout the process, which is the optimal value for the strain's maximum bioluminescence production. Samples were drawn periodically to monitor growth kinetics (assessed by OD_{600nm}) and luminescence (measured in photons), using a Synergy H1 - Agilent spectrophotometer in 96-well plate (Kasvi), transparent and black, respectively. Additionally, periodic photographs were captured to provide visual confirmation of the bacterial light emission during the cultivation period. Figure 2 shows the beginning of the process, just after the inoculum.

Results

During the process, the operational conditions of temperature at 28 °C and the buffered pH at 6.50 were rigorously maintained, as were the stirrer (100 min⁻¹), total flow (0.5 L min⁻¹). The pO₂ (dissolved oxygen) is a key parameter for bioluminescent organisms, as oxygen is an essential co-substrate for the luciferase reaction. Regarding the Initial behavior (0-5 hours), a high rate of initial consumption was observed, coinciding with the peak of luciferase activity. This validates that oxygen was available but was rapidly consumed due to the high initial metabolic activity of the strain.

After the initial drop, maintaining adequate oxygen levels demonstrated that the limitation of bioluminescence was not due to a lack of the element, but rather to internal metabolic repression. Starting from 92 hours, the oxygen level begins to rise, indicating cell death (Figure 3).



Figure 2. *Aliivibrio* sp. (ref. 1485) from REGENERA BANK inoculated in Minifors 2 bioreactor (INFORS HT).

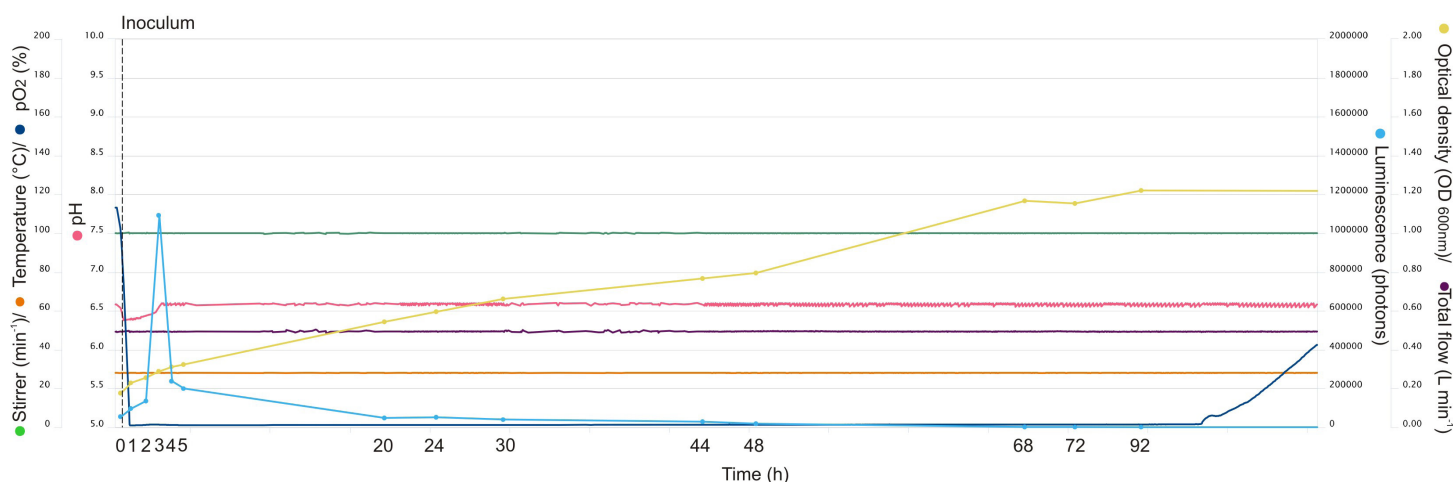


Figure 3. *Aliivibrio* sp. (ref. 1485) culture parameters recorded by eve® bioprocess platform software (2025 H1 R1 - 1.149.11.1) (INFORS HT).

The results of the scaling-up of the *Aliivibrio* sp. (ref. 1485) strain in the Minifors 2 bioreactor are presented in Figure 4, for both cell growth (OD_{600nm}) and bioluminescence (photons) production. Both endpoints show statistically significant differences (One-way ANOVA) between the observation times, $F(13, 28) = 416.5, p=0.0000$ e $F(13, 28) = 1495.6, p=0.0000$, respectively (Figure 4).

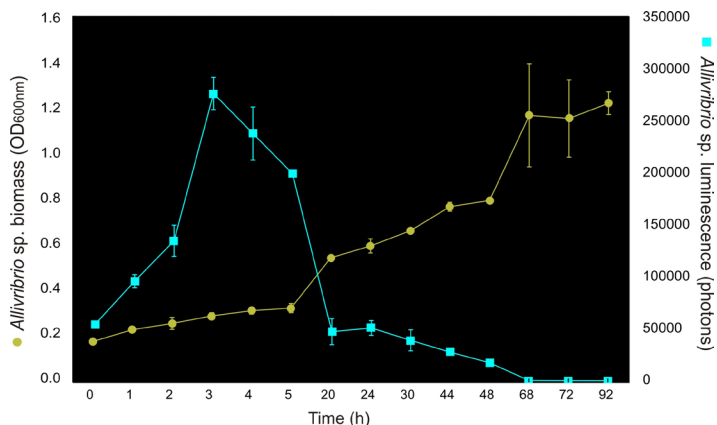


Figure 4. Representative graph of growth and bioluminescence kinetics of *Aliivibrio* sp. (ref. 1485). Vertical bars denote 0.95 confidence intervals.

The analysis of the Optical Density (OD_{600nm}) data revealed that the culture demonstrated a lag phase (latency) between 0h and 5h, indicating that the inoculum and the culture medium (MB) were adequate for biomass production. The exponential growth phase began approximately after 5 hours and continued until around 68 hours of cultivation. The culture reached the stationary phase (OD maximum) at approximately 68 hours, achieving a maximum value of around 1.2, where the stationary phase is presumed to have commenced.

The analysis of the Luminescence (photons) data revealed that the bioluminescence emission followed the opposite pattern of cell growth, demonstrating an inverse proportionality. The maximum luminescent emission peak was reached just 3 hours after the inoculum was performed, recording 276,000 photons. Following the peak, luminescence showed a decrease, concurrently with the biomass beginning to increase (OD > 0.5), starting from 20 hours into the process. Visibly, bioluminescence was observable between 0 hours and 48 hours (> 18,000 photons) (Figure 5). The scaling-up protocol was successfully validated. The operational conditions of temperature at 28 °C and the buffered pH at 6.50 were effective, allowing for robust and rapid growth (stationary phase at 68 hours) of the marine *Aliivibrio* strain.

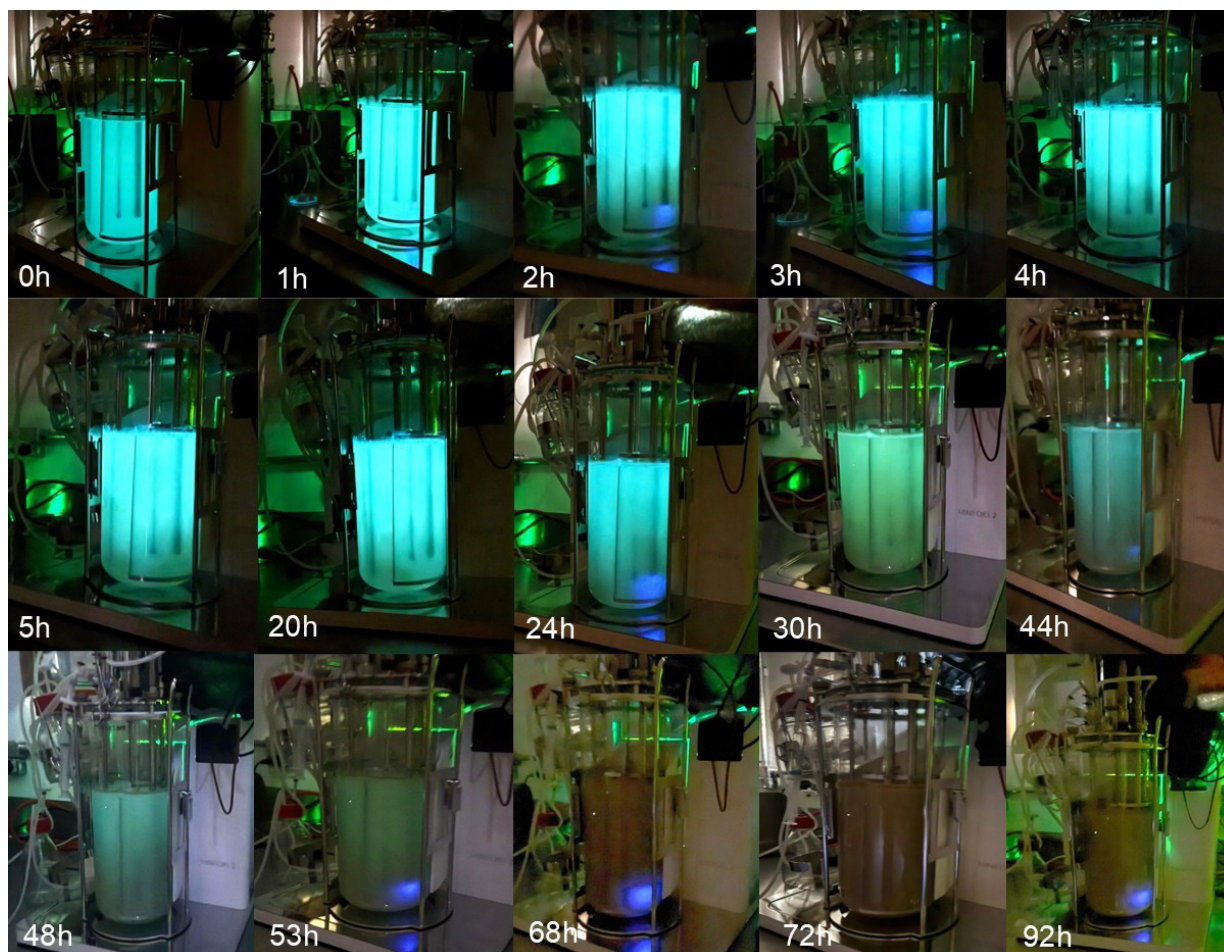


Figure 5. Images of the *Aliivibrio* sp. (ref. 1485) bioprocess over time.

The observed dissociation between the maximum biomass (between 68 and 92 hours) and the luminescence peak (between 0 and 5 hours) is a fundamental result. Maintaining the at pH 6.50, a critical abiotic factor for luciferase activity, ensured that the metabolism was directed towards light production, as expected for *Aliivibrio* bacteria [9]. The obtained data confirm that the ideal harvesting window for this strain in the medium is immediately after inoculation to capture maximum luminescent performance.

The bioluminescence kinetic results diverge from the canonical behavior of *Aliivibrio fischeri* (formerly *Vibrio fischeri*), which typically regulates light emission via Quorum Sensing (QS), reaching the peak in the stationary phase [10]. The observed kinetics suggest a distinct metabolic control in the REGENERA BANK strain: the light peak at 3 hours, well before massive growth, indicates that the initial pool of precursors or the enzymatic state of luciferase (lux) was highly active at the time of inoculation. The drop in luminescence, which coincides with the increase in biomass (OD = 0.5 starting from 20 hours), suggests catabolite repression or inhibition by metabolic by products released at high concentration during the exponential phase [11]. This “switching off” effect at high density, instead of “switching on” (typical of QS), requires further investigation but has direct implications for the application.

The commercial medium used compromises the long-term stability of the bioluminescence, making it unsuitable for the final purpose of sustainable signaling [5]. To overcome this limitation, REGENERA, through Design of Experiments (DoE) [12], developed medium BO1485, which is currently under industrial secrecy. This medium is specifically designed to retard cell growth and maintain viable bioluminescence for up to 100 days.

Therefore, the next strategic step is to apply the scaling-up protocol validated in this study using medium BO1485. Furthermore, an optimization study involving different temperatures and stirrer must be carried out, as temperature is another primary factor regulating luciferase activity and the static condition can slow down the biomass growth, can maximizing the bioluminescence and the longevity of the strain activity.

Conclusion

The scaling-up protocol for the marine bioluminescent wild strain *Aliivibrio* sp. (ref. 1485) was successfully validated in the Minifors 2 bioreactor, establishing an efficient and reproducible method for the mass production of the biomaterial. The rigorous maintenance of operational parameters (pH 6.50, 28 °C, and pO₂ control) confirmed the suitability of the culture system for the genus.

The main kinetic result lies in the fundamental dissociation between maximum biomass (reached at 68 hours) and the luminescence peak (reached early at 3 hours). This kinetics is atypical for *Aliivibrio* and diverges from classic QS control, suggesting that light expression is rapidly repressed by metabolic factors intrinsic to the strain at high density. The direct implication is that the ideal harvesting window in the MB medium is immediately after inoculation (0-5 hours) to capture maximum performance.

However, the commercial medium is inadequate for the development of sustainable signaling systems due to long-term bioluminescence instability. REGENERA’s next strategic step is the transition to medium BO1485 (under industrial secrecy), specifically developed to retard cell growth and ensure luminescent viability for up to 100 days. Additionally, a temperature, aeration cascade and stirrer optimization studies is recommended to maximize the strain’s longevity, thus solidifying the foundation for the formulation of long-lasting bioluminescent signaling devices.

Integrated with the eve® bioprocess platform, the Minifors 2 bioreactor enabled precise real-time control of temperature, pH, and dissolved oxygen, allowing REGENERA MOLÉCULAS DO MAR to identify the strain’s early luminescence peak and validate a reproducible scalable protocol for sustainable bioluminescent technologies.



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Conflicts of interest

V.O.A. and J.S. are REGENERA BIOTECNOLOGIA S.A. employees; M.L.C.F.J., and A.J.M. are REGENERA BIOTECNOLOGIA S.A. shareholders; A.M.S is ECOVIAS CERRADO S.A. employee. The remaining authors declare no competing interests.

Funding

This work was carried out with the support of Technological Development Resources (RDT) [50500.252331/2022-84], provided by the Brazilian Concessionaire ECOVIAS CERRADO, under the regulation of the National Land Transportation Agency (ANTT).

Acknowledgments

We thank the National Land Transportation Agency (ANTT), Technological Development Resources (RDT), and ECOVIAS CERRADO road concessionaire for their assistance during the project's development.

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