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Bioremediation and biomass production by Chlorella sp. isolated from effluent treatment station of a parboiled rice industry

Parboiling rice is a high-water demand process that generates approximately 2 L of effluent per kilogram of processed rice. This effluent is recognized for its high concentrations of phosphorus, organic matter, and nitrogen, and if not treated correctly, it can potentially contaminate the environment. Bioremediation methods offer a sustainable and low-cost alternative to support treatment stations. In this paper, we report on the successful isolation of a microalgae strain from a tertiary treatment station of the rice effluent industry. We also investigate the bioremediation and biomass potential of the strain after cultivating it in parboiled effluent without dilution or supplement. The cultures were carried out in a photobioreactor using parboiled effluent (PE) at 28 ºC, pH 7, a 16:8 light/dark cycle, and 2000 lux for 14 days. The highest concentration of Chlorella sp. biomass was obtained on the 14th day, reaching 2.4 g L-1 in PE. Additionally, removals ranged from 39 to 95% of total Kjedahl nitrogen (TKN) and 66 to 82% of chemical oxygen demand (COD). These results suggest that treatment stations are a promising source of adapted microorganisms, and that PE can be used as culture medium to produce biomass and obtain bioproducts of high commercial value while simultaneously treating PE

Palavras-chave: Biomass; Bioremediation; Cell viability; Effluent; Microalgae.

Biorremediação e produção de biomassa por Chlorella sp. isolado de estação de tratamento de efluentes de uma indústria de arroz parboilizado

A parboilização do arroz é um processo de alta demanda de água que gera aproximadamente 2 L de efluente por quilograma de arroz processado. Esse efluente é reconhecido por suas altas concentrações de fósforo, matéria orgânica e nitrogênio e, se não for tratado corretamente, pode potencialmente contaminar o meio ambiente. Os métodos de biorremediação oferecem uma alternativa sustentável e de baixo custo para apoiar estações de tratamento. Neste artigo, relatamos o isolamento bem-sucedido de uma cepa de microalgas de uma estação de tratamento terciário da indústria de efluentes de arroz. Também investigamos o potencial de biorremediação e biomassa da cepa após cultivá-la em efluente parboilizado sem diluição ou suplemento. Os cultivos foram realizados em fotobiorreator com efluente parboilizado (PE) a 28 ºC, pH 7, ciclo claro/escuro 16:8 e 2.000 lux por 14 dias. A maior concentração de Chlorella sp. a biomassa foi obtida no 14º dia, atingindo 2,4 g L-1 em PE. Além disso, as remoções variaram de 39 a 95% do nitrogênio total de Kjedahl (TKN) e de 66 a 82% da demanda química de oxigênio (DQO). Estes resultados sugerem que as estações de tratamento são uma fonte promissora de microrganismos adaptados, e que o PE pode ser utilizado como meio de cultura para produzir biomassa e obter bioprodutos de alto valor comercial e, ao mesmo tempo, tratar o PE.

Keywords: Biomassa; Biorremediação; Viabilidade celular; Efluente; Microalgas.

Topic: **Biotecnologia**

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INTRODUCTION

Rice is an essential and widely consumed cereal worldwide. According to last report from the Food and Agriculture Organization (FAO), global rice production reached a staggering 520.8 million tons in 2021/2022 (FAO, 2022), with Brazil contributing of 8 million metric tonnes (USDA, 2023). Nearly 25% of Brazilian rice production is designated to parboiled rice industry (PARAGINSKY et al., 2014). Parboiled rice is renowned for its distinct quality, lower calorie content, and higher levels of protein and fiber compared to white rice. Through the processes of soaking and steaming, rice starch is gelatinized, and cracks on grain sealed, enhancing it durability. The parboiling process demands a high volume of water and generates an average volume of 2 liters of effluent (PE) per kg of rice (GABOARDI et al., 2018). This hydrothermal process transfer nutrients from the inner part of grain to the PE, resulting in elevated levels of organic compounds, nitrogen and phosphorus. To prevent the release of highly nutritious effluent that can disrupt the environmental balance, leading to water eutrophication, reduced dissolved oxygen and harm to aquatic biodiversity, an effective treatment method is crucial.

PE is typically treated in effluent treatment stations using physicochemical and biological methods with anaerobic/aerobic steps, which may require a significant volume of chemicals, complex monitoring, and operational costs. The PE treatment station is often structured into four stages of treatment: preliminary, primary, secondary, and tertiary treatment. During the preliminary treatment, solids that can cause clogging are removed by passing the effluent through bar screens. In the primary treatment, physicochemical methods such as clarifiers and aeration are employed to remove solid materials, thereby reducing the biological oxygen demand (BOD). The secondary treatment involves the use of aerobic treatments, such as activated sludge, to further enhance BOD removal. Finally, in the tertiary treatment or polishing step, remaining suspended solids in the PE are eliminated. This stage employs disinfection methods like UV, ozone, and chlorination to remove persistent parameters such as phosphorus and nitrogen.

The need for efficient and greener alternatives are required to ensure environmental safety and sustainability of industry sector. Biological methods present a promising alternative for treating industrial effluent and have been widely employed in various matrices, including textile wastewater (ARAÚJO et al., 2022), petrochemical wastewater (KARDENA et al., 2017) and landfill leachate (ER et al., 2018). Microorganisms exhibit remarkable adaptability and can efficiently break down complex matrices to obtain energy. Additionally, valuable compounds can be obtained from its metabolism such as cell biomass, while simultaneously mitigating the high levels of nutrients in a sustainable matter, aspects that are hard to achieve while applying complex chemicals at the treatment plant (GERBER et al., 2018). The search for alternatives in PE treatment has led to the emergence of several research studies on biological treatment systems, including the use of cyanobacteria and microalgae (BASTOS et al., 2014) (MUKHERJEE et al., 2016), yeast (SANTOS et al., 2012) (FEHRENBACH et al., 2022), and enzymatic pre-treatment of PE prior to yeast culture (FEHRENBACH et al., 2021).

Microalgae are photosynthetic microorganisms with several interesting characteristics such as rapid

growth, the ability to incorporate complex nutrients like phosphorus and nitrogen, and wide-ranging applications of the generated microalgae biomass, such as CO2 fixation and energy recovery (ABDEL-RAOUF et al., 2012). As a result, microalgae have been utilized in the treatment of residues from piggery farms (MARTIN et al. 1985a) agricultural wastes (PHANG et al., 1988), effluent from food processing factories (RODRIGUES et al., 1987), toxic minerals (SOEDER et al., 1978) and parboiled effluent (MUKHERJEE et al., 2016).

The isolation of wild microorganisms from effluent treatment stations or contaminated sites is particularly interesting due to their adaptation to nutrient levels and the specific environment, which enhances their bioremediation potential. Conversely, exogenous microorganisms generally fail or exhibit low activity in bioremediation processes due to the differing conditions found in controlled laboratory environments (DÍAZ et al., 2003). Consequently, the tertiary treatment station of parboiled rice effluent represents an ideal niche for microalgae development due to nutrient concentrations and environment conditions. The objective of this study was to isolate a microalgae strain from effluent treatment plant capable of using PE as source of nutrients, test it on direct treatment of parboiled effluent by analysing the removals of total nitrogen and organic matter, and its potential for biomass production as a valuable coproduct.

METHODOLOGY

Parboiled rice effluent

Parboiled rice effluent (PE) was obtained from a local industry at the city of Pelotas – Brazil (latitude 31.646790, longitude 52.340367). Samples from rice parboiling process were collected directly from maceration tanks and conditioned in sterile plastic vials. PE was autoclaved for 25 min and stored at 4 °C until used.

Microalgae isolation and experimental conditions

The isolation of microalgae species from effluent treatment station was performed in 4 major steps (Figure 1). First, a sample from last pond of treatment station was collected in sterilised vials of 50 mL and immediately transported at room temperature to Microbiology Laboratory – UFPEL (Brazil). Secondly, BG11 agar plates (Himedia) were prepared and inoculated with 60 µL of collected sample, and plates incubated for 7 days at 28 °C, light/dark cycle of 16:8, and 2000 lux. This step was repeated several times until obtention of pure colonies.

To test the applicability of isolated strains on effluent treatment, a third step consisted of inoculating 0.5 g L^{-1} and 20% v/v of microalgae in baffled flasks containing parboiled effluent. The microalgae cell viability was determined by counting on Neubauer chamber, and the strain with the highest cell cell viability was selected and applied on the upscaling of cultures in 4 L column photo-reactor of PE for 14 days at 28 °C, air injection, light/dark cycle of 16:8 and 2000 lux (step 4), followed by determination of parameters of chemical

oxygen demand (COD), total Kjedahl nitrogen (TKN) and biomass. The pH level was measured every 48h and adjusted to 7. A culture in Watanabe medium with the following reagents and concentrations was also realized as used as control of biomass at same experimental conditions as PE: 1.25 g L^{-1} KNO_3 (NEON), 1.25 g L^{-1} KH_2PO_4, 20 mg L^{-1} $MgSO_4$, 20 mg L^{-1} $FeSO_4$ (Synth) and 1 ml L^{-1} of A5 solution (2.9 g L^{-1} H_3B_3O, 1.81 g L^{-1} $MnCl_2$, 0.08 g L^{-1} $CuSO_4$, 0.018 g L^{-1} $_3(NH_4)O_7MoO_3$ and 0.11 g L^{-1} $ZnCl_2$).

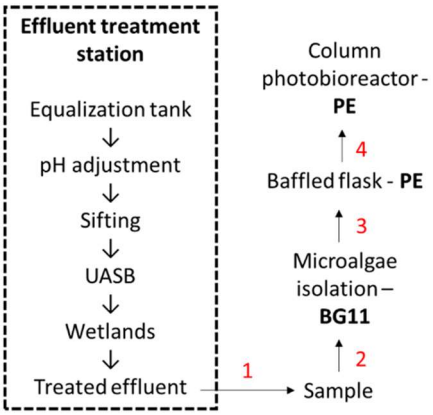

Figure 1: The several steps involved in the isolation and culture of microalgae strain. [1]

Analytical determination of total nitrogen and chemical oxygen demand

Triplicate samples of 1 mL from PE were collected for total Kjedahl nitrogen (TKN) and chemical oxygen demand (COD). TKN and COD were determined at 0, 2, 6, 10 and 14 d. Samples were stored frozen until analysis. At room temperature, samples were centrifuged at 3000 *g* (Kubota - KR 600) for 5 min and supernatant was collected for analysis.

Total nitrogen was determined by HI93767B-50 Hanna TKN kit, transferring 500 µL and potassium persulfate to digestion tube and heated in a heated block (Hanna HI839800) at 105 °C for 30 min. Then, at room temperature sodium metabisulfite was added and mixed. After 3 min of reaction, total nitrogen reagent was added, and tube was left reacting for 2 min. The colorimetric reagent was mixed with 2 mL digested sample and the absorbance was analysed in a photometer Hanna HI8339902.

COD was determined by HI93754C-25 Hanna COD kit. To the digestion tubes containing dichromate, 200 µL of sample were transferred and heated to 150 °C for 2 h. Colorimetric reaction was analysed in a photometer Hanna HI8339902.

Biomass

Microalgal biomass (g) was quantified collecting a triplicate sample of 10 mL from each media at intervals of 48 h and transferring to sterile vials. Then, samples were centrifuged at 2000 *g* (Kubota - KR 600) for 10 min and pellet washed two times with sterile water before dried at 60 ºC until constant weight. The dried pellets were weighted in analytical balance and biomass calculated directly (Shimadzu - ATY224).

[1] 1) first step involved collecting samples from the last pond of the effluent treatment station; 2) samples were processed to isolate individual microalgae species; 3) once the microalgae species were isolated, they were introduced into culture systems containing parboiled effluent; 4) to enhance the cultivation of isolate microalgae species a culture in column photobioreactor was performed.

Statistical analysis

The results were analysed on Statistica software version 10 (Stat Soft) by student's t-test comparing means and $p< 0.05$ was considered significant. All experiments were done, at least three times in triplicates.

DISCUSSION

Microalgae isolation

A wide variety of species has been observed on BG11 agar plates after culturing a sample from the last effluent treatment pond. Four passages were required to isolate the microalgae species, that posteriorly were expanded to Watanabe broth. As expected, the mild concentration of nitrogen, phosphorus and organic matter in the pond were suitable for microalgae growth, and the real applicability would have to be tested directly in parboiled effluent (PE). In this way, all the obtained species were centrifuged and transferred to baffled flasks containing PE. Only two isolates have grown and were identified by comparing the observed morphology to data available in the algae bank (GUIRY et al., 2019), identified as *Chlorella* sp. (Figure 2) and *Closterium* sp.

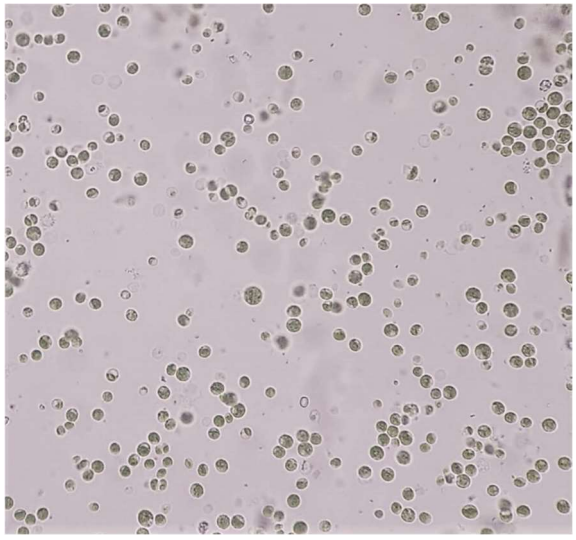

Figure 2: Light microscope image of *Chlorella* sp. isolated from tertiary effluent treatment station of parboiled rice industry.

The selection of the most suitable species for upscaling was based on biomass results, among all the samples collected during a 6-day culture, *Chlorella* sp. consistently demonstrated higher yields. The biomass yields for *Chlorella* sp. were as follows*:* 1st (1.88%), 2nd (32.51%), 3rd (47.95%), 4th (51.13%), 5th (52.4%) and 6th (55.65%). The pre-culturing in PE not only allowed for the identification of most promising microalgae, but it also contributed to their enhanced. This find aligns with the study conducted by Mukherjee et al. (2016), who reported better results with pre-acclimatized microalgae and cyanobacteria. At this stage, the focus of the research was not on identifying the obtained strain to the subspecies level. Instead, the main objective was to obtain a photosynthetic organism capable of thriving in raw effluent, producing biomass, and reducing the levels of main environmental parameters. These improvements are essential for ensuring compliance with the discharge limits of the effluent.

Biomass production

Chlorella sp. biomass in PE and Watanabe medium are shown in Fig. 3. Biomass results in PE were higher than standard medium from 0 to 12 d. Statistical significance ($p<0.05$) was observed at 0 ($p= 0.0109$), 2 ($p= 0.0138$) and 4 ($p= 0.0019$). From 12 to 14, biomass in Watanabe was higher with no statistical significance. The maximum biomass obtained for PE was at 14 d of culture (2.43 g L^{-1}) and higher biomass tax between the intervals of 0-2 (21.25 mg h^{-1}) and 10-12 days (14.27 mg h^{-1}). The increasement of biomass in control medium Watanabe reached the highest concentration of 2.75 g L^{-1} at 18 d.

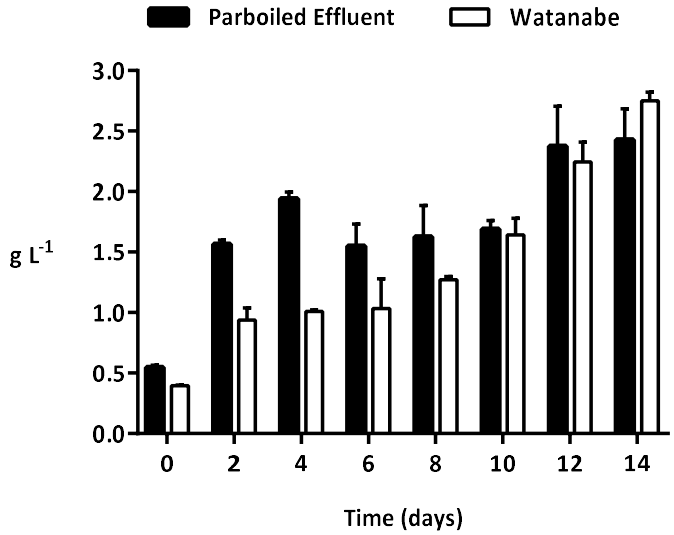

Figure 3: Biomass in parboiled rice effluent and Watanabe medium. The data represents the biomass (mean +/- SD) at 0, 2, 4, 6, 8, 10, 12 and 14 d in parboiled effluent and Watanabe inoculated with *Chlorella* sp.

The microalgal biomass obtained in the parboiled effluent exceeded the average expectations for culturing in standard media such as BG11 (XIN et al., 2010). This observation indicates that the PE was highly efficient in providing nutrients to support the growth of *Chlorella* sp. and did not exhibit signs of toxicity. In comparison, lower biomasses were reported when culturing *Chlorella ellipsoidea* in secondary domestic effluent of activated sludge treatment, with a maximum biomass of 0.425 g L^{-1} observed after 18 d of culturing (YAN et al., 2011). Similarly, Kim et al. (2010) achieved a maximum biomass of 0.13 g L^{-1} of *Chlorella vulgaris* in 9 d using wastewater effluent as a culture medium. *Chlorella* is widely recognized as one of the most popular microalgae for both feed production and extraction of valuable components. This suggests that parboiled effluent can be explored as culture media with no adequation, or high controlled conditions required to ensure the microalgae multiplication and biomass production. Furthermore, the highest biomass yield rates in this study were observed between 0-2 d (21.25 mg h^{-1}) and 10-12 d (14.27 mg h^{-1}), indicating rapid biomass accumulation within a short culture period.

Environmental Parameters COD and TKN

Total Kjedahl nitrogen (TKN) and carbon organic demand (COD) removals at 2, 6, 10, and 14 d are presented in Figure 4. The maximum removals occurred at day 6 for COD (82.48%) and at day 14 for TKN (94.9%).

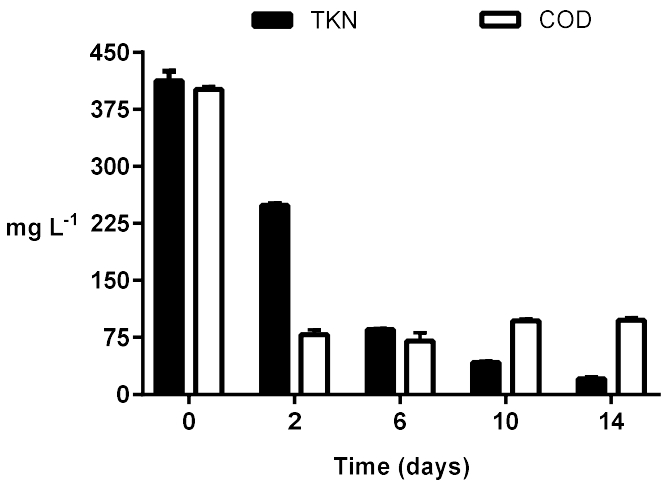

Figure 4: Total Kjedahl nitrogen and carbon organic demand removals (%). The removals are related to 0 d concentration of TKN and COD, not cumulative, at 2, 6, 10 and 14 d.

The ability to consume different forms of nutrients is essential for the applicability of microalgae species on effluent treatment stations. Nitrogen and phosphorus are essential for microalgae growth and required in adequate concentrations and chemical forms. *Chlorella* species are primary producers in different ecosystems, easily adapted, versatile in mass cultivation systems and widely applicated biotechnologically (SAFI et al., 2014) (KRIENITZ et al., 2012). The economic interest on *Chlorella* biomass and marketability depends on costs associated to production. Microalgae co-products represent a sustainable and green alternative to chemical methods to obtain pigments, nutraceuticals, supplements, and vitamins (CHACÓN-LEE et al., 2010).

When compared to standard mediums, waste-grown microalgae show as non-expensive system to produce biomass remediation and simultaneously reduce the high nutrient levels in industrial effluent. Converting by the maximum biomass obtained on our study, the volume of PE generated daily could 2.362,5 kg of *Chlorella* sp. biomass. However, subsequent studies are needed to upscale for total volume treatment, improve the nutrient removal, and direct the *Chlorella* sp. metabolism to accumulate specific compounds of commercial interest. Mature cells of *Chlorella vulgaris* can accumulate a total protein content between 42-58% of biomass dry weight, depending on growth conditions (SERVAITES et al., 2012), and are comparable or even better emulsifying agents than commercial ingredients (URSU et al., 2014). In optimal conditions, *C. vulgaris* can reach 5-40% lipids per dry weight of biomass, with high potential to be applied on biodiesel (BECKER, 1994). In low concentrations of nitrogen, total carbohydrates can reach 12-55% dry weight (BRANYIKOVA et al., 2011). The wide variety of products that can be extracted from *Chlorella* biomass includes pigments (chlorophyll, carotenoids, astaxanthin, and others), minerals (potassium, magnesium, zinc and others) and vitamins (B1, B2, B3, B5, B6, B7, B9, B12, E and A) (CHACÓN-LEE et al., 2010) (KITADA et al., 2009).

CONSEMA, the state environmental agency of Rio Grande do Sul, Brazil, establishes more stringent limits for effluent and emission levels compared to the federal environmental agency CONAMA. The PE used in this study was collected from a local industry that generates a daily volume of 750.000 L of effluent. The

discharge limits set by CONSEMA (Resolution nº 355/2017) for COD and TKN are 300 mg L^{-1} and 20 mg L^{-1}, respectively, for this volume of effluent.

Chlorella sp. demonstrated the ability to consistently reduce COD levels under 300 mg L^{-1} throughout the sampling period. The microalgae effectively assimilated the organic matter, as indicated by the higher growth rate observed between 0-2 d, followed by significant COD removal. The decrease in biomass at day 4 suggests a change of organic source, which was followed by a stable COD removal efficiency ranging from 82% to 75%.

In terms of total nitrogen, measured using the TKN method, *Chlorella* sp. exhibited substantial removal efficiency. After 14 d of cultivation, the concentration of total nitrogen was only 1 mg L^{-1} higher than the discharge limit of 20 mg L^{-1}, demonstrating a removal efficiency of 94.9%. These findings support the application of *Chlorella* sp. in effluent treatment stations to aid in nutrient removal and ensure compliance with discharge limits.

Overall, the results indicate that *Chlorella* sp. can be a co-product from rice productive chain and effectively contribute to the treatment of PE, helping to mitigate nutrient levels and ensure compliance with environmental regulations.

CONCLUSIONS

We have achieved successful isolation of two microalgae strains from the tertiary treatment station of parboiled rice effluent (PE). The most promising candidate was selected based on its biomass yield when cultured in PE. The isolated strain has been identified as Chlorella sp. through morphological analysis. The biomass yield in PE surpassed that achieved in standard medium, indicating that Chlorella sp. can be effectively cultivated in PE, offering the opportunity to generate valuable biomass for commercial purposes while simultaneously treating the effluent. We also confirm that effluent treatment stations are potential sources of adapted microorganisms as utilizing the isolate strain, organic matter and nitrogen levels were reduced, even under relatively low-controlled conditions.

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