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Combined treatment with α-amylase and *Saccharomyces cerevisiae* culture for nutrient removal and biomass production in effluent from rice parboilization

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ABSTRACT

In this study, we report a non-supplemented and biomass productive treatment for parboiled rice effluent (PE) combining a pre-treatment with α-amylase followed by *Saccharomyces cerevisiae* culture. PE with amylase (PEA) was pre-treated with 5 mg L^{-1} of α-amylase for 12.5 min at 50°C – a non-enzymatic culture with PE was used as the negative control. Both cultures were inoculated with 10% v/v of *S. cerevisiae* at a concentration of 1.2×10^3 CFU mL^{-1} for 48 h. The highest level of glucose was 1.75 g L^{-1} and obtained in PEA culture, 40% higher than in the control PE. CFU and biomass followed the glucose levels and were 73% and 62.9% higher than in PE, with a maximum of 3.35×10^8 CFU mL^{-1} and 2 g L^{-1}, respectively. The highest removals were obtained in PEA for phosphorus (40.68%), PE for organic matter (70%), and 100% of removal for total nitrogen in both cultures. The low cost of the method (0.45$ per m^3), nutrient removals (40.68% P, 100% TKN, 62% COD), cell viability (3.35×10^8 CFU mL^{-1}) and biomass (2 g L^{-1}) suggests that α-amylase improves the bioremediation potential of *S. cerevisiae* cultured in PE and can provide a widely marketable biomass as by-product.

Keywords: Effluent treatment; Enzymatic treatment; Bioremediation; Bioproducts; Parboiling process

1. Introduction

Rice is a cereal crop consumed by over half of the world's population and is currently the second most important cereal crop. The world rice consumption is still increasing and in 2020/2021 was estimated at 504 million metric tons, the largest on record. Brazil is the largest consumer and producer after the Asian continent, totalizing an annual average production of 10.56 million – 9 million tons on the southern in the regions of Santa Catarina and the Rio Grande do Sul [1,2]. Parboiled rice is obtained by partially cooking the rough rice in water or steam at ±58°C followed by a slow drying process. This process modifies the rice amide and preserves the vitamins and minerals, that not only increases the shelf life but also produces a healthier rice and more nutritive than white and polished types [3].

The parboilization process requires a high volume of water, generating 1–1.2 L of a complex effluent per kilogram of rice produced [4], and represents a relevant cost and environmental risk when compared to the processes of other rice varieties. If not treated adequately, the parboiled rice effluent (PE) can easily overload rivers and lakes with high concentrations of organic matter, nitrogen and phosphorus, reducing the dissolved oxygen and leading to aquatic death [5]. The usual effluent treatment methods for nitrogen, organic matter and phosphorus removal in PE employ expensive steps of anaerobic digestion, chemical precipitation [6], anaerobic reactors of ascendant flow [7],

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wetlands [8], nitrification and denitrification [9]. The PE composition is still not clear and treatment methods were not focused on the determination of how compounds are available or complexed in the effluent. The necessity for an alternative, efficient and sustainable technology for PE treatment have supported the development of biological approaches, historically applied on the treatment of complex residues, petrochemical effluents and environmental disasters, not only for polishing but also representing a potential alternative for the stablished effluent treatment stations [10].

Yeasts are unicellular fungi widely used in the food industry, beverage and prebiotics, and its versatility motivated the application in waste treatment [11]. Gil de los Santos et al. [12] and Gaboardi et al. [13] reported the application of *Saccharomyces boulardii* and *Pichia pastoris* X-33 in supplemented cultures for PE treatment. Posteriorly, prebiotic and immune modulator action in poultry and quail was also verified for a *Saccharomyces boulardii* culture in PE [14]. In all these studies an extra carbon source was necessary, increasing the costs and complexity of application. The increasement of nutrient availability by single-step pre-treatments as enzymatic digestion can improve the nutrient uptake, and consequently lead to higher yeast viability and biomass. In this paper we report a combined treatment for nutrient removal and biomass production in effluent from rice parboilization, consisting of an enzymatic pre-treatment using α-amylase previously to the inoculation of *S. cerevisiae*.

2. Material and methods

2.1. Sampling

Effluent from rice parboiling process was collected from a local industry in Pelotas – Brazil (latitude: –31.646790 longitude: –52.340367) every 3 months for a year and stored in sterile flasks at –4°C.

2.2. Enzymatic reaction, glucose standard curve and quantification

First, the parboiled rice effluent (PE) collected from the rice industry was pre-treated with a non-purified fungal α-amylase (ART Alimentos) of 40.000 SKB. This enzyme has a low cost and is widely used in the bakery industry. The working concentration of α-amylase was determined in non-published results, where 5 mg L^{-1} delivered the higher levels of glucose and lower cost when compared to 10, 50 and 100 mg L^{-1}. The parameters of enzymatic reaction as pH (5.5, 6, and 6.5), temperature (40°C, 50°C, and 60°C), and incubation time (5, 12.5, and 20 min) were analysed to determine the optimal conditions in the PE matrix based on levels of total glucose.

A glucose (Kasvi) standard solution of 5 g L^{-1} was prepared to generate a standard curve with 9 concentrations from 0.125 to 2 g L^{-1}. The DNS method [15] was used to quantify the glucose concentration, transferring 1 mL from enzymatic reaction and 1 mL of DNS to glass tubes for 15 min at 100°C. The absorbance (ABS) was measured on spectrophotometer (Shimadzu) at 540 nm after cooling the glass tubes to room temperature. The standard curve and the experimental procedure described for glucose quantification at preliminary assays were also employed for glucose quantification in parboiled rice effluent pre-treated with amylase (PEA) and parboiled rice effluent (PE), both inoculated with *S. cerevisiae*.

2.3. Inoculum and S. cerevisiae culture

Parboiled rice effluent pre-treated with amylase (PEA) and parboiled rice effluent (PE) were inoculated with *S. cerevisiae* strain YT01 (Centro de Biotecnologia – Federal University of Pelotas), prepared from a yeast colony transferred to 50 mL of Yeast Medium (YM) (Sigma-Aldrich), and incubated in shaker (Quimis) at 180 rpm and 28°C for 18 h. The initial concentration of *S. cerevisiae* was 1.2×10^3 colonies forming units per mL (CFU mL^{-1}) in 10% v/v. The experiments were performed in 5 L flasks (Fig. 1) with 4 L of non-sterilised parboiled rice effluent with 1% vvm of aeration, pH 5.5 and 28°C for 48 h.

2.4. Cell viability and biomass determination

Cell viability was expressed in terms of colony forming units per millilitre (CFU mL^{-1}) of culture medium, and assessed by collecting triplicates of 500 μL from parboiled rice effluent pre-treated with amylase (PEA) and parboiled rice effluent (PE) cultures at 0, 2, 4, 6, 8, 12, 24, 36 and 48 h. The samples collected for counting were serially diluted and transferred to yeast medium plates (YM), then incubated for 48 h at 28°C.

Biomass quantification (g) was realised collecting 10 mL samples of PEA and PE flasks at 0, 6, 8, 24, 36 and 48 h. Samples were centrifuged at 2,500 × g (Kubota) for 20 min and the pellet dried in stove at 60°C until constant weight, verified by sequential measures on analytical balance (Shimadzu).

2.5. Nutrient removal

The main environmental parameters of total phosphorus (P), total nitrogen (TKN) and chemical oxygen demand

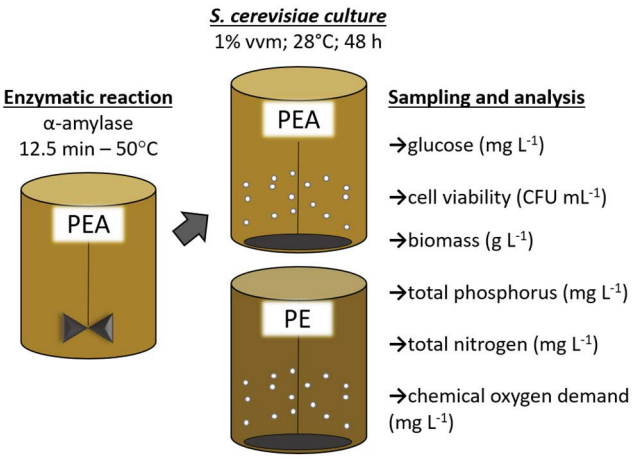

Fig. 1. Experimental setup. Parboiled rice effluent pre-treated with α-amylase (PEA) and inoculation of *Saccharomyces cerevisiae* in PEA and parboiled rice effluent (PE), followed by culture in 5 L flasks at 28°C and aeration of 1% vvm.

(COD) were analysed for nutrient removal. Triplicate samples of 500 μL were collected from parboiled rice effluent (PE) and parboiled rice effluent with amylase (PEA) at 0, 8, 24, 36 and 48 h. Samples were stored frozen until analysis, then thawed at room temperature (25°C), and centrifuged (Kubota) at 3,000 g for 5 min. Finally, the supernatant was gently collected for analysis.

Total phosphorus was determined by colorimetric method of phosphate vanadium-molibdate (NBR 12772–1992), transferring 2 mL of molibdate-antimonium (LabSynth) to 10 mL volumetric flask followed by respective sample (500 μL) and dilution. A phosphate standard solution (1,000 mg L^{-1}) was prepared and used to generate a standard curve at 1, 2.5, 5, 7.5 and 10 mg L^{-1}. The determinations were made in triplicate absorbances measured in UV-VIS (Kasuaki) at 400 nm, quantifying the colorimetric intensity of complex antimonium-phosphate-molibdate. Total phosphorus (mg L^{-1}) was determined by conversion of phosphate molecular weight into phosphorus.

Total nitrogen was determined using a Hanna TKN kit. A sample of 500 μL was transferred to a reaction tube and heated to 105°C for 30 min. Sodium metabisulfite was added to the reaction tube after cooling at room temperature, mixed, and the reaction tube was incubated at room temperature for 3 min. Then, total nitrogen reagent was added and left for 2 min. A 2 mL sample was collected and analysed in a photometer (Hanna).

COD was determined using a COD kit (Hanna). A volume of 200 μL of sample were transfer to digestion tubes containing dichromate and incubated at 150°C for 2 h. The colorimetric reaction was analysed in photometer (Hanna).

2.6. Statistical analysis

Statistical analyses were performed on software Statistica v.10 (Statsoft, USA). Experimental data were analysed and the averages were compared using Student's t-test with a 5% level of significance.

3. Results

3.1. Enzymatic reaction

The standard curve of glucose at concentrations from 0.25 to 2 g L^{-1} was $y = 1.7731x–0.1465$, where y corresponds to absorbance at 540 nm and x the concentration of glucose, with a regression coefficient of 0.9985. Results of glucose from the preliminary enzymatic assays at different levels of pH, temperature, and incubation time are presented in Table 1.

Treatment 15 had the highest concentration of glucose, reaching 520.83 mg L^{-1}, corresponding to 12.5 min of reaction at 50°C and pH 5.16; on the other side, reaction 9 (20 min, 60°C, and pH 6.5) resulted in 453.75 mg L^{-1} of glucose. The conditions presented by reaction 15 were selected for the following experiments with *S. cerevisiae*, after considering the levels of glucose obtained in the preliminary enzymatic assay, similarity to optimal pH for yeast culture, temperature, and volume required to perform the enzymatic reaction.

Table 1
Glucose (mg L^{-1}) in different levels of pH, temperature and incubation time

Reaction	Incubation time (min)	Temperature (°C)	pH	Glucose (mg L^{-1})
1	5	40	5.5	483.05
2	5	60	6.5	457.1
3	20	40	6.5	456.54
4	20	60	5.5	480.23
5	12.5	50	6	479.1
6	5	40	6.5	466.69
7	5	60	5.5	509
8	20	40	5.5	507.86
9	20	60	6.5	453.75
10	12.5	50	6	488.69
11	20	50	6	487.56
12	25	50	6	471.2
13	12.5	33.26	6	504.48
14	12.5	66.73	6	514.63
15	12.5	50	5.16	520.83
16	12.5	50	6.83	501.66
17	12.5	50	6	509

3.2. Cell viability, glucose, and biomass

Cell viability, glucose, and biomass results for parboiled rice effluent with amylase (PEA) and parboiled rice effluent (PE) are presented in Fig. 2.

The highest cell viability and biomass were observed in PEA at 48 h, and glucose at 8 h. Statistical significance ($p \leq 0.05$) between the averages in PEA and PE was observed for cell viability (8, 24, 36, and 48 h), biomass (6, 8, 24, 36, and 48 h), and glucose (0, 2, 4, 6, and 8 h).

3.3. Nutrient removal

The removals (%) of total phosphorus (mg L^{-1}), chemical oxygen demand (mg L^{-1}) and total nitrogen (mg L^{-1}) in parboiled rice effluent with amylase (PEA) and parboiled rice effluent (PE) are presented in Fig. 3.

The highest removals of P were 40.7% in PEA and 32.9% in PE, both at 48 h. The averages of COD removals in PEA and PE were statistically significant at 8, 24, 36 and 48 h, with highest removals of 70.7% in PE and 59.6% in PEA at 48 h. TKN was completed consumed for both cultures after 48 h, and reaching 100% at 36 h in PE.

3.4. Discussion

In this paper we report a method for the pre-treatment of parboiled rice effluent (PE) applying α-amylase to improve the carbon availability for *S. cerevisiae* culture. The method does not require a previous sterilisation or supplementation of the effluent, and also reports the presence of amide in the composition of PE. The pre-treatment improved the cell viability of *S. cerevisiae*, biomass and

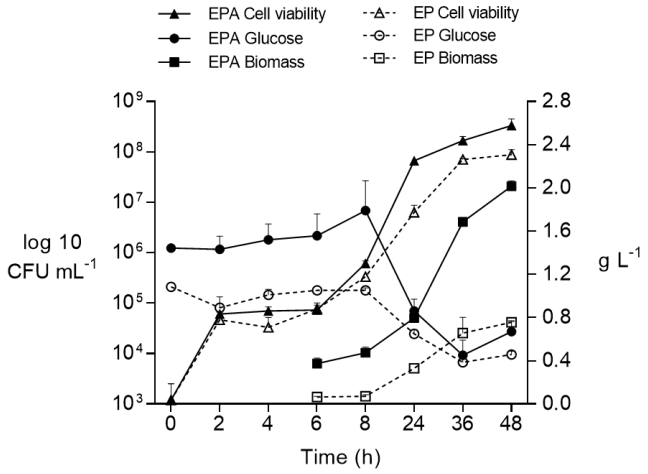

Fig. 2. Results of cell viability (log CFU mL^{-1}) and glucose (g L^{-1}) at 0, 2, 4, 8, 24, 36, 48 h, and biomass at 0, 6, 8, 24, 36, 48 h. Curves indicate mean ± standard deviation.

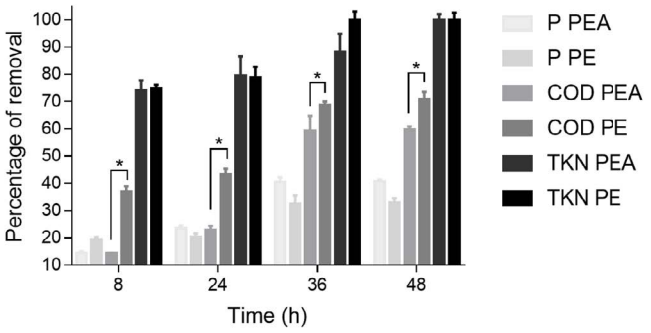

Fig. 3. Removal (%) of total phosphorus (P), chemical oxygen demand (COD) and nitrogen (TKN) in parboiled rice effluent pre-treated with amylase (PEA) and parboiled rice effluent (PE). Bars indicate mean ± standard deviation. Asterisk (*) indicate statistical significance ($p < 0.05$) of COD between PEA and PE at 8, 24, 36, and 48 h.

removals of P, COD and TKN. As far as we aware this is the first time that amide is detected in PE. Previous studies have reported the requirement of an external supplement for PE bioremediation to improve the nutrient availability for microorganism multiplication, biomass production and reduction of environmental parameters [12,13]. The method we describe is a straight-forward approach, modifying PE with a robust enzyme followed by *S. cerevisiae* culture, representing an innovative approach to bioremediation technologies where pre-treatments can be applied to support microorganism development and efficiency, instead of using external sources of nutrients.

Application feasibility and market competitivity of bioremediation methods relies principally on costs. On this way, the high stability and simple production process confer a low cost to the selected fungal α-amylase, besides the versatility and high stability in non-controlled mediums [16]. Amylase can be obtained from various sources as fungi, bacteria, yeast, actinomycetes and is widely applied by industry on hydrolysis reaction to obtain glucose [17]. The breakage of glycosidic bonds α-1,4 by α-amylase result in products of low molecular weight as maltose, maltotriose and glucose [18] – major carbon sources for *S. cerevisiae* metabolism [19]. Furthermore, fungal α-amylase is widely used on bread and brewing process due to low-grade of purification and consequently low cost, and representing to this method a cost of 0.45$ per m^3 of treated PE.

The sampling of PE was realised in a full year basis to ensure the reproducibility of the method, where no alterations were observed on parameters removal and yeast growth. The analysis of pH, temperature and time of enzymatic treatment were essential due to unprecedent methodology applying α-amylase in PE pre-treatment followed by *S. cerevisiae* inoculation. After identifying the best conditions at laboratory scale, the industrial conditions were also considered on enzymatic and culture experiments. The temperature range (40°C, 50°C, 60°C – 33.26°C/66.73°C axials) was analysed due to reports of α-amylase optimal activity and the temperature that PE is discharged from parboiling process, ensuring a lower cost when upscaled. The parboiling conditions can vary between the industries. However, the effluent leaves the parboiling process in a temperature range of 35°C–48°C [20]. The selected temperature of 50°C not only follows the highest glucose level presented in Table 1 by treatment 15 but also agrees with the working range. The pH levels (5.5, 6, 6.5 – 5.16/6.83 axials) were analysed as the effluent has an acidic pH after parboiling process, which also suits the yeast metabolism. *S. cerevisiae* is an acidophilic organism and in non-published results we identified the higher viabilities and biomass at the pH 5.5. Similar conditions as acidic medium and temperatures from 30 to 80°C have been also used on treatment and re-use of food waste as culture medium [21,22].

The highest concentration of glucose detected was 1.75 g L^{-1} and observed in PEA at 8 h, 67% superior to glucose in PE (Fig. 2), what suggests that amylase activity continued after the pre-treatment. In accordance with these findings, the averages of cell viability and biomass were statistically significant. The levels of glucose have declined after 8 h of culture, probably due to consumption, reduction of enzymatic activity after a long exposure to effluent, depletion of starch, structural modifications, and pH fluctuation in the media [23]. The similarity between PEA and PE after 8 h suggests that the extra carbon source from the pre-treatment was totally consumed. The higher glucose concentration in PEA than PE probably induced the cell viability of *S. cerevisiae* (Fig. 2), where PEA was superior to PE in all sampling times and statistically significant at 8, 24, 36 and 48 h.

Yeast biomass is widely used as animal or human protein supplement in human nutrition or animal feeding [24]. The usual industrial processes for biomass production occur by upscaling reactors volume, using agar and sugar as principal compounds in medium [25], what represents a high cost to industries. The application of PE as a non-cost culture medium for *S. cerevisiae* it is an interesting alternative to reduce the effluent treatment cost in rice industry and environmental risk due to effluent complexity. Depending on the purification degree, yeast biomass can be found from 4$ to 209.74$ per kg [26,27]. In our study, the usage of PE as potential culture medium was improved

by the enzymatic treatment, reaching 2 g L^{-1} in 48 h of culture in non-stirred 5 L flasks using a non-sterilised effluent, superior ($p \leq 0.05$) to PE in all sampling times. This result is similar to the 2.1 g L^{-1} biomass of *Pichia pastoris* X-33 after 70 h presented by Gil de los Santos et al. [12] in PE supplemented with 15 g L^{-1} of biodiesel glycerol. In a similar attempt to improve nutrient removal and biomass production, Gaboardi et al. [13] supplemented PE with 1% sucrose and cultured *Saccharomyces boulardii* in bioreactor, improving the culture conditions and biomass production to 3.8 g L^{-1} in 48 h, nonetheless, with higher costs due to use of bioreactor and supplement. Our method shows a clear advantage when comparing with supplemented and bioreactors cultures, with a further biomass production when applying an enzymatic pre-treatment in PE. Moreover, other cultivation parameters need be considered as noted by Kucharczyk and Tuszyński [28], reporting the importance of aeration for biomass vitality and yeast resistance against stress conditions associated with concentration of glycogen and trialose, that allows the yeast to absorb nutrients from medium and realize selective metabolic exchanges. The absence of supplementation and simple culture procedure in flasks it is a remarkable achievement of yeast application in bioremediation of industrial effluents. Biomass production is also favoured by the high volume of PE available – in ideal conditions, 1.300 kg of *S. cerevisiae* could be produced in PEA within 48 h of culture, representing a co-product from the effluent treatment.

The usefulness of *S. cerevisiae* biomass produced on PE treatment is not limited to a source of nutrients in fertilizers or animal feeding, but also in higher value applications as probiotics [24,29]. For this, cell viability is an interesting assay as measures the ability of grow and generate colonies, where a minimum therapeutic level can be obtained at 10^6 CFU g^{-1} [30]. The higher levels of glucose in PEA induced the *S. cerevisiae* cell viability (Fig. 2), where PEA was superior to PE in all sampling times, statistically significant at 8, 24, 36, 48 h and a maximum cell viability 26% higher than PE. The logarithmic phase of both cultures occurred from 6 to 24 h, coinciding with higher increases in biomass and glucose reduction. The maximum cell viability of 3.35×10^8 CFU mL^{-1} was 28.3% higher than the reported in PE supplemented with 1% sucrose in a bioreactor culture [13] with a high cost to be implemented in the industry, since a regular parboiled industry produces 1–1.2 L of effluent per kilogram of rice and would require a large industrial structure [4].

Total phosphorus (P), COD and total nitrogen removals (TKN) were determined at 8, 24, 36 and 48 h in PEA and PE. COD is a common approach to quantify biodegradable and non-biodegradable compounds in effluent samples. The highest COD removals in PEA and PE were 59.64% and 70.74% (Fig. 3), respectively. The COD removals in PE were unexpectedly higher ($p \leq 0.05$) than PEA in all sampling times. The higher concentration of glucose in PEA due to enzymatic activity would favour the conversion of organic matter in biomass. However, this fact can be explained by the instability of organic wastes to chemical and biochemical oxidations where an interference from enzymatic products could be associated with lower COD removals in PEA [31]. Nevertheless, the experimental setup without supplementation, sterilisation and culture in flasks was simpler than reported by Gil de los Santos et al. [12] and Gaboardi et al. [13] – maximum COD removals reported by these authors were 20% lower and 14% higher in 48 h, respectively. TKN is a method for nitrogen quantification and widely applied in complex matrices, considering ammonia, ammonium and nitrogen present in organic substances. Nitrogen uptake reached levels higher than 70% in both cultures in 8 h, 100% in PE at 36 h and later in PEA at 48 h. Nitrogen was completely consumed in the cultures and therefore can be considered as limiting nutrient for nutrient uptake. The TKN uptake was followed by reduction in COD and P at 8 and 24 h. A similar behaviour was observed at 36 and 48 h – TKN removals have not changed expressively and the same happened with P and COD. This fact reinforce the importance of C:N:P ratio on yeast metabolism and nutrient uptake. The C/N ratio at 0 h was 5.41 and lower than required for an adequate development of micro-organisms. For example, Rončević et al. [32] reported a C:N:P ratio of 50:3.5:1.3 for maximum bioethanol production in the process of fermentation using immobilised *S. cerevisiae*. P is considered one of the hardest nutrients to be removed from industrial effluents. The application of *S. cerevisiae* in P removal not only shows as an alternative for nutrient uptake, but also recover and concentrate a mineral of growing limitation on global scales [33].

On this research, the highest removals were mostly obtained in PEA at 24, 36 and 48 h, reaching 40.7% in PEA and 32.9% in PE. The efficacy of this treatment is observed when comparing with similar approaches, applying yeast in supplemented PE obtaining 52% of removal in 70 h [12] and 93% of removal in 36 d culture with microalgae and cyanobacteria [34].

4. Conclusions

We report in this paper the potential use of amide in parboiled rice effluent as a carbon source for bioremediation process with *S. cerevisiae* after a pre-treatment with α-amylase. The optimal conditions for enzymatic treatment and *S. cerevisiae* culture were determined based on industrial characteristics and outputs as biomass, parameters removal and glucose levels. The suggested method increased in 40% the glucose concentration for *S. cerevisiae* culture, 73% the biomass weight and 62.9% cell viability when compared to control. The method is also an alternative for nutrient removal as uptakes of 40.7%, 62%, and 100% were reported for phosphorus, COD ad TKN, respectively. This simple and environmentally friendly method can be expanded for different matrices, exploring the composition of industrial effluent previously to bioremediation step and enhancing the treatment with yeasts.

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The authors have declared no conflict of interests.

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