



Spent yeast reuse as inoculum: a strategy to boost cellulosic ethanol productivity

Mariana S. T. Amândio^{1,2} · Manuel J. A. Gonçalves¹ · Luísa S. Serafim¹ · Jorge M. S. Rocha² · Ana M. R. B. Xavier¹

Received: 19 July 2024 / Revised: 24 October 2024 / Accepted: 29 October 2024 / Published online: 11 November 2024
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Abstract

For the implementation of an integrated biorefinery into a pulping mill, the valorization of bark through cellulosic ethanol production and simultaneously the valorization of the residual yeast from each fermentation run should be considered to increase sustainability and pulp and paper profitability. Spent yeast recycling during cellulosic ethanol production is an opportunity to reduce waste according to environmental issues for disposal at the industrial level, boosting the circular economy. This work evaluated the reuse of spent yeast in successive fermentation experiments at Erlenmeyer and bioreactor scales for cellulosic ethanol from *Eucalyptus globulus* bark. The spent yeast recovered through centrifugation at the end of each experiment was used as inoculum for subsequent batch fermentation. Across five sequential fermentations, wherein only a fraction of spent yeast was recycled, a discernible decline in ethanol volumetric productivity was observed throughout successive fermentation cycles. Conversely, employing the complete spent yeast for recycling along 11 consecutive trials resulted in a notable enhancement of ethanol productivity, as evidenced by a reduction in fermentation duration to approximately one-third remaining the conversion efficiency above 70%. Four successive experimental trials were conducted at the bioreactor scale, resulting in volumetric productivities ranging from 2.00 to 5.83 g L⁻¹ h⁻¹. The reuse of spent yeast in consecutive fermentation cycles demonstrably augmented productivity, thereby validating a promising strategy for the industrial-scale production of cellulosic ethanol. This significantly contributes to advances in the circular economy principles within the overarching framework of biorefinery operations.

Keywords Cell reuse · Cellulosic ethanol · Circular economy · Inoculum · Spent yeast · Yeast recycling

Highlights

- Spent yeast reuse is successful in cellulosic ethanol production from bark residues.
- Reusing a fraction of spent yeast in five consecutive batches provided good results.
- Complete spent yeast reuse was successful and reduced fermentation time to 1/3.
- Complete reuse at the bioreactor provided conversion efficiencies higher than 75%.
- Maximum volumetric productivity of 5.83 g L⁻¹ h⁻¹ was reached at bioreactor scale.

✉ Ana M. R. B. Xavier
abx@ua.pt

¹ CICECO—Aveiro Institute of Materials, Chemistry Department, University of Aveiro, Campus Universitario de Santiago, 3810-193, Aveiro, Portugal

² CERES, Department of Chemical Engineering, Faculty of Sciences and Technology, University of Coimbra, Rua Sílvio Lima, Polo II 3030-790, Coimbra, Portugal

1 Introduction

Spent yeast is one of the main by-products of fermentation processes, corresponding to the residual or surplus yeast that is no longer useful for the process and must be disposed of. This sub-product, also known as residual yeast or surplus yeast, is a valuable source of nitrogen and proteins [1, 2]. However, its application has been constrained to low-value end-use, such as animal nutrition or feed for livestock [1, 3]. Frequently, the residual yeast is considered organic waste, disposed of in landfills or incinerated, negatively impacting the environment. On the other hand, the high moisture content and the chemical composition of post-fermentation biomass promote fast degradation, hindering its storage under environmental conditions [2]. These concerns are particularly relevant at the industrial level due to the high volumes involved, microbiological safety, environmental restrictions, and potential profitability [4, 5].

Considering the increasingly stricter environmental regulations, a new design of the industrial processes is demanded by the circular economy model to improve materials' life cycle by reusing and recycling to reduce waste and simultaneously promote multi-valorization of the by-products [4–6]. Therefore, spent yeast released from industrial processes should be re-directed to an integrated biorefinery in the existing industrial plants instead of being discarded. Converting the by-products into valuable products is becoming increasingly crucial. Spent yeast can be valorized if employed as yeasts or as its bioactive compounds for food additives, animal feed, or innovative fertilizers [3, 7]. This subproduct could also be employed for yeast extract production since it is a valuable source of nitrogen and proteins, which includes all the essential amino acids [1, 2], namely for replacing traditional protein nutrients and specific food applications, as in the case of replacing egg whites [8]. These yeasts, mainly belonging to the *Saccharomyces* genera are generally recognized as safe (GRAS) as producers of food products [9, 10]. Growing demand for alternative protein sources to meet the needs of vegetarian and vegan populations is also driving interest [11]. Spent yeast is also utilized to produce several food additives and nutraceuticals based on its content of β -glucans, which improve food properties by emulsifying, water-holding, and thickening effects, and some authors also already identified antioxidant, antimicrobial, and anti-tumor properties [12, 13]. Chattaraj et al. [3] and Pasquet et al. [7] summarized the recent advances and novel applications for brewers' spent yeast.

The reuse of spent yeast as inoculum in consecutive fermentation batches is also a promising approach to valorization. This is already a common strategy used for first-generation bioethanol mainly in Brazilian biorefineries which are the largest producers of ethanol from sugarcane [14–18]. In that process, about 90–95% of the yeast cells are reused many times during 6 to 8 months [14–17]. Some authors have also focused on optimizing brewing processes by evaluating the quality of recycled yeast cells by determining cell viability, reproduction capacity reduction, cell damage, and decreased metabolic activity. Although it is common practice in first-generation ethanol biorefineries and breweries, this approach has not been widely exploited for cellulosic ethanol production [14–16]. The process of second-generation bioethanol production is undoubtedly more challenging due to the complexity and recalcitrance of lignocellulosic materials [19]. Moreover, the long-term viability in cellulosic ethanol fermentations is expected to be lower than in the first-generation processes due to the presence of fermentation inhibitors typically present in lignocellulosic hydrolysates [20]. This approach is aligned with the biorefinery

concept by efficiently converting biomass-based sources into a wide range of bioproducts, contributing to improving commercial competitiveness, which is still one of the major challenges of second-generation bioethanol [21–23].

In the present work, it was shown for the first time that the spent yeast derived from cellulosic ethanol production from pretreated *E. globulus* bark can be successfully reused as inoculum in consecutive fermentation experiments. *Eucalyptus globulus* bark is an industrial residue widely available in the pulp and paper sector, which has a significant impact on the national economy. Since bioethanol is not produced in Portugal yet, there is a great interest in using the bark residues in a sugar-based biofuel production platform in a pulping mill-integrated biorefinery. However, there are also concerns about the waste and effluent generated. This work supported the circular economy concept since combining the bark as raw material (a large volume residue from the pulp and paper industry) with the reuse of spent yeast (a by-product of the fermentation process) could contribute to making cellulosic ethanol production more cost-competitive. Therefore, this work studied the spent yeast reuse as inoculum as a strategy to promote its valorization but also to boost ethanol productivity. The experimental assays were carried out using Ethanol Red®, a commercial strain of *Saccharomyces cerevisiae* developed for industrial cellulosic ethanol. The assays were conducted at Erlenmeyer and bioreactor scales, and their performance was evaluated based on ethanol concentration, productivity, and conversion efficiency. For each set of experiments, the first fermentation started with fresh inoculum and subsequent assays were inoculated with the spent yeast. To our knowledge, this is the first time that spent yeast derived from cellulosic ethanol production from pretreated *E. globulus* bark was evaluated as inoculum in consecutive fermentation experiments.

2 Materials and methods

2.1 Raw material and pretreatment

The *Eucalyptus globulus* bark was previously submitted to kraft pulping pretreatment (170 °C, 75 min using an active alkali of 24%). The kraft pulp is mainly composed of cellulose (79.8 ± 3.8 wt%), hemicelluloses (15.5 ± 0.6 wt%), and lignin (2.6 ± 0.3 wt%) [24]. This raw material was kindly provided by RAIZ—Instituto de Investigação da Floresta e do Papel (Portugal), and the respective composition was determined following National Renewable Energy Laboratory (NREL) standard protocols [25]. Enzymatic inhibitors (namely levulinic, furfural, and hydroxymethylfurfural) were not detected in this raw material.

2.2 Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated *E. globulus* bark followed a fed-batch strategy using Cellic® CTec2, a commercial cellulases consortium acquired from Sigma-Aldrich. The enzymes consortium was fed all at once initially with a dosage of 25 FPU $\text{g}_{\text{carbohydrates}}^{-1}$ whose filter paper unit (FPU) was 168.7 FPU mL^{-1} based on the NREL standard procedure. The reaction lasted 24 h at 50 °C and 150 rpm, with pH controlled between 4.5 and 5.5 (adjusted by adding H_2SO_4 2 M and NaOH 2 M solutions). The hydrolysate produced contained about 136 g L^{-1} of glucose and 25 g L^{-1} of xylose. More detailed information about the enzymatic hydrolysis process can be consulted in [26].

2.3 Pre-inocula and inocula

Pre-inocula and inocula were prepared in duplicate for the initial batch of each set of experiments. The commercial yeast Ethanol Red® gently supplied by Leaf, France, was maintained at 4 °C on Petri dishes with solid yeast medium (YM)–containing glucose (10 g L^{-1}), peptone (5 g L^{-1}), malt extract (3 g L^{-1}), yeast extract (3 g L^{-1}), and agar (20 g L^{-1}).

2.3.1 Erlenmeyer experiments

Pre-inocula were prepared by transferring one colony from a maintenance YM Petri dish to a 50-mL Falcon tube containing 10 mL of liquid YM (similar to solid YM, except agar) and then incubated at 180 rpm and 28 °C for 24 h (Stuart SI500).

Inocula were prepared by transferring the volume of pre-inoculum to 40-mL fresh liquid YM that ensured an optical density ($\text{OD}_{620\text{ nm}}$) of about 0.400, corresponding to an initial biomass concentration of about 1 g L^{-1} . A calibration curve of $\text{OD}_{620\text{ nm}}$ vs. dry weight biomass concentration (g L^{-1}) was previously made.

Inocula were incubated at 180 rpm and 28 °C for 14 h using 100-mL Erlenmeyer flasks [24].

2.3.2 Bioreactor experiments

Pre-inocula were prepared by transferring 2–3 colonies from a maintenance YM Petri dish to a 100-mL Erlenmeyer flask containing 40 mL of liquid YM and then incubated at 180 rpm and 28 °C for 24 h.

Inocula were prepared by transferring the volume of pre-inoculum to 200-mL fresh liquid YM following the procedure described in the previous Section 2.3.1.

Inocula were incubated at 180 rpm and 28 °C for 14 h using 500-mL Erlenmeyer flasks [24].

2.4 Fermentation assays with spent yeast reuse

The spent yeast was reused throughout consecutive fermentation assays at the Erlenmeyer and bioreactor scale. Before starting the following fermentation cycle, glucose depletion was confirmed by high-performance liquid chromatography (HPLC) analysis at least for one replicate (if applicable). This was the criterion used to continue reusing the yeast and move on to the next cycle.

The fermentation medium was composed of 85% (v v^{-1}) hydrolysate, 10% (v v^{-1}) inoculum, and 5% (v v^{-1}) of supplementation composed of 2.5 g L^{-1} yeast extract, 2.0 g L^{-1} $(\text{NH}_4)_2\text{HPO}_4$, 1.0 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, and 0.50 g L^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ [27].

2.4.1 Erlenmeyer experiments

Five successive experiments (E5) were performed in duplicate in 250-mL Erlenmeyer flasks with a working volume of 100 mL incubated at 180 rpm and 28 °C. At the end of each fermentation assay, after glucose depletion and except for the last one, the culture broth was centrifuged at 5000 rpm for 10 min and 4 °C in 50-mL Falcon tubes. The supernatant was discarded, and a certain amount of biomass pellet was resuspended in YM to ensure an initial biomass concentration of around 1.0 g L^{-1} . This procedure lasted about 1 h. Furthermore, in a few cases, the overnight period did not allow for monitoring the progress of the fermentation and/or immediately proceeding with reusing the inoculum.

The complete reuse of the spent yeast was evaluated through eleven consecutive fermentation assays (E11) at the Erlenmeyer scale. A similar procedure was followed but all the spent yeast was collected and used to re-inoculate the following fermentation assay. Moreover, due to its limited availability, the hydrolysate was diluted to obtain a volume that allowed as many consecutive fermentations as possible with a glucose concentration of at least 1/3 of the value in the original hydrolysate. Still, these successive fermentations were carried out as proof of concept, aiming to demonstrate that the cells can maintain their ability to produce ethanol even after several cycles of reuse.

For the Erlenmeyer experiments, the pH was monitored using a Hach sensION + MM340 benchtop meter (United States of America (USA)) with an electrode InPro 3030/200 (Mettler Toledo, USA).

2.4.2 Bioreactor experiments

Four consecutive experiments (B4) were carried out in a 5-L BIOSTAT A plus bioreactor (Sartorius Stedim Biotech,

Germany) with a working volume of 1 L and automatic control of stirring, temperature and pH by micro-DCU software and data acquisition by MFCS/DA 3.0 system (Sartorius Stedim Systems, Germany). The temperature was controlled at 28 °C, and the stirring was set at 180 rpm using two six-bladed Rushton turbines. In the case of bioreactor experiments, the pH was automatically controlled to 5.50 ± 0.05 by adding KOH 5 M and H₂SO₄ 1 M, being measured using an electrode EasyFerm Plus K8 325 (Hamilton, USA).

After 22 h of the initial batch, the fermented broth was pumped from the bioreactor through the outlet pipe using a peristaltic pump Sci Q 400 (Watson Marlow, UK) and collected under sterile conditions. Then, the fermented broth was centrifuged at 5000 rpm for 10 min and 4 °C in a 400-mL centrifuge bottles. After that, the supernatant was discarded, and the total pellet containing the biomass was resuspended in YM medium (used as a fresh nutrient source) to a final volume that corresponded to 10% (v v⁻¹) of the total working volume and was used to re-inoculate the fermenter containing already 850 mL of hydrolysate and 50 mL of supplementation solution. The procedure was repeated along consecutive experiments.

2.5 Analytical methods

2.5.1 Biomass concentration

Biomass concentration was determined using a calibration curve of optical density versus biomass dry weight. After diluting the samples with NaCl 0.9% (w v⁻¹), the optical density was measured at 620 nm using a Shimadzu UVmini-1240 spectrophotometer (Kyoto, Japan).

2.5.2 High performance liquid chromatography

HPLC was used to determine the concentration of glucose, xylose, ethanol, glycerol and acetic acid. After properly diluted and filtered (0.2-μm pore size), 10 μL of each sample was injected on a Rezex ROA-Organic Acid H⁺ (8%) 300 × 7.8 mm ion-exchange column (Phenomenex, USA) at 65 °C (oven Gecko 2000) connected to a refraction index detector L-2490 (Hitachi, Japan). The mobile phase was H₂SO₄ 0.005 N, using a flow rate of 0.500 mL min⁻¹ (Hitachi pump L-2130, Japan).

Standards in the 0–5 g/L range for sugars, ethanol, glycerol, and acetic acid were used to obtain standard calibration curves.

2.6 Calculations

The volumetric glucose consumption rate, r_{glucose} (g L⁻¹ h⁻¹), was calculated from the module of the linear regression slope of glucose concentration over time.

The volumetric ethanol productivity, Prod_{vol} (g_{ethanol} L⁻¹ h⁻¹) was calculated according to Eq. (1), considering the differences (Δ) between the maximum ethanol concentration achieved and the initial ethanol of the fermentation.

$$\text{Prod}_{\text{vol}} (\text{g}_{\text{ethanol}} \text{L}^{-1} \text{h}^{-1}) = \frac{\Delta[\text{Ethanol}]}{\Delta t} \quad (1)$$

The specific ethanol productivity ($\text{Prod}_{\text{spec}}$) was calculated by the ratio between the volumetric productivity (Prod_{vol}) and the biomass concentration ($[\text{Biomass}]_{\text{max}}$) at the time when ethanol concentration was maximum. This parameter was calculated based on Eq. (2).

$$\text{Prod}_{\text{spec}} (\text{g}_{\text{ethanol}} \text{g}_{\text{biomass}}^{-1} \text{h}^{-1}) = \frac{\text{Prod}_{\text{vol}}}{[\text{Biomass}]_{\text{max}}} \quad (2)$$

The ethanol yield, $Y_{\text{ethanol/substrate}}$ (g_{ethanol} g_{substrate}⁻¹) was determined by Eq. (3), considering both glucose and xylose as the substrates and the maximum ethanol concentration. Substrate concentration differences (Δ) were calculated from the beginning of the fermentation until the maximum ethanol concentration was reached.

$$Y_{\text{ethanol/substrate}} (\text{g}_{\text{ethanol}} \text{g}_{\text{substrate}}^{-1}) = \frac{\Delta[\text{Ethanol}]}{\Delta[\text{Substrate}]} \quad (3)$$

The conversion efficiency was calculated based on Eq. (4), considering the maximum theoretical ethanol yield of 0.511 g_{ethanol} g_{sugars}⁻¹ [28]:

$$\text{Conversion efficiency (\%)} = \frac{Y_{\text{ethanol/substrate}}}{0.511} \times 100 \quad (4)$$

2.7 Statistical analysis

The statistical data analysis was conducted using the analysis of variance (one-way ANOVA) with a 95% confidence level. For that purpose, Microsoft Excel with the support of the analysis toolpack add-in was used.

The initial batch of each round of tests was not included in the statistical analysis as it was started with fresh inoculum.

3 Results and discussion

3.1 Spent yeast reuse as fermentation inoculum

3.1.1 Erlenmeyer scale—reuse only a fraction of the spent yeast

The potential of cell reuse in five consecutive fermentations (E5), where only the initial batch was inoculated with fresh inoculum, was evaluated at the Erlenmeyer scale. The concentration profile of metabolites and biomass in Fig. 1 (as

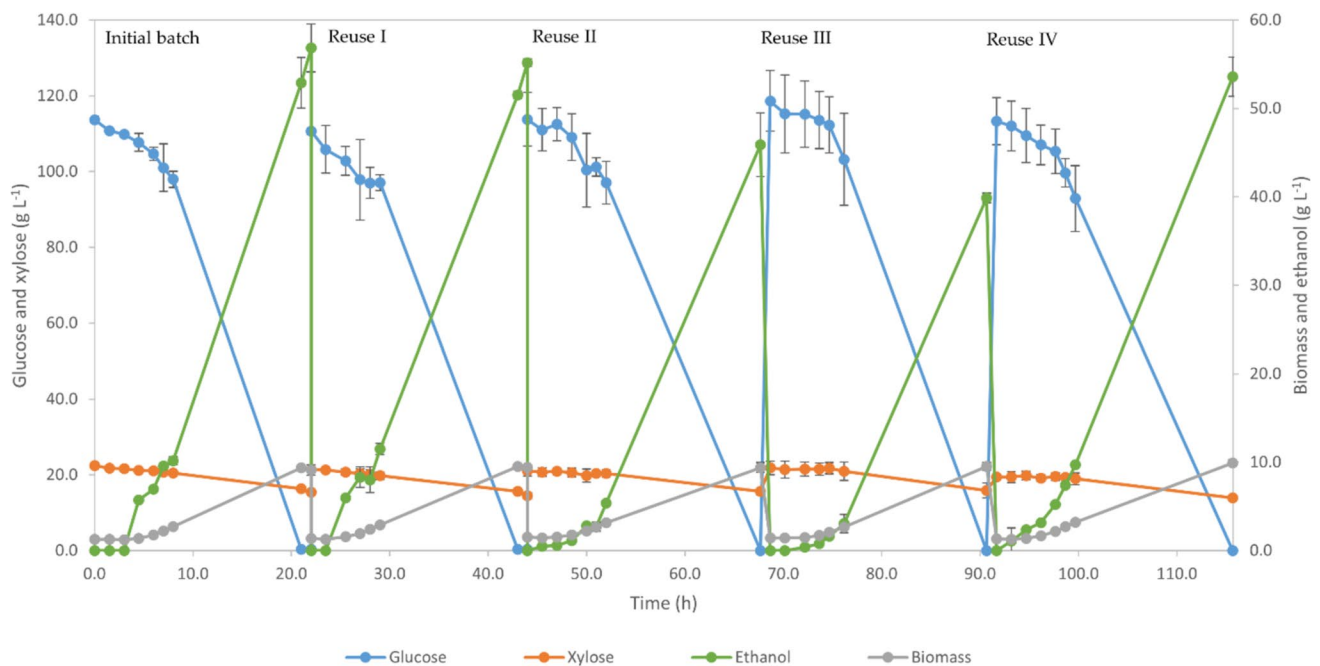


Fig. 1 Profile of glucose, xylose, ethanol, and biomass concentrations (g/L) for five consecutive fermentation experiments at Erlenmeyer scale (E5) with reuse of only a fraction of the spent yeast as inoculum

well as in Fig. 3) was plotted continuously to simplify the interpretation of the results. However, there was an interval of about 1 h between each cycle due to the time required for the collection, centrifugation, and re-inoculation.

Glucose consumption started to be detected after 4.5 h, at a volumetric rate of $6.85 \pm 0.46 \text{ g L}^{-1} \text{ h}^{-1}$ until 21.0 h. The maximum ethanol concentration of $56.9 \pm 1.1 \text{ g L}^{-1}$ was achieved at 22.0 h corresponding to a volumetric productivity and conversion efficiency of $2.58 \pm 0.05 \text{ g L}^{-1} \text{ h}^{-1}$ and $92.5 \pm 0.7\%$, respectively. Specific productivity was about $0.28 \pm 0.01 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$. Glycerol and acetic acid formation were almost negligible. Similar performance concerning biomass growth and ethanol production was found for the following batch, reuse I. Bioethanol concentration achieved a maximum of $55.2 \pm 0.4 \text{ g L}^{-1}$ after 22.0 h with a volumetric productivity of $2.51 \pm 0.02 \text{ g L}^{-1} \text{ h}^{-1}$ and $92.6 \pm 1.6\%$ conversion efficiency. It was found a slightly reduced performance in the reuse II, with a production of ethanol of $45.9 \pm 3.6 \text{ g L}^{-1}$. Volumetric productivity and conversion efficiency decreased to $1.94 \pm 0.15 \text{ g L}^{-1} \text{ h}^{-1}$ and $75.3 \pm 1.1\%$, respectively.

Reuse III assay presented a longer lag phase of 6 h, resulting in a lower conversion efficiency of $54.0 \pm 4.0\%$, with a maximum ethanol concentration of $39.9 \pm 7.5 \text{ g L}^{-1}$. Specific productivity also decreased to $0.19 \pm 0.04 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$. A considerable difference between replicates was found for initial glucose concentration but also for ethanol production, which can justify the high standard deviation.

In the last experiment (reuse IV), glucose started to be consumed after 3 h of the beginning, demonstrating a shorter lag phase. The maximum biomass concentration was higher than in the previous runs, reaching $9.94 \pm 0.38 \text{ g L}^{-1}$. Ethanol production began simultaneously with glucose consumption and reached its maximum of $53.6 \pm 2.2 \text{ g L}^{-1}$ after 24 h, with a volumetric productivity of $2.23 \pm 0.09 \text{ g L}^{-1} \text{ h}^{-1}$ and a conversion efficiency of $88.3 \pm 1.0\%$. Therefore, the fermentation performance seems to have improved from the fourth to the fifth experiment. Similar findings were reported by Pereira et al. [29] by performing a cell recycle batch fermentation for 15 consecutive cycles using a synthetic medium with a high glucose concentration (300 to 400 g L^{-1}). During the first five fermentation cycles, the conversion efficiency and productivity ranged from 78 to 82% and 3.45 to $4.59 \text{ g L}^{-1} \text{ h}^{-1}$, respectively. Then, from cycles 5 to 6, only a small fraction of the spent yeast was transferred, which restored viability from 27 to 49% and from 42 to 77% between cycles 12 to 13 [29].

Overall, these five consecutive fermentations started with almost the same initial biomass concentration (between 1.26 and 1.51 g L^{-1}) and reached a similar maximum biomass concentration. Ethanol production ranged from about 40 to 57 g L^{-1} , with volumetric productivity varying from 1.81 to $2.58 \text{ g L}^{-1} \text{ h}^{-1}$ and specific productivity ranging from 0.19 to $0.28 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$. As expected, the highest specific productivity was achieved for the initial batch and reuse I, while a slight decline in overall performance was observed for the remaining experiments certainly due to an increase

of the non-viable or at least non-productive fraction of cells after successive reuses. A similar observation was found by Fan et al. [30], showing lower volumetric productivity in the third cycle. These authors attributed this effect to the decrease of viable cells due to stress conditions caused by the high ethanol concentration or other inhibitors for a long time. Pereira et al. [29] also observed a severe decrease in cell viability after the fifth fermentation cycle, reducing conversion efficiency. Indeed, the conversion efficiency reduction should be related to the higher energy requirements for cell maintenance, mainly because cells were exposed to hydrolysate components or other stressing conditions for extended periods (namely low pH, high ethanol concentration, among others) [15, 29, 31].

There is no significant difference regarding volumetric productivity (p -value 0.072), specific productivity (p -value 0.095), and maximum ethanol production (p -value 0.068). However, a significant difference was found regarding the conversion efficiency (p -value 0.0002).

3.1.2 Erlenmeyer scale—complete reuse of the spent yeast

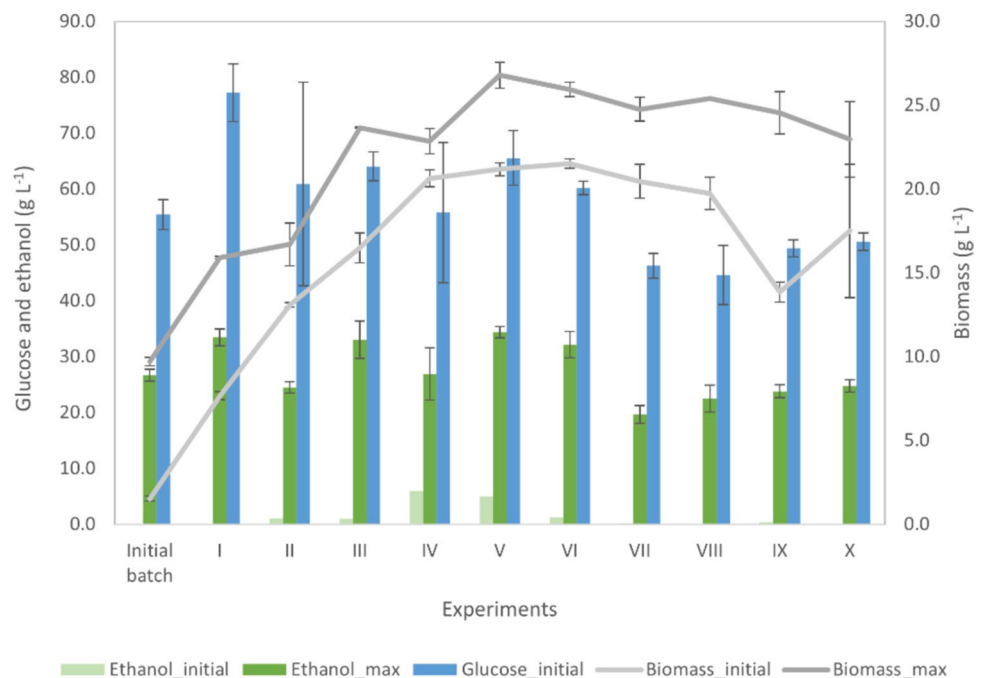
The complete reuse of the spent yeast was evaluated through eleven consecutive fermentation assays at the Erlenmeyer scale (E11). The initial batch started with a biomass concentration of about $1.52 \pm 0.15 \text{ g L}^{-1}$, and the lag phase lasted about 4.5 h. After that, glucose started to be consumed, and ethanol was produced (Fig. 2). The maximum ethanol concentration of $26.7 \pm 1.1 \text{ g L}^{-1}$ was detected at 22.8 h, corresponding to a conversion efficiency of $82.5 \pm 0.4\%$. As expected, these values were lower than in the set of

experiments E5 due to the lower sugar concentration supplied. A volumetric productivity of $1.17 \pm 0.05 \text{ g L}^{-1} \text{ h}^{-1}$ was determined. However, this volumetric productivity value might be underestimated. Considering the glucose consumption rate of $8.67 \pm 0.32 \text{ g L}^{-1} \text{ h}^{-1}$, observed between 6 and 8 h, its depletion may have occurred around 11.5 h. Considering that the maximum ethanol concentration of 26.7 g L^{-1} occurred simultaneously, this translated to an estimated productivity of about twice, $2.32 \text{ g L}^{-1} \text{ h}^{-1}$.

In the reuse I, the initial biomass concentration was about fivefold higher than in the initial batch. The spent yeast reuse assay was faster than the initial batch since the lag phase was not detected. During the first 4 h, about half of the initial glucose concentration was already consumed. In contrast, almost no glucose consumption was observed in the initial batch in the same period. At 4 h, ethanol production of about $13.8 \pm 5.5 \text{ g L}^{-1}$ was detected, reaching its maximum of $33.4 \pm 1.5 \text{ g L}^{-1}$ after 7 h. A significant volumetric productivity improvement was observed, attaining $4.78 \pm 0.22 \text{ g L}^{-1} \text{ h}^{-1}$.

The reuse II was even faster, consuming all the glucose in only 4 h. In this case, the overall time process was shorter than the lag phase of the initial batch. After this reuse II experiment, the volumetric productivity was always increased being higher than $6 \text{ g L}^{-1} \text{ h}^{-1}$ and achieving its maximum of $9.2 \text{ g L}^{-1} \text{ h}^{-1}$ at reuse VI. These results evidenced the adaptability of the yeast strain, minimizing the lag phase. Moreover, a significant increase in the initial biomass concentration was observed along the reuse cycles. Indeed, this high initial biomass concentration also shortened the overall process time. While the initial batch was

Fig. 2 Biomass, glucose, and produced ethanol concentrations (g L^{-1}) for eleven consecutive fermentation experiments at the Erlenmeyer scale (E11) with complete reuse of the spent yeast as inoculum. The maximum ethanol concentration value was calculated discounting the initial ethanol concentration. Maximum biomass concentration corresponds to the biomass concentration when maximum ethanol concentration was detected (these times are shown in Table 1)



started with a biomass concentration of around 1.5 g L^{-1} , reuses I and II started with 7.7 and 13.1 g L^{-1} , respectively. The initial biomass concentration increased considerably from the initial batch until reuse IV (Fig. 2). The reuse VI started with a biomass concentration of 21.5 g L^{-1} , achieving the highest maximum of 26.7 g L^{-1} . In the reuse IV, the initial biomass concentration stabilized at around 20 g L^{-1} , clearly showing that the growing capacity of the cells was already limited certainly due to the high increase of the non-viable or at least non-active cells fraction versus the active and non-inhibited resistant cells fraction.

Even after ten cycles of spent yeast reuse, a good fermentation performance was observed, with a maximum ethanol concentration of $24.8 \pm 1.1 \text{ g L}^{-1}$ detected just after 3 h. This production corresponded to a volumetric and specific productivity of $8.25 \pm 0.38 \text{ g L}^{-1} \text{ h}^{-1}$ and $0.36 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$, respectively, and the conversion efficiency was higher than 90%.

Overall, ethanol production ranged between about 20 and 34 g L^{-1} with a conversion efficiency higher than 70% during ten cycles of spent yeast reuse. Volumetric and specific productivities varied from 1.17 to $9.18 \text{ g L}^{-1} \text{ h}^{-1}$ and from 0.24 to $0.37 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$, with the minimum values obtained for the initial batch. Even considering that glucose depletion occurred after 11.5 h in the initial batch, this means that spent yeast reuse experiments allowed an operation time reduction to about one-third.

To compare these experiments, the specific productivity was calculated at the time when maximum ethanol concentration was attained. According to the results shown in Table 1, the lowest specific productivity was obtained for the initial batch ($0.24 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$), indicating the highest number of viable cells at the beginning of the fermentation. This value was calculated based on estimated productivity and maximum biomass concentration. Even so, the specific productivity clearly increased compared to the

Table 1 Summary table of fermentation parameters for the three rounds of experiments of spent yeast reuse

Experiments		Fermentation parameters				
		[Biomass] _{max} ^a (g L^{-1})	$\Delta[\text{Ethanol}]^b$ (g L^{-1})	Prod _{vol} ^c ($\text{g}_{\text{ethanol}} \text{ L}^{-1} \text{ h}^{-1}$)	Prod _{spec} ^d ($\text{g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$)	Conversion efficiency (%)
Erlenmeyer scale—reuse of a fraction of the spent yeast (E5)	Initial batch	9.47 ± 0.08	56.9 ± 1.1 (22.0 h)	2.58 ± 0.05	0.28 ± 0.01	92.5 ± 0.7
	Reuse I	9.52 ± 0.24	55.2 ± 0.4 (22.0 h)	2.51 ± 0.02	0.27 ± 0.01	92.6 ± 1.6
	Reuse II	9.49 ± 0.42	45.9 ± 3.6 (23.7 h)	1.94 ± 0.15	0.21 ± 0.00	75.3 ± 1.1
	Reuse III	9.51 ± 0.45	39.9 ± 7.5 (22.0 h)	1.81 ± 0.34	0.19 ± 0.04	54.0 ± 4.0
	Reuse IV	9.94 ± 0.38	53.6 ± 2.2 (24.0 h)	2.23 ± 0.09	0.22 ± 0.00	88.3 ± 1.0
Erlenmeyer scale—complete reuse of the spent yeast (E11)	Initial batch	9.71 ± 0.25	26.7 ± 1.1 (11.5 h) ^e	2.32 ± 0.05^f	0.24 ± 0.00^g	82.5 ± 0.4
	Reuse I	16.4 ± 0.6	33.4 ± 1.5 (7.0 h)	4.78 ± 0.22	0.30 ± 0.01	77.8 ± 11.0
	Reuse II	17.9 ± 1.8	24.5 ± 1.0 (4.0 h)	6.12 ± 0.25	0.37 ± 0.04	73.3 ± 17.8
	Reuse III	23.7 ± 0.0	33.1 ± 3.3 (5.0 h)	6.61 ± 0.67	0.28 ± 0.03	88.6 ± 6.1
	Reuse IV	22.9 ± 0.8	26.9 ± 4.7 (4.0 h)	6.72 ± 1.16	0.29 ± 0.04	81.9 ± 3.0
	Reuse V	23.7 ± 0.0	34.4 ± 1.1 (4.0 h)	8.59 ± 0.26	0.32 ± 0.02	89.8 ± 10.4
	Reuse VI	26.7 ± 0.3	32.1 ± 2.4 (3.5 h)	9.18 ± 0.68	0.35 ± 0.03	93.7 ± 5.9
	Reuse VII	24.8 ± 0.7	19.6 ± 1.6 (2.5 h)	7.85 ± 0.64	0.32 ± 0.04	79.4 ± 4.3
	Reuse VIII	25.4 ± 0.0	22.5 ± 2.4 (3.0 h)	7.50 ± 0.79	0.30 ± 0.03	96.8 ± 1.5
	Reuse IX	24.5 ± 1.3	23.8 ± 1.1 (3.0 h)	7.93 ± 0.38	0.32 ± 0.00	92.4 ± 7.5
Bioreactor scale—complete reuse of the spent yeast (B4)	Reuse X	23.8 ± 2.2	24.8 ± 1.1 (3.0 h)	8.25 ± 0.38	0.36 ± 0.02	92.8 ± 7.6
	Initial batch	12.1	43.9 (22.0 h)	2.00	0.17	78.2
	Reuse I	16.5	40.5 (7.2 h) ^h	5.64 ⁱ	0.34 ^j	80.7
	Reuse II	20.3	40.8 (7.0 h)	5.83	0.29	75.7
	Reuse III	20.5	45.5 (8.0 h)	5.69	0.28	93.2

^a[Biomass]_{max} (g L^{-1}) corresponds to the biomass concentration at the time when the maximum ethanol concentration was detected. ^b $\Delta[\text{Ethanol}]$ (g L^{-1}) corresponds to the difference between the maximum and initial ethanol concentration. ^cProd_{vol} was calculated based on Eq. (1); ^dProd_{spec} was calculated based on Eq. (2). ^eTime at which all glucose is estimated to have been depleted based on glucose volumetric consumption rate between 6 and 8 h. ^fEstimated volumetric productivity considering that maximum ethanol concentration of 26.7 g L^{-1} occurred at 11.5 h, time at which all glucose is estimated to have been depleted. ^gEstimated Prod_{spec} considering estimated volumetric productivity and maximum biomass concentration of 9.71 g L^{-1} . ^hTime at which all glucose is estimated to have been depleted based on glucose volumetric consumption rate between 3 and 5 h. ⁱEstimated Prod_{vol} considering that the maximum ethanol concentration of 40.5 g L^{-1} occurred at 7.2 h, the time at which all glucose is estimated to have been depleted. ^jEstimated Prod_{spec} considering estimated volumetric productivity and maximum biomass concentration of 16.5 g L^{-1} .

initial batch with fresh inoculum, with an almost constant value of around $0.30 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$ for the remaining experiments. The increase in specific productivity compared to the initial batch translates into a greater redirection of carbon source consumption for ethanol production instead of cell growth. Considering that substrate consumption can be directed to three main paths, namely cell growth, product formation, and cell maintenance, this means that the initial batch with fresh inoculum was the one that consumed more substrate for biomass growth. Compared to the previous strategy, complete reuse of the spent yeast seems to have favored not only volumetric productivity but also specific productivity, evidencing the high adaptability of the cells to the fermentation broth. There is a significant difference in volumetric productivity (p -value 0.0009). On the other hand, there is no significant difference in specific productivity (p -value 0.17) since this parameter is already normalized considering the increase in biomass concentration. It is important to mention that in these experiments, it was used diluted hydrolysate, which could also contribute to better results. For that reason, in the following section, consecutive fermentations at the bioreactor scale were carried out with complete spent yeast reuse using original (non-diluted) hydrolysate.

These two strategies evaluated at the Erlenmeyer scale demonstrated the success of spent yeast reuse as inoculum. On the one hand, it was observed that the fermentation performance was maintained over the reuse cycles by using only a fraction of the spent yeast. In these experiments, the initial biomass concentration was kept almost constant in all assays, ranging between 1.26 and 1.51 g L^{-1} . The maximum biomass concentration in the first set of experiments E5 did not exceed 10 g L^{-1} . On the other hand, when a complete reuse of the spent yeast was carried out, the initial biomass significantly increased from 1.5 to 20.6 g L^{-1} from the initial batch until reuse IV (Fig. 2). After reuse IV, the initial biomass concentration stabilized at around 20 g L^{-1} . Due to high cell density, there was virtually no adaptation phase, with ethanol production detected immediately after half an hour. The maximum volumetric productivity was more than threefold when the spent yeast was completely reused instead of just a fraction. Volumetric productivity significantly improved along reusing cycles suggesting the high adaptability of the yeasts to the medium. Similar findings were reported by Silva et al. [14], who performed five consecutive cycles of cell reuse batch fermentation using a hydrolysate derived from sugarcane bagasse pulp with *S. cerevisiae* PE-2. While the first batch took about 24 h, the second batch required about 12 h and the last three batches lasted only 8 h. The ethanol volumetric productivity of the first batch was about 1.94 $\text{g L}^{-1} \text{ h}^{-1}$, whereas for the second cycle was 4.19 $\text{g L}^{-1} \text{ h}^{-1}$ and for the remaining batches was higher than 5.60 $\text{g L}^{-1} \text{ h}^{-1}$. Maximum volumetric

productivity of 5.81 $\text{g L}^{-1} \text{ h}^{-1}$ was attained for the fifth batch, and an average conversion efficiency of 87.4% was reached [14]. To be well compared to the average value presented by these authors, the conversion efficiencies of the present work were recalculated considering only glucose and not xylose as substrate, which resulted in an increase in conversion efficiency from 86 to 94%, exhibiting superiority even in eleven consecutive experiments. A similar average conversion efficiency of 88% was achieved by Fan et al. [30] for ethanol production from corncob residues hydrolysate using an adapted strain *Pichia guilliermondii*. However, average productivity (0.99 $\text{g L}^{-1} \text{ h}^{-1}$) was significantly lower compared to the present work (6.72 $\text{g L}^{-1} \text{ h}^{-1}$).

Regarding the ethanol-specific productivity, its increase was significant in the experiments in which complete reuse of the spent yeast was performed (Table 1), showing that the biomass concentration of the inoculum has a strong effect on ethanol productivity and glucose utilization. Summarizing, the yeast maintained its fermentative capacity throughout eleven successive fermentations, exhibiting high tolerance to ethanol and resistance to the imposed stress conditions such as centrifugation.

3.1.3 Bioreactor scale—complete reuse of the spent yeast

To validate the complete reuse of the spent yeast as inoculum, four successive fermentations were carried out in a 5-L bioreactor using 1 L of working volume (B4). Initial glucose and xylose concentrations were about 100 and 20 g L^{-1} , respectively (Fig. 3).

The initial batch started with a fresh inoculum, with a biomass concentration of about 1.09 g L^{-1} and presented a lag phase of 6 h (Fig. 3). The end of the assay was at 22 h when all the glucose was consumed and attained the maximum ethanol concentration of 43.9 g L^{-1} , corresponding to an ethanol volumetric productivity of 2.00 $\text{g L}^{-1} \text{ h}^{-1}$ and a conversion efficiency of 78.2%. The reuse I assay was initiated with a biomass concentration of 8.8 g L^{-1} . The adaptation phase was shorter since about 8.70 g L^{-1} of glucose were already consumed as soon as 1.5 h after the beginning. A glucose consumption rate of about 16.5 $\text{g L}^{-1} \text{ h}^{-1}$ was calculated from the linear regression slope of glucose concentration between 3 and 5 h after the beginning. Accordingly, it was estimated that glucose depletion occurred after 7.2 h (between 29 and 30 h), although it was detected only 18 h after the beginning (around 40.9 h). At the same time, it was noticed the maximum ethanol concentration of 49.5 g L^{-1} . However, only 40.5 g L^{-1} of ethanol were effectively produced since about 8.9 g L^{-1} was noticed in the fermentation medium at the beginning of the operation. This ethanol came together with biomass suspension, collected, and re-inoculated, meaning that just one centrifugation step may not be enough to separate the fermented broth with bioethanol

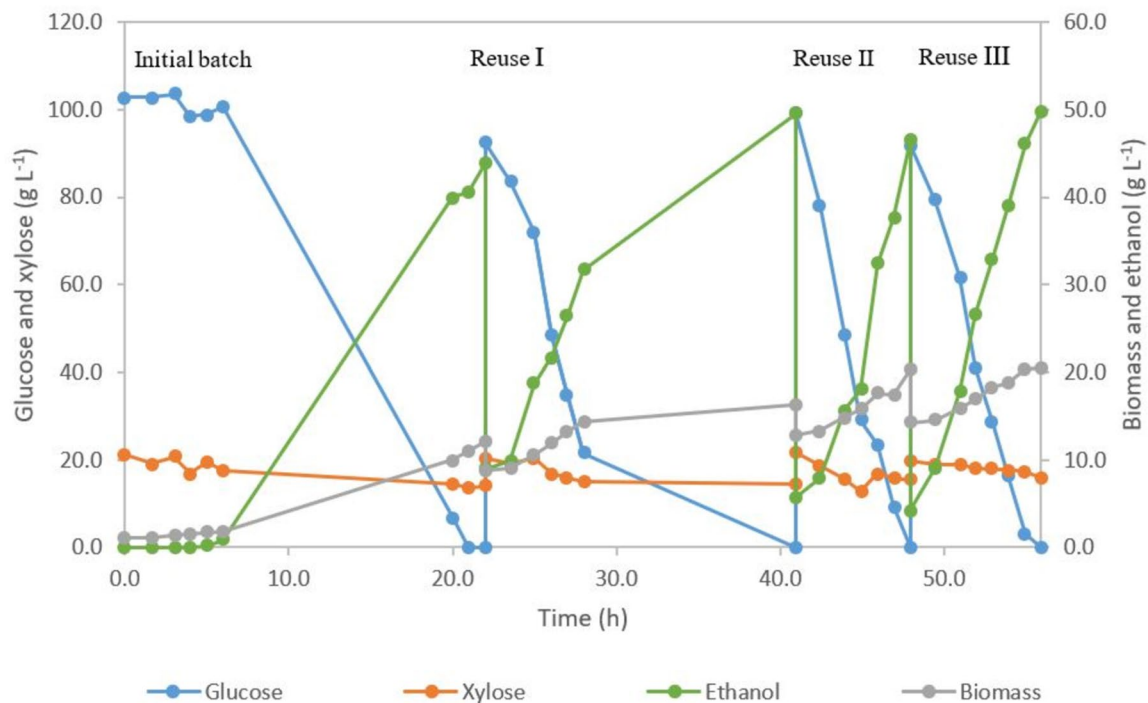


Fig. 3 Profile of glucose, xylose, ethanol, and biomass concentrations (g L^{-1}) for four consecutive fermentation assays at bioreactor scale (B4) with complete reuse of the spent yeast as inoculum

from the microbial biomass, before its reuse. The ethanol production corresponds to a conversion efficiency of 80.7% and volumetric productivity of $2.14 \text{ g L}^{-1} \text{ h}^{-1}$. Considering that the maximum ethanol concentration should have been achieved simultaneously with the glucose depletion, after 7.2 h, estimated volumetric productivity could reach $5.64 \text{ g L}^{-1} \text{ h}^{-1}$.

The reuse II assay started with a biomass concentration of about 12.9 g L^{-1} and almost 20 g L^{-1} of glucose were consumed in the first 1.5 h of the fermentation, indicating that the lag phase was almost imperceptible. Glucose exhaustion was detected after 7.0 h of operation (around 47.9 h), reducing the time cycle to about one-third compared to the initial batch (22.0 h). A maximum ethanol concentration of 46.6 g L^{-1} was accomplished, increasing the volumetric productivity from 2.00 to $5.83 \text{ g L}^{-1} \text{ h}^{-1}$. The volumetric productivity and conversion efficiency calculation considered the actual ethanol production (40.8 g L^{-1}), deducting the initial ethanol concentration of 5.7 g L^{-1} . A conversion efficiency of 75.7% was achieved, which is a similar result to the previous experiment.

The last assay had an initial biomass concentration of 14.3 g L^{-1} . This experiment followed a similar trend to the previous one, with glucose depletion and maximum ethanol concentration occurring after 8.0 h of the beginning, at around 55.9 h. During the first 3.0 h of fermentation, 30% of the initial glucose concentration was consumed, resulting in

13.5 g L^{-1} of ethanol which contrasts with the concentration in the first 7 h of operation of the initial batch lower than 1 g L^{-1} . The maximum ethanol concentration was about 49.8 g L^{-1} , corresponding to 45.5 g L^{-1} produced (4.31 g L^{-1} already at the beginning of the assay). The conversion efficiency and volumetric productivity reached 93.2% and $5.69 \text{ g L}^{-1} \text{ h}^{-1}$, respectively. The results of all experiments are summarized in Table 1.

The significant improvement in volumetric productivity is associated with high cell concentration [14]. The last assay started with a biomass concentration of 14.26 g L^{-1} while the initial batch was initiated with 1.09 g L^{-1} . Consequently, the fermentation time was curtailed to nearly 7.5 h, contrasting with 22 h for the initial batch.

The specific productivity of reuse I of bioreactor assay (B4) is estimated to be around $0.34 \text{ g}_{\text{ethanol}} \text{ g}_{\text{biomass}}^{-1} \text{ h}^{-1}$, meaning that it is similar to the reuse experiments at the previous Erlenmeyer assay (E11), demonstrating the reproducibility of this approach. This was also proved by statistical analysis that demonstrated that no significant differences were found for the previous reuse I, II, and III from E11 assay when compared to the correspondent reuse I, II, and III from B4 considering volumetric productivity (p -value 0.84), specific productivity (p -value 0.63) and conversion efficiency (p -value 0.57).

Overall, spent yeast reuse experiments resulted in ethanol production ranging from 40 to 46 g L^{-1} with conversion

efficiency varying from 75 to 93% and volumetric productivity from 2.00 to 5.83 g L⁻¹ h⁻¹. It was observed that the specific cell growth rate decreased along reuse batches, evidencing the redirection of sugars towards ethanol production instead of biomass growth. The spent yeast reuse also contributed to the robustness of the yeast strain since it could consume glucose readily, even with a slight amount of ethanol early on. It was also concluded that initial biomass concentration significantly impacts fermentation performance. Another advantage of spent yeast reuse is related to avoiding the time-consuming labor tasks related to yeast propagation, such as pre-inoculum and inoculum preparation [15, 29].

Therefore, it was found that the complete reuse of spent yeast led to higher cell density, shortened, or even practically eliminated adaptation and cell growth, resulting in a considerably faster fermentation. When cells are recycled, more sugar diverges towards ethanol production instead of microbial cell growth. These observations are similar to those reported for first-generation ethanol [15, 16]. Adaptive evolution induced by repeated fermentations improves the performance of microbial strains since tolerance of the recycled cells and forward generations to stressful conditions is boosted, developing a selective pressure effect in the cells [15, 16]. Consequently, there was an increase in the non-productive fraction of cells (mutants) and, simultaneously, a trigger to the appearance of mutations in those cells with a higher adaptation capacity (mutants) that could survive and eventually increase their production. The natural selection mechanism based on the adaptive capacity has been applied by some authors as a strategy to perform microbial evolutionary engineering through long-term continuous fermentations with specific selective pressures to obtain more resistant and stable mutants [32].

There are still few works published applying spent yeast reuse in cellulosic ethanol production [20]. Most of the works reported in the literature do not mention specific productivity, based exclusively on cell viability as a criterion for performance evaluation. However, this parameter indicates the reproductive capacity of the cells, not their fermentative ability and ethanol production. Therefore, for ethanol production processes yeast cells' reutilization, both volumetric and specific productivity and also conversion efficiency are required to generate an effective assessment.

This work stands out for excellent results in terms of volumetric productivity and conversion efficiency. The maximum volumetric productivity reported in the literature varies from 1.0 to 5.9 g L⁻¹ h⁻¹ and in contrast, a maximum value of 9.6 g L⁻¹ h⁻¹ was accomplished in the present work for the Erlenmeyer scale. The average conversion efficiency for eleven consecutive fermentations with complete reuse of spent yeast was 86.3% similar to values reported in the literature. Therefore, the present work demonstrated the success of the complete reuse of spent yeast along eleven

consecutive fermentation experiments at the Erlenmeyer flask, maintaining excellent performance. Some of the most extensive experiments reported in the literature evaluated until five reuse cycles [14, 30], whereas fifteen reuses were only reported with synthetic medium [29]. Furthermore, this concept was also feasible at the bioreactor scale with 1 L of working volume along five successive experiments where the maximum volumetric productivity and average conversion efficiency were about 5.8 g L⁻¹ h⁻¹ and 82%, respectively. Therefore, our results evidenced the great potential of the spent yeast reuse for boosting ethanol productivity from the fermentation of hydrolysate of kraft pulp from *E. globulus* bark.

However, there are still improvement opportunities. For future work, it is crucial to consider some issues related to prolonged cell reuse, namely decline in cell viability, cell inactivation or even cell death reducing ethanol-specific productivity besides contamination issues [20, 33]. Moreover, the centrifugation procedure for collecting the cells may also have a negative impact on their viability [20]. Therefore, appropriate methodologies must be applied along the consecutive batches to infer cell viability and robustness. Pereira et al. [29] noticed a drastic decrease in cell viability after the fifth fermentation cycle, reducing conversion efficiency, even using a synthetic medium. These authors restored the cell viability by transferring only a small fraction of the spent yeast to the following assay when cell viability was lower than 50% [29]. Therefore, if this strategy would be applied to the present work, the remaining spent yeast should still be considered as a potential nutritional source for complementing the fermentation broth [34, 35].

Another promising strategy is to follow the spent yeast reuse approach within the integrated configuration. Some authors have already assessed ethanol production from simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP) from corn stover and rice straw [36, 37]. In the integrated configuration SSCF, the unhydrolyzed solids and enzymatic consortium were recycled along with cells from one cycle to the following [36]. These studies were conducted on a small scale, and the productivity obtained was very low compared to our results, so scaling up may be challenging. On an industrial scale, the harvesting methodology will depend upon the yeast strain adopted and the bioreactor configuration. The separation of biomass cells from the fermentation broth is commonly carried out through centrifugation, sedimentation (mainly for flocculent strains) or membrane filtration [17, 38]. For example, conical-bottomed ferments typically used for brewing greatly improve the efficiency of harvesting yeast, allowing an easy discharge [39]. Moreover, at industrial scale, cells are usually further washed with diluted sulfuric acid at pH between 2.0 to 2.5 for 1–3 h before reuse to reduce bacterial contamination [15, 40]. Furthermore, agitation may

become more complex and some mass and heat transfer limitations could occur due to the higher viscosity of the fermentation medium. High cell-density fermentations are only worthwhile when higher volumetric productivity offsets the decline in specific productivity and the extra costs associated to cell separation processes [17].

4 Conclusions

The present work demonstrated that spent yeast can be successfully used as inoculum in consecutive fermentation experiments for cellulosic ethanol production from pretreated *Eucalyptus globulus* bark. Starting by reusing only a fraction of spent yeast, a slight decline in ethanol volumetric productivity was observed throughout five successive fermentation experiments. In contrast, the complete reuse of the spent yeast tripled ethanol productivity, minimizing the yeasts' adaptation phase, and promoting fast glucose consumption. Fermentation performance was successfully maintained over eleven consecutive fermentations at the Erlenmeyer scale with a conversion efficiency higher than 70%. Maximum ethanol volumetric productivity of $9.2 \text{ g L}^{-1} \text{ h}^{-1}$ was accomplished by reuse VI assay, the highest value reported in the literature so far. A similar strategy was followed through four consecutive batch fermentation experiments at a 5 L bioreactor with a working volume of 1 L, resulting in a conversion efficiency ranging from 75.7 to 92.3%. Fermentation time was reduced to about one-third, significantly improving the volumetric productivity from 2.00 to $5.83 \text{ g L}^{-1} \text{ h}^{-1}$. Therefore, the spent yeast reuse significantly improved volumetric productivity, allowing faster and more robust fermentation processes. It was also found that initial biomass concentration significantly impacts fermentation performance. Finally, the potential integration of this approach into a pulp and paper biorefinery has a high potential to reduce capital costs, which is still one of the main challenges associated with cellulosic ethanol production at an industrial scale. For future work, it is crucial to infer cell viability and robustness along the consecutive batches.

Author contributions All authors contributed to the study's conception and design. Material preparation, experiments, data collection, and analysis were performed by Mariana S. T. Amândio. The first draft of the manuscript was written by Mariana S. T. Amândio, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by FCTIFCCN (b-on). This work was carried out under the Project InPaCTus—Innovative Products and Technologies from Eucalyptus. Project No. 21874 funded by Portugal 2020 through European Regional Development Fund (ERDF) in the frame of COMPETE 2020 n°246/AXIS II/2017. This

work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 (<https://doi.org/10.54499/UIDB/50011/2020>), UIDP/50011/2020 (<https://doi.org/10.54499/UIDP/50011/2020>), and LA/P/0006/2020 (<https://doi.org/10.54499/LA/P/0006/2020>), financed by national funds through the FCT/MCTES (PIDDAC). The authors acknowledge to FCT (Fundação para a Ciência e Tecnologia, Portugal) for the financial support to CERES, the base (<https://doi.org/10.54499/UIDB/00102/2020>) and programmatic (<https://doi.org/10.54499/UIDP/00102/2020>) budgets. Mariana S. T. Amândio acknowledges the support by the Project InPaCTus for her PhD grant, PO-CI-01–0247-FEDER-021874.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

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