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ORIGINAL ARTICLE

Towards industrial application of fungal pretreatment in 2G biorefinery: scale-up of solid-state fermentation of wheat straw

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Abstract

Fungal pretreatment of lignocellulosic biomass for bioethanol production is an environmental-friendly alternative to steam explosion. However, this biological pretreatment has been tested on a small scale, where most of the typical problems of solid-state fermentations (SSF), such as limited aeration or temperature control, are not observed. The main objective of this study was to assess the feasibility of the fungal pretreatment of lignocellulosic biomass (wheat straw) at a demonstration scale using the white-rot fungus *Irpex lacteus* to improve straw digestibility. Different configurations were evaluated for the design of a 22 L SSF reactor, but a versatile vertical design that can operate as a packed-bed and as a tray reactor was selected. The wheat straw digestibility obtained in the SSF bioreactor after 21 days of pretreatment (60.6%) was similar to that achieved on a small scale (57.9%). In addition, the most common online monitoring variables (temperature and CO_2 production) correlate with the fungal action on wheat straw. As well as the weight loss, obtaining comparable results at flask and reactor scale (30 and 34.5%, respectively).

Keywords Bioethanol · Lignocellulosic biomass pretreatment · Ligninolytic fungus · Solid-state fermentation · Bioprocess scale-up

1 Introduction

The energy sector is heavily dependent on non-renewable fossil fuels, which has led to energy instability and environmental problems associated with greenhouse gas emissions. In order to tackle climate change, the EU has a target to reduce CO_2 emissions by at least 40% by 2030 [1]. In line with this target, the search for more environmental-friendly renewable energy sources has gained particular prominence in recent years, with the aim of securing the supply of more efficient and less costly energy sources. Bioethanol is mainly produced in first-generation (1G) biorefineries through the fermentation of sugars obtained from grains or crops. However, this bioethanol production system has the disadvantage of competition with food crops [2]. For this reason, current bioethanol production studies focus on second-generation (2G) bioethanol, which uses lignocellulosic materials such as agricultural and forestry residues [3]. However, the transformation of these renewable resources requires a conditioning or pretreatment step because this type of feedstock has a complex lignocellulosic structure, which requires the subsequent hydrolysis of cellulose and hemicellulose [4].

Highlights
- Fungal pretreatment of wheat straw was successfully scaled up to a 22-L bioreactor.
- Cellulose digestibilities achieved in the SSF reactor and in the flasks were similar.
- Versatile configuration allows to use the bioreactor in packed-bed and tray modes.
- Online monitored parameters correlated with fungal growth and pretreatment progress.

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Thermal and chemical pretreatments have proven to be effective methods to enhance the enzymatic hydrolysis of lignocellulose [5, 6], with steam explosion (SE) as the most widely applied process for the pretreatment of lignocellulosic biomass (LCB) [7]. However, these techniques often involve side reactions and produce some inhibitory compounds to the microorganisms used in fermentation [8, 9].

An alternative pretreatment is based on the use of white-rot fungi (WRF), which have received renewed interest due to their ability to break down and mineralise lignin. This ability is provided by the oxidative activity of their ligninolytic enzymes. The most outstanding advantages of biological pretreatment are the lower waste production, the production or recovery of value-added co-products, and the low energy requirements [10]. These features can be achieved through solid-state fermentation (SSF), which employs a natural substrate or an inert substrate as solid support for the microorganism growth in an environment with absence or very low levels of water [11]. SSF has been widely studied in the last decades to develop processes concerning the production of enzymes, biopulping, biomolecules synthesis, or biomass pretreatment [12–15]. Concretely, the biological biomass pretreatment employing SSF with fungi (brown-rot, white-rot and soft-rot fungi) has been proposed as an eco-friendly alternative to enhance the biorefinery process due to its ability to delignify lignocellulosic substrates [16].

Among the different types of WRFs commonly used in biological treatment, *Irpex lacteus* seems to be one of the most efficient to ensure high saccharification yields of agricultural residues [17, 18], as there are different references showing that the pretreatment of some lignocellulosic substrates such as corn stover, wheat straw, or olive biomass by this fungus managed to increase the digestibility of the material [19–21].

However, the most important drawbacks of WRF pretreatment are the long treatment time and the limited levels in fermentable sugars obtained in most of the biological pretreatments reported so far. Furthermore, pretreatment of LCB using white-rot fungi has been demonstrated only on a small scale in SSF carried out in flasks [3, 18]. On a laboratory scale, no limitations of mass and energy transfer are observed in SSF. It should be noted that the main bottleneck of SSF pretreatment is the scale-up of the process, as certain limitations must be taken into account, such as oxygen limitation, substrate inhomogeneity, overheating due to microbial activity, water loss, or CO_2 accumulation in the system [22]. From an economic perspective, taking into account the long time required to perform SSF, it is a pretreatment that could be discarded at first sight. However, it is important to consider its advantages compared to other types of pretreatments such as the use of milder pressure and temperature conditions, reduction of reagents consumption, and avoiding the formation of inhibitory compounds that may interfere in later stages [23]. If this pretreatment is proposed as an alternative for further development of industrial scale treatments, the technological feasibility of scaling up from small flasks to demonstration or pilot plant scale reactors must first be demonstrated.

Considering the specific characteristics of the biological system, many types of SSF reactors have been proposed, but only few options are commercially available, such as the small-scale 5 L CSFS-07-SA05P (Cleaver Scientific Ltd., UK). The other designs are prototypes designed and tailor-made for a specific application. Some SSF bioreactor designs have been shown to work well at laboratory scale, e.g., the submerged column designs described in the ORSTOM patent [24], chamber trays [25, 26], or rotating chamber reactors [27]. On a larger scale, the Plafractor™ reactor can be considered as the most complete and advanced design, although its use was intended to produce high-value, low-volume products [28] rather than low-value, high-volume products such as bioethanol. Kumar and Gomes [29] conducted an extensive review to select the best SSF reactor configuration to increase the carbohydrate fraction in wheat straw that can be utilised by ruminant gut biota by fungal pretreatment with the white rot fungus *Phanerochaete chrysosporium*. After analysing different alternatives, they selected a vertical bed reactor (1200 L) as the best design.

Other aspects to be taken into account in the design of the bioreactor are closely related to the culture requirements of the microorganism, e.g., the need for agitation and aeration, usually passive or forced. The evaluation of fungal pretreatment on a demonstration scale can also allow studying the influence of some operational variables on the pretreatment time that cannot be tested on a small scale such as mass transfer, aeration rate, pressure drop, and temperature gradients. In addition, it could also be used to determine the effect of these variables on the final digestibility and their effect on the kinetics of the process. Based on previous studies using wheat straw on *I. lacteus*, it is known that the biological pretreatment is an aerobic process characterised by a low fungal biomass growth rate with a typical duration between 15 and 21 days [10, 20]. Different limitations have been described for this type of culture such as air diffusion, moisture of the solid substrate, or heat transfer. The humidity of the solid substrate must be kept high enough to ensure the growth phase as well as to maintain the viability of the culture. At the same time, the temperature should be kept in the range of 25–32 °C, which is optimal for fungal growth. At low temperatures, fungal growth slows down, while operating at higher temperatures could completely stop fungal metabolism. Due to the low growth rate of *I. lacteus* and the potential presence of competing microorganisms, it is necessary to ensure sterilisation of the culture medium.

In this work, a 22-L SSF reactor was designed, constructed, and operated using as initial operating conditions those previously tested on a small scale [20]. For the design of the bioreactor, different aspects were considered: (a) previous studies reported in the literature, (b) preliminary experimental tests performed using laboratory equipment with a similar configuration, and (c) characteristics of the specific microorganism and the lignocellulosic substrate to be pretreated. A versatile design was considered to test two different airflow configurations: basket reactor with passive aeration and packed-bed reactor with forced aeration. Key control parameters for fungal growth, such as bed temperature, air flow, and CO_2 content of the outlet gas, were monitored. In order to compare the results of the reactor operations with the results obtained at low scale, different parameters related to wheat straw composition and enzyme production were assessed to determine whether the proposed SSF reactor configurations are optimal for increasing wheat straw digestibility.

2 Materials and methods

2.1 Mechanical size reduction and thermal sterilisation of wheat straw

Raw wheat straw (provided by ABENGOA S.A, Bavilafuente, Salamanca, Spain) was chopped in a blade mill to reduce its particle size to 1–5 cm. Once sterilised in an autoclave, it was placed in the containment basket of the reactor or directly inside the reactor, depending on the configuration selected. To study whether the initial content of sugars assimilated by the fungus influences its growth, different sterilisation programmes were studied. For the experiments in which the reactor was operated as a basket reactor, a one-stage sterilisation was applied in the first experiment and a two-stage sterilisation in the second experiment, these experiments being referred as Basket 1 and Basket 2, respectively. In the Basket 1 experiment, the mixture of wheat straw and tap water (300 g of wheat straw per L of water) was sterilised under standard conditions (121 °C for 20 min) in an autoclave (RAYPA AES-75, Barcelona, Spain). Two-stage thermal sterilisation consisting of two consecutive autoclaving steps of 115 °C for 20 min and 121 °C for 20 min was applied to the Basket 2 experiment. In the case of the experiment where the reactor was operated as a packed bed reactor, the same sterilisation schedule was used as in the Basket 2 experiment.

2.2 Fungal culture

Irpex lacteus (Fr. 238 617/93) was obtained from the Culture Collection of Basidiomycetes (CCBAS) of the Academy of Sciences of the Czech Republic, Prague. The fungus was transferred from slant tubes (kept at 4 °C) to agar plates containing malt-based extract medium. Three plugs of active mycelia from Petri plates were added to Fernbach flasks with 200 mL of CSS medium, containing (per L) $FeSO_4$ 0.4 g, $(NH_4)_2SO_4$ 9 g, KH_2PO_4 4 g, glucose 50 g, corn steep solids (CSS) 26.3 g, $CaCO_3$ 7 g, and soybean oil 2.8 mL. Static cultures of the fungus at 28 °C allowed the formation of mycelium on the surface of the liquid. After 7 days, the fungal culture broth was then crushed in a blender and used as inoculum (10% v/v) in shake fermentation flasks with CSS medium. The flasks were incubated at 28 °C and 165 rpm for seven days until the mycelial pellets were transferred as inoculum to the SSF cultures.

2.3 Solid-state fermentation

2.3.1 Flasks

Flasks with thermally sterilised wheat straw (6 g of straw and 20 mL of tap water) were inoculated with 0.5 mL of inoculum per gram of straw (corresponding to 1.5 mg fungal biomass/g wheat straw) and incubated at 28 °C. Three replicates were withdrawn and analysed at 0 and 21 days in order to characterise the extract (enzyme activity and sugar composition) and the solid fraction (composition and digestibility) for comparison with the results obtained at reactor scale.

2.3.2 Reactor

Description of the equipment and ancillaries for fermentation monitoring The SSF bioreactor, constructed of stainless steel and Pyrex glass (9 mm) and with 22 L of total volume, has a cylindrical geometry and vertical orientation (Fig. SM1). The cylindrical glass vessel has a height to diameter ratio of 2 and is mounted on a stainless-steel support frame. At the top and bottom of the reactor, two plates hold the ports for the different sensors, as well as the gas inlet and outlet ports (Fig. SM2). The top and bottom plates are connected to the support frame with six bolted screws. The top plate contains four ¾″ and three ½″ ports, while the bottom plate has two ports, one for air inlet and one for condensate drainage. For safety reasons, the reactor includes a spring-loaded pressure safety valve (set pressure 2 atm) mounted on the top plate, and a perforated stainless-steel shield, which covers the glass vessel during autoclaving.

Operation of the reactor The reactor was sterilised by passing steam generated in a mini autoclave (Raypa AES-8, Spain) at 115 °C for 1 h. During sterilisation, the outlet gas valve is slightly open to keep the steam flowing through the vessel. The connections, filters, and humidification bottle were sterilised at 121 °C for 20 min, and the probes were washed with ethanol and irradiated with UV light. Thereafter, wheat straw inoculated with the fungus (5.35 kg) was introduced into the reactor, and fermentation was continued for 21 days, with continuous injection of humidified air at controlled temperature. Table 1 summarises the conditions for the three experiments. Three replicates of initial and final samples were analysed. The bioreactor was designed to use two aeration configurations: (a) passive aeration, with mass and heat being transferred through the side walls of the bed, and (b) forced aeration, with air blown forcefully through the bed. These two configurations are depicted in Fig. 1.

2.4 Sample processing

After the biological pretreatment, the moisture content was determined by drying a sample of known weight at 105 °C to reach a constant weight. The ash content was determined by incineration in a muffle furnace at 550 °C. Extraction was performed by suspending 26 g of the sample in 60 mL of deionised water, followed by incubation for 1 h in a rotary shaker (160 rpm) at 25 °C. After filtration, the substrate was extracted again with 60 mL of water. The two liquid extracts were combined to measure sugar content and enzyme activities. Glucose concentration was measured with a glucose oxidase kit (GOD-PAP/Trimer, Spinreact). The concentration of total reducing sugars (TRS) was determined by the DNS method [30]. Manganese peroxidase (MnP) and laccase activities were measured by DMP oxidation [31]. Finally, the solid fraction was lyophilised and used for digestibility and composition analyses.

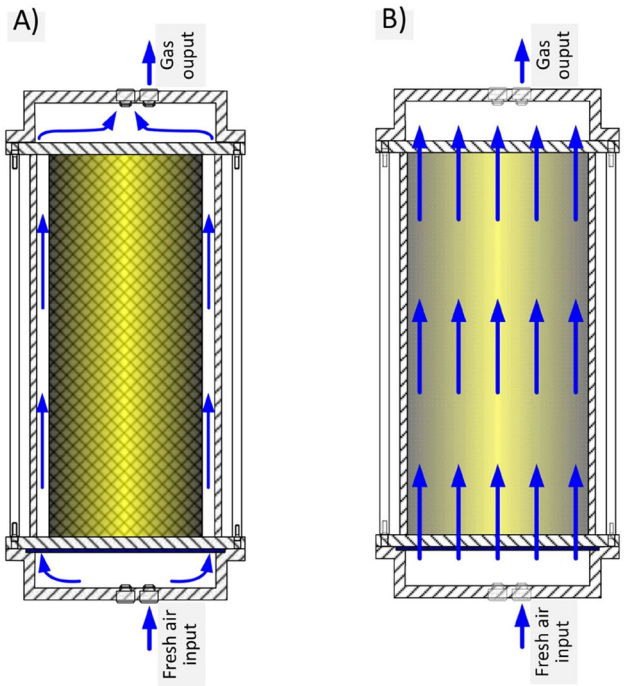

Fig. 1 Two different reactor configurations according to the air flow: **A** basket reactor with passive aeration and **B** packed bed reactor with forced aeration through the biomass

2.5 Enzymatic hydrolysis

Prior to enzymatic hydrolysis, a mild alkaline wash was performed on the freeze-dried wheat straw, as described by López-Abelairas et al. [20]. A modified version of the NREL (National Renewable Energy Laboratory, Golden, USA) protocol was used to carry out the enzymatic hydrolysis [10]. The enzyme doses of cellulase and xylanases were 15 FPU/g cellulose (Celluclast and NS50010) and 15 IU/g hemicellulose (NS 50,013 and NS 50,030), respectively. Commercial enzymatic cocktails were kindly provided by Novozymes.

Table 1 Main operational conditions of the SSF fermentations conducted in the SSF bioreactor using basket and packed-bed configurations

	Experiment B1	Experiment B2	Experiment PB
Reactor configuration	Basket	Basket	Packed bed
Type of aeration	Passive	Passive	Forced
Aeration rate (L/min)	2 (days 0–9) 2.7 (days 10–21)	2.25	3.2
Temperature (°C)	28	28 (days 0.5–2) 30 (days 2–5) 32 (days 5–8) 28 (days 8–12) 30 (days 12–15) 34 (days 15–18) 35 (days 18–21)	30
Thermal sterilisation	20 min at 121 °C	20 min at 115 °C and 20 min at 121 °C	20 min at 115 °C and 20 min at 121 °C

The hydrolysis was carried out in 0.1 M citrate buffer, pH 4.8, with a 5% (w/v) solid loading for 48 h on an orbital shaker at 50 °C and 165 rpm. Antibiotics (tetracycline and cycloheximide) were added to prevent contamination. Cellulose and total digestibility were calculated with Eqs. 1 and 2 [32].

$$\text{Cellulose digestibility}(\%) = \frac{\text{glucose in liquid phase(g)} \times 0.9}{\text{cellulose in solid phase before hydrolysis(g)}} \times 100 \quad (1)$$

$$\text{Total digestibility}(\%) = \frac{\text{TRS in liquid phase(g)} \times 0.89}{\text{cellulose + hemicellulose in solid phase before hydrolysis(g)}} \times 100 \quad (2)$$

Correction factors of 0.9 and 0.89, corresponding to hexoses and a mixture of hexoses and pentoses, were used in the calculations to estimate the fraction of water in the hydrolysis of each glycosidic bond. The yield of fermentable sugars was defined as the percentage of reducing sugars after enzymatic hydrolysis referred to the initial sugars in cellulose and hemicellulose expressed as monomers.

$$\text{Sugars yield}(\%) = \frac{\text{TRS after hydrolysis(g)}}{\left(\frac{\text{cellulose in initial solid(g)}}{0.9} + \frac{\text{hemicellulose in initial solid(g)}}{0.88}\right)} \times 100 \quad (3)$$

2.6 Biomass weight loss

Biomass weight loss was determined at the end of fermentation by (a) direct measurement of the final total solids and (b) from the amount of CO_2 released during cultivation. The direct measurement of weight loss was determined by calculating the difference between the weight of the dry biomass initially loaded into the SSF bioreactor and that of the dry biomass at the end of fermentation. The final value was calculated by directly weighing the total final biomass and determining its moisture content.

Loss weight was also estimated using on-line data of CO_2 concentration in the exit gas (AI-125 sensor) and air flow (V-105 valve). To obtain the amount of CO_2 generated in the bioreactor, the CO_2 concentration in the inlet air was subtracted from the online measured value to consider only the CO_2 generated by the fungal metabolism. With these data, it is possible to calculate the volume of CO_2 released between the initial time and a given time by applying Eq. 4, then integrating this function between times 0 and n gives the cumulative CO_2 produced.

$$CO_2(L) = \sum_{t=0}^{n} \left[Q\left(\frac{L}{h}\right) \times (t_n - t_{n-1})(h) \times \left(\frac{CO_2(\%)_{t_n} + CO_2(\%)_{t_{n-1}}}{2} \right) \cdot \frac{1}{100} \right] \quad (4)$$

The C weight loss was calculated from the volume of CO_2 released, applying the ideal gas law. Finally, for the calculation of loss weight in terms of elemental C, Eq. 5 was applied.

$$\text{Loss weight}(\%) = \frac{\text{mg } C_{\text{released}}}{\text{mg } C_{\text{wheat straw}}} \cdot 100 \quad (5)$$

2.7 Statistical analysis

The software R (version 4.0.5, R Core Team, 2021) was used in the statistical analysis of the experimental results. A one-way analysis of variance (ANOVA) was applied to determine if there were significant differences between the experimental values obtained using the two SSF bioreactor configurations and at flask scale. The parameters evaluated were initial sugar concentration, MnP and laccase activity, biomass humidity, total weight loss, lignin content reduction, lignin selectivity removal, and cellulose and total digestibility. If the ANOVA confirmed the difference, a post hoc analysis (Tukey's HSD) was applied to determine between which values the difference was significant. In all the statistical analyses, a significance level (α) of 0.05 was considered. The output of the statistical analysis, including ANOVA tables, Levene's test for homogeneity of variance, TukeyHSD analysis, and figures (box plots and Tukey's HDS pair comparisons), was included as Supplementary Material (Statistical analysis).

3 Results and discussion

3.1 Design of the SSF bioreactor

Scaling up solid-state fermentations is not as straightforward as that of submerged cultures. Many issues must be tackled before selecting the best bioreactor design. These include growth kinetics of the microorganisms, physical properties of the solid substrate, agitation requirements, sterility conditions, and mechanical stress produced by the agitation. Besides, for the industrial scale applications of SSF, some modifications should be made, especially, in the bioreactor design, mathematical modelling, and controlling the process parameters [33]. In the selection of the SSF bioreactor design, a rotating drum bioreactor (RDB) or any other design with agitation was ruled out because we have previously observed that *I. lacteus* tends to agglomerate into wheat straw during fungal colonisation, so periodic agitation of the solid matrix content in the SSF would be unfeasible. In addition, agitation would make the design a complex mechanical solution to build and scale-up. It has also been reported that the use of agitation in SSF with white-rot fungi may have a negative effect due to mechanical stress as reported by Rodriguez-Couto et al. [34], who evaluated three different SSF bioreactor designs for the production of laccases with the white-rot fungus

Trametes versicolor. They reported that a tray bioreactor led to higher laccase activities than the other two types of agitated bioreactors evaluated, indicating that agitation may have caused mechanical stress to the fungus, repressing laccase production.

One of the main challenges of SSF is temperature increase due to heat derived from the metabolism and growth of the microorganism. This is particularly important in non-stirred reactors, as it can lead to temperature gradients in the bed and moisture loss [22]. However, in slow-growing kinetic processes, such as fungal pretreatment of wheat straw, heat generation does not seem critical, and the need for internal heat exchangers is not foreseen. In such cases, heat dissipation is usually done through aeration: air is blown into the system to force out the generated heat through a gas outlet. Therefore, aeration is a key parameter in SSF reactors, not only because it fulfils the oxygen requirements in aerobic processes, but also because it allows indirectly influencing various aspects of the process by acting as a vehicle for mass and energy transfer. In this sense, air flow, temperature, and humidity were selected as process control variables to maintain the bed at the desired conditions.

Due to the heterogeneity of SSF, it is practically impossible to take equal samples from different bioreactor zones to characterise the biomass and determine microbial growth. Furthermore, in fermentations involving filamentous fungi, the fungal mycelium penetrates deep into the substrate, making it impractical to use a direct technique, such as the dry weight method, to estimate fungal growth. Indirect methods, such as oxygen consumption or carbon dioxide production, can be used to monitor growth [22]. Metabolic activity was estimated by monitoring the evolution of carbon dioxide in the gas. Carbon compounds within the substrate are metabolised during growth to produce CO_2 and fungal biomass. CO_2 production causes the weight of the fermentation substrate to decrease, and weight loss could be correlated with fungal growth. Estimation of growth based on CO_2 release assumes that metabolism is fully associated with growth. Other important factors considered for bioreactor design include the need for sterilisation, particularly important in processes with slow growth kinetics and long operating periods, and monitoring of the pressure drop, as high-pressure gradients would make the process unfeasible.

Taking into account the above considerations, the designed bioreactor consists of an autoclavable vertical cylindrical glass column with a capacity of 22 L (Fig. SM1 and SM2). The process and instrumentation diagram (P&ID) of the SSF bioreactor is shown in Fig. SM3. The main elements and control strategies used in the monitoring and control system are summarised in Table SM1. In the SSF pretreatment, the two most important controlled process variables are temperature and bed moisture. These are closely related to the conditions of the air entering the bioreactor, which are humidity, temperature, pressure, and mass flow. A temperature controller was installed to maintain the biomass temperature (the average value of the two sensors, TI-116 and TI-117) between two pre-set values (low and high set points) by switching on and off the connection of the air heating and cooling devices to modify the inlet air temperature. To prevent the substrate from drying out, the air was forced to pass through a water bubbling system. The humidity of the inlet and outlet air (HI-113 and HI-115 sensors) can be modified manually by regulating a valve that controls the air flow through the humidification device.

Since aeration is a critical parameter in SSF design, two different bioreactor configurations with passive and forced aeration were considered (Fig. 1). When operating with passive aeration, the substrate is placed in a wire mesh basket. In this configuration, air flows through the empty space between the inner wall of the glass vessel and the basket, while air is transferred from this gas stream into the substrate by passive diffusion. Alternative modes of operation of this configuration to enhance gas and heat transfer are the use of internal air recirculation or air pulses, instead of continuous flow. In the packed-bed configuration, the substrate occupies the entire cross-sectional area of the glass vessel, so air is forced to flow through the substrate. Furthermore, in the case of operating the SSF bioreactor in forced aeration configuration, the pressure difference between the upper (PI-116) and lower (PI-114) chambers can be directly correlated with the fungal colonisation of the substrate. Another parameter that gives information about the fungal growth rate is the CO_2 concentration in the outlet gas. These two parameters were monitored in the SSF bioreactor using pressure sensors P1 and P2 placed in the upper and lower chambers, while an IR mass sensor (AI-124) was placed in the gas outlet line to measure the CO_2 concentration on-line.

3.2 Operation and monitoring of the SSF bioreactor

As mentioned above, aeration is a crucial parameter in the design of an SSF bioreactor. Substrate moisture, as well as mass and heat transfer, can be controlled by modifying the water content, flow rate, and inlet air temperature. Forced aeration favours the uptake of oxygen by the fungus and the release of CO_2 and heat from the substrate. In order to evaluate this critical aspect of fungal pretreatment of wheat straw, the SSF bioreactor was operated by (a) passive aeration, with mass and heat being transferred through the side walls of the bed, and (b) forced aeration, with air blown forcefully through the bed (Fig. 1). Two experiments were carried out using the basket configuration with passive aeration, denoted as B1 and B2, differing in a more severe thermal sterilisation of the substrate for B2 and the

set-point temperature of the bed, according to Table 1. The forced aeration scheme was performed in the absence of the basket and was referred to as packed bed fermentation (PB). In addition, a flask scale experiment was performed as a control to compare the results at both scales. Figure 2 depicts the time evolution of the morphological aspect of wheat straw treated in both reactor configurations. It is observed that wheat straw is a suitable feedstock for WRF, as previously reported [35, 36]. However, when comparing the wheat straw colonisation on day 4, it is evident that *I. lacteus* grew faster in the packed bed configuration.

The air and bed temperature and the CO_2 concentration of the exhaust gases were recorded online (Fig. 3). The average bed temperature in B1 was 28 °C, which shows that the control system, by modifying the inlet air temperature, was robust. As can be seen in Fig. 3A, at the beginning of the operation, the differences between air and bed temperature are evident, while they are minimised at the end of the fermentation. This is indicative of a decrease in heat generation by fungal metabolism from day 10 onwards. In experiment B2, the temperature setpoint was modified during the bioreactor operation (Table 1), trying to establish a correlation with the CO_2 release. It was observed that an increase in temperature up to a certain level can mitigate the progressive decrease in CO_2 release observed after the first week. For example, when the temperature was set at 32 °C on day 5, an increase in CO_2 concentration was observed (Fig. 3A, B). However, a high temperature in the bed can stop fungal metabolism.

The temperature in the packed bed reactor was controlled at 30 °C during the 3 weeks of fermentation. The vertical temperature gradient in the packed bed configuration was practically zero. As observed in the B1 fermentation, the differences between air and biomass temperatures

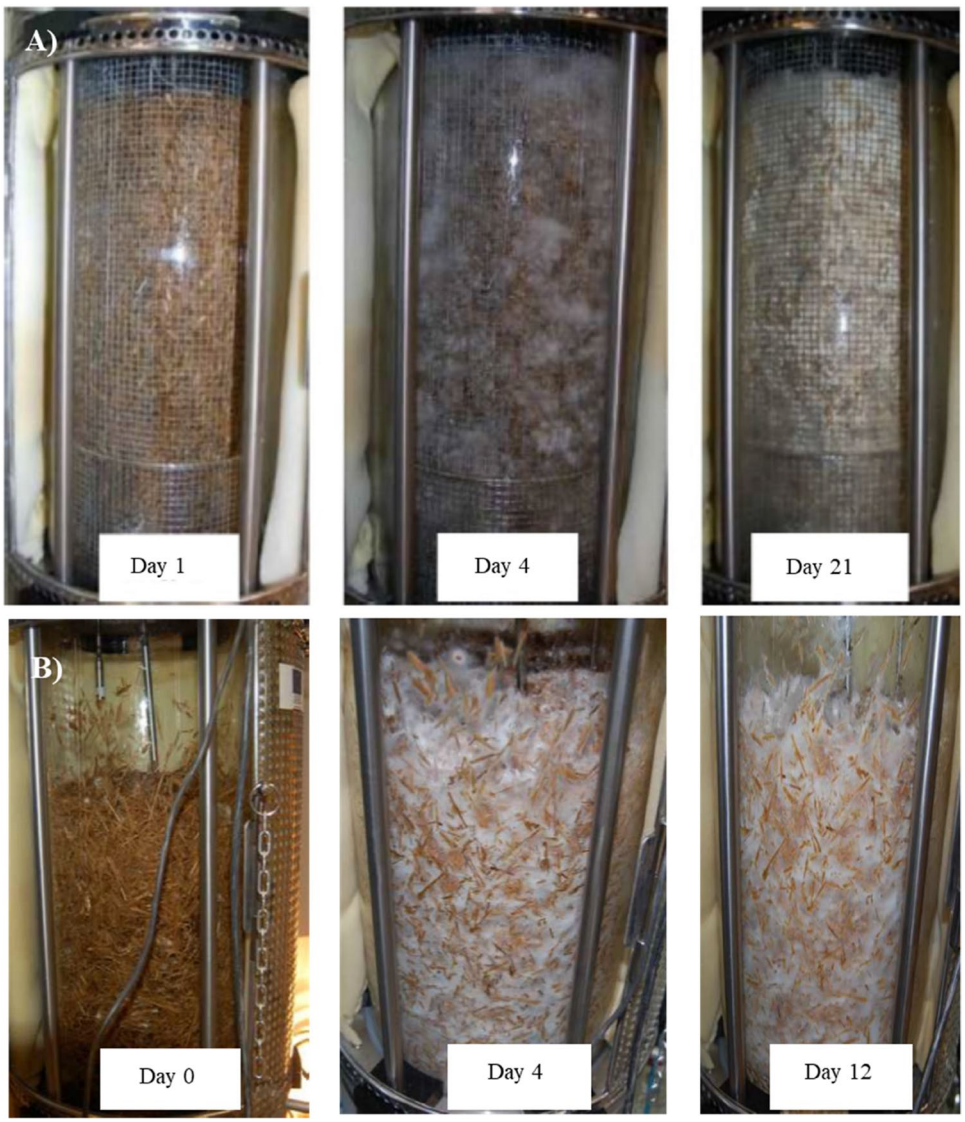

Fig. 2 Evolution of wheat straw during biological pretreatment with *I. lacteus* in the SSF reactor using the basket (**A**) and packed bed (**B**) configurations

decreased with operating time, indicating that heat generation decreases with time, particularly during the week of fermentation. In the case of CO_2 production, it increases sharply after the first day of operation, which is the time necessary for the fungus to adapt to the substrate and start its metabolic activity.

The faster growth observed in Fig. 3C for the packed bed configuration was also reflected in the CO_2 released. Botella et al. [37] have explored the use of CO_2 evolution rate (CER) to estimate fungal growth during SSF. Then, based on the calculated fungal growth, they were able to estimate the total dry weight. The CO_2 content reached values close to 1% in the packed bed configuration (day 1), while the maximum CO_2 concentration was 4 times lower when passive aeration was used in B1 and B2. Considering the air flow in each of the systems, maximum CO_2 flow rates of 30 mL CO_2/min and 5 mL CO_2/min were achieved for the packed bed and the basket reactor, respectively. After this initial peak, the concentration progressively increased to 0.18% and 0.55% for the basket and packed bed configurations, respectively, on day 6 of operation. Then, the CO_2 content decreased to 0.1% and 0.3% at the end of the fermentation, representing 3 mL CO_2/min, 4.5 mL CO_2/min, and 10 mL CO_2/min, for fermentations B1, B2, and PB, respectively. An important parameter of the packed bed configuration is the pressure gradient, which has to be considered in the process scale-up. A pressure drop of less than 10 mbar across the bed was observed, which would imply a minimal pumping cost.

3.3 Characterisation of the extract

3.3.1 Sugar composition

In order to characterise the liquid extract, glucose and TRS were determined at the beginning and at the end of the experiments. As seen in Fig. 4, after 21 days of treatment, the evolution of sugars in the three bioreactor experiments was totally different. In experiment B1, the initial sugar content is significantly lower than in B2 ($p = 2.0 \times 10^{-5}$), which may be the result of the use of a single-stage sterilisation. An increase in sugars was observed at the end of the experiment, especially total reducing sugars. These results demonstrate the ability of *I. lacteus* to degrade wheat straw polysaccharides under the specified conditions, especially

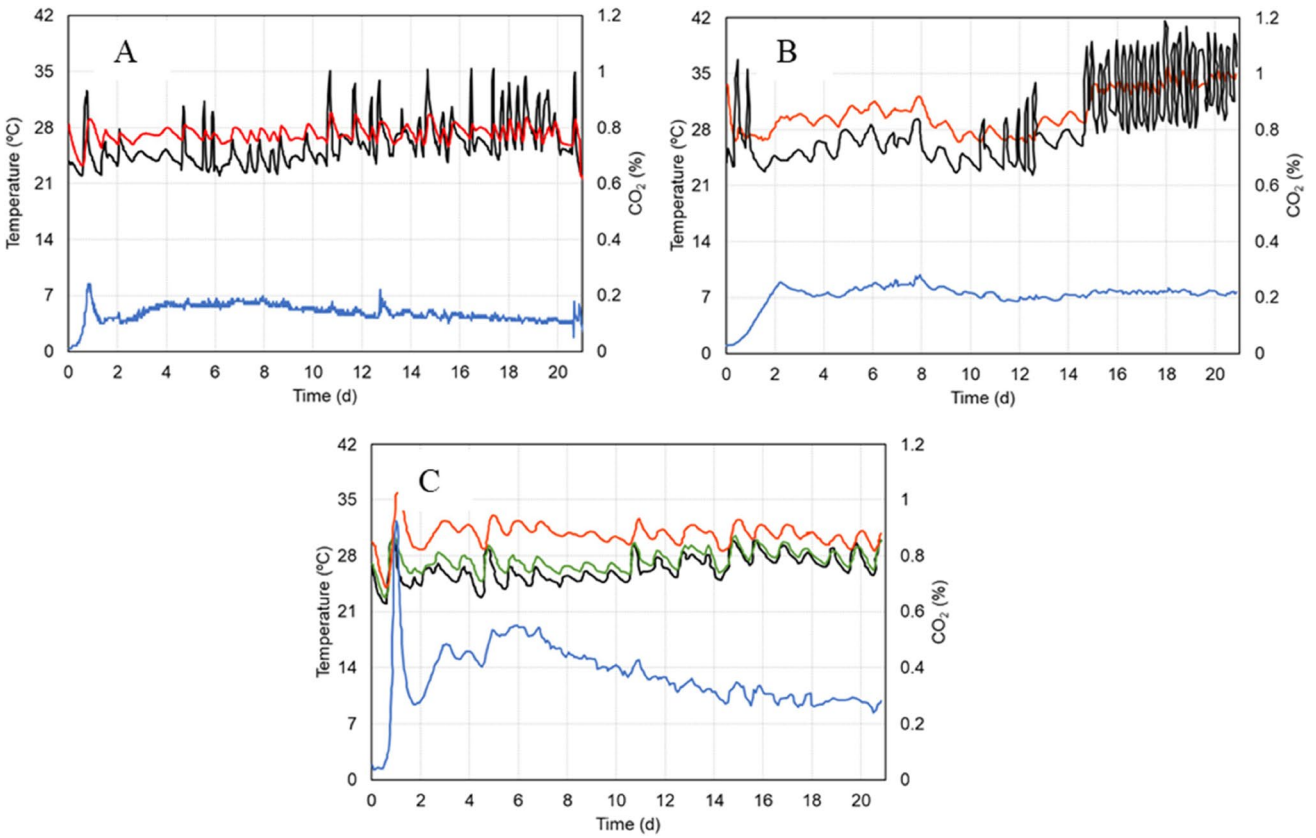

Fig. 3 Monitoring of inlet air temperature (black), outlet air temperature (green) temperature of the bed (red) and CO_2 concentration (blue) of the gas leaving the reactor in the B1 (**A**), B2 (**B**), and PB (**C**) fermentations

in the absence of initial reducing sugars to incorporate into its metabolism.

In contrast, in experiment B2, at the end of fermentation, the concentration of all sugars in the extract was lower than the initial content. Sterilisation in this experiment was carried out in two stages, which allowed a high release of sugars that could be assimilated by the fungus in the first days. In the PB experiment, a small increase in sugar content can be observed, similar to that observed in the flask experiment.

3.3.2 Ligninolytic enzyme activity

Laccase and MnP activities were measured at the beginning and at the end of the cultures. *I. lacteus* is known to produce mainly MnP when grown in SSF [38]. In all fermentations, it was observed that the initial ligninolytic activity was negligible and that laccase production was lower than MnP production. This could be also attributed to the typical profile of the expression of both enzymes, which are triggered sequentially, with laccase being expressed first [20]. Therefore, laccase might have peaked first, so that the final remaining activity is lower than that of MnP.

In experiment B1, the final activities of MnP and laccase reached 1.67 U/g and 0.32 U/g wheat straw, respectively. This higher MnP activity may be related to the lower sugar content at the beginning of the experiment, which may induce the expression of ligninolytic enzymes. In experiment B2, MnP and laccase activities of 0.23 and 0.12 U/g wheat straw, respectively, were detected. The lower MnP activity could be caused by the higher bed temperature reached at the last stage of fermentation (37 °C), which could partially inactivate the enzymes. Some studies have shown that MnP from *I. lacteus* can be active up to 45–50 °C, but with a consequent reduction of its activity [39, 40].

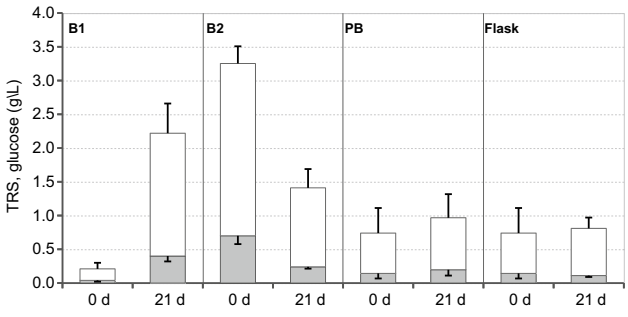

Fig. 4 TRS (white) and glucose (grey) content at days 0 and 21 of the B1, B2, and PB experiments, carried out in the SSF reactor, and in the flask culture

The MnP activity obtained in the packed bed configuration was comparable to that obtained in the flask fermentation ($p = 0.994$). The final laccase activities were similar in all the fermentations ($p = 0.189$). The MnP and laccase activities achieved in this study (Table 2) are similar to those obtained by other researchers in the pretreatment of wheat straw at flask scale. Lopéz-Abelairas et al. [20] detected maximum MnP and laccase activities of 0.74 and 0.34 U/g in the SSF of wheat straw with *I. lacteus* under optimal conditions. In a screening study to evaluate WRF performance in wheat straw, Salvachúa et al. [10] found higher activities of 3.5 and 0.25 U/g of MnP and laccase, respectively with *I. lacteus*. However, they did not find a direct relationship between the level of ligninolytic enzyme activity and increased digestibility of wheat straw.

3.4 Characterisation of the solid

3.4.1 Humidity, ash content, and weight loss

Humidity during the operation of the SSF bioreactor slightly decreased after the 21 days of fermentation, varying from 79.18 ± 0.54 to $73.02 \pm 0.15\%$ ($p = 4.5 \times 10^{-5}$) in experiment B1, from 78.27 ± 0.40 to $73.95 \pm 0.26\%$ ($p = 9.7 \times 10^{-5}$) in experiment B2, and from 82.42 ± 0.26 to $79.34 \pm 0.92\%$ ($p = 0.005$) in experiment PB. This demonstrates that the strategy of saturating the inlet air with water succeeded in maintaining the wheat straw humidity in ranges that support fungal growth. At the same time, the percentage of ash increased in all the pretreatments. Since ash is an inert compound that is not metabolised by the fungus, the increase of ash content indicates that part of the remaining fraction of wheat straw was mineralised due to the fungal action and converted to CO_2. The lower and higher increments of ash contents were obtained in the B1 (10.07 to 11.53%) and PB (7.07 to 8.12%) experiments, respectively.

The weight loss of wheat straw observed in the SSF experiments determined by using two different methods is summarised in Table 3. The direct weight difference is the most reliable method, but only gives information at the end of the experiment. On the contrary, an estimation of the weight loss can be monitored during the fermentation

Table 2 Laccase and MnP activities reached at the end of the SSF experiments

Experiment	MnP (U/g)	Laccase (U/g)
Basket 1	1.67 ± 0.09	0.32 ± 0.21
Basket 2	0.23 ± 0.09	0.12 ± 0.05
Packed bed	0.63 ± 0.17	0.12 ± 0.05
Flask	0.66 ± 0.20	0.23 ± 0.08

Table 3 Weight loss (%) estimated in the experiments

Calculation basis	B1	B2	PB	Flask
Weight difference	10.2	25.0	30.0	30.0
CO_2 release	11.1	23.4	39.0	ND
Average weight loss	10.7	24.2	34.5	30.0

by applying the elemental C method based on CO_2 released measured on-line.

Similar weight loss values were obtained with the two methods, demonstrating that CO_2 release measurement is a simple online tool to monitor fermentation without the need to withdraw the sample from the reactor. In terms of the values achieved, the average weight loss of experiment B1 was significantly lower than that of the other experiments ($0.0006 < p < 0.0107$). On the other hand, the highest average weight loss was observed in the PB experiment. In this case, the weight loss determined by mass balance was identical to that obtained for the flask experiment ($p = 0.288$).

During its growth, the fungus consumes part of the carbohydrates present in the raw material, causing the observed weight loss. The weight loss of experiments B2 and PB are similar to those found in previous studies of fungal pretreatment of wheat straw using *I. lacteus*, which ranged between 30 and 38% [3, 20], and also similar to that reached by Zuo et al. [17] for the pretreatment of corn stover with *I. lacteus* (33%) after 42 days of fermentation.

3.4.2 Solid fraction composition

The composition of the solid fraction was determined to evaluate the impact of the fermentation in the raw material. Figure 5 shows the evolution of the glucan, xylan, lignin, and ash fractions, as well as the total weight loss with respect to the initial weight of wheat straw. The variation of each fraction was calculated taking into account its composition as well as the reduction of total weight loss. The initial and final composition of wheat straw, as well as the total weight loss achieved after 21 days is shown in Table SM2. In experiment B1, the most significant differences can be observed in the lignin and sugar fractions. A high reduction of lignin (26.5%) is noticeable, which is in accordance with the major ligninolytic enzyme production in this experiment. The other parameters also decrease, apart from ash, which increases.

The decrease of lignin in experiment B2 ($34.90 \pm 0.87\%$) was higher than that of experiment B1 ($26.7 \pm 3.08\%$, $p = 0.001$), whereas the sugar percentage barely presents differences between day 0 and 21. In the PB fermentation, an increase was observed for all compounds except lignin, which decreased by $52.3 \pm 0.69\%$. This was the largest reduction of any biomass component achieved in the three SSF reactor experiments. A similar lignin reduction was reached in the flask-scale pretreatment ($53.2 \pm 0.60\%$, $p = 0.901$). This is indicative of the selectivity of the fungus for lignin degradation. To better compare lignin degradation, the parameter 'lignin selectivity removal' (defined as the ratio between lignin loss and total weight loss) was calculated [3]. This parameter relates lignin loss and total weight loss. The highest lignin selectivity removal was obtained in the B1 pretreatment (2.34 ± 0.03, $2.3 \times 10^{-5} < p < 0.0076$), followed by the PB ($1.74 \pm 0.02$) and flask experiments (1.77 ± 0.02), which reached similar values ($p = 0.995$). The lowest value (1.01 ± 0.03) was reached in the B2 retreatment. This lignin selectivity removal correlates with the higher ligninolytic enzyme activity shown in Table 2.

3.4.3 Enzymatic digestibility of wheat straw

Figure 6 shows the increase of digestibility of the four experiments. In experiment B1, cellulose digestibility increased from 27.6 ± 3.53 to $44.8 \pm 4.16\%$ ($p = 0.005$) after 21 days of operation, while total digestibility increased from 26.9 ± 2.12 to $37.5 \pm 3.56\%$ ($p = 0.011$). The increase in

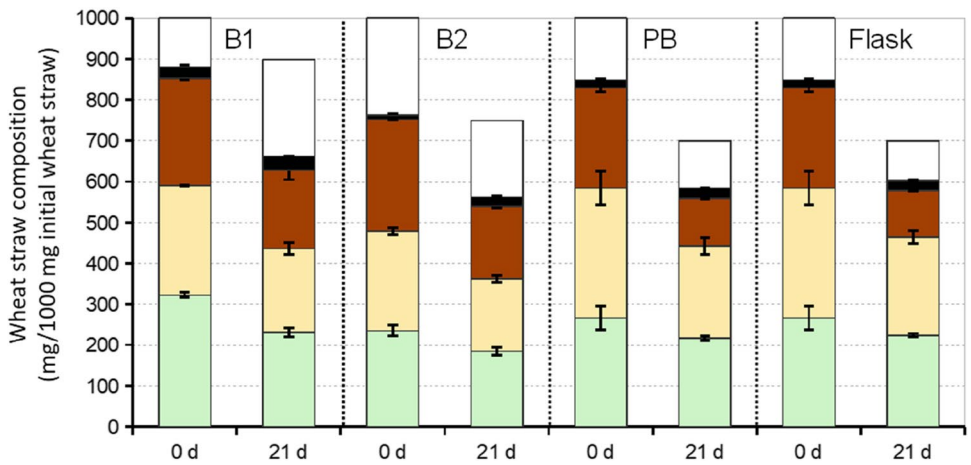

Fig. 5 Solid fraction composition at days 0 and 21 of the experiment B1, B2, PB, and flask. The content of cellulose (), hemicellulose (), lignin () and ash (□) are shown. The total height of the bars represents the total dried biomass weight

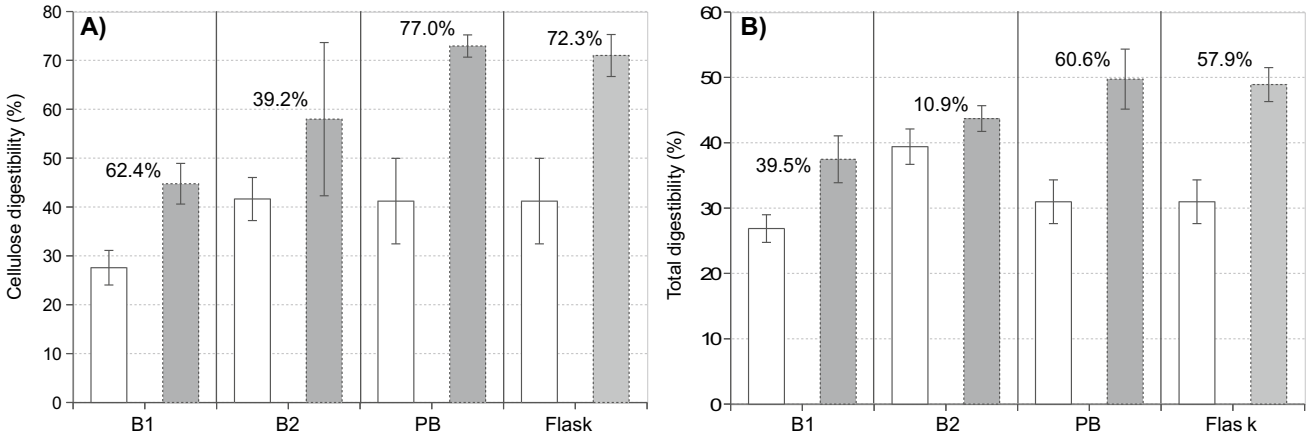

Fig. 6 Cellulose (**A**) and total (**B**) digestibility of wheat straw at days 0 (white) and 21 (grey) of the experiments B1, B2, and PB, carried out in the SSF reactor, and in the flask culture. The value next to the grey column shows the percentage of digestibility increase

cellulose digestibility correlates with a decrease in the lignin content. In experiment B2, cellulose digestibility varied from 41.7 ± 4.4 to 58 ± 15.5% ($p = 0.155$), while total digestibility from 39.4 ± 2.7 to 43.7 ± 2.0% ($p = 0.092$). However, these increases are not significant because they presented a high deviation. Although lignin attack is essential to the efficiency of the enzymatic hydrolysis, the highest lignin degradation is not always positively correlated with the highest levels of cellulose and hemicellulose digestibility [41]. The highest increase of total digestibility (60.6 ± 4.16%) in the SSF reactor was reached in the PB experiment ($8.0 \times 10^{-5} < p < 0.020$), which was not significantly different to that achieved at flask scale (57.9 ± 10.62%, $p = 0.958$). When comparing the digestibility achieved in the flask and SSF bioreactor using the PB configuration, it is observed that the performance is similar at both scales, being slightly higher at the reactor scale for cellulose ($p = 0.547$) and total digestibility ($p = 0.799$). Final cellulose digestibility is also similar to that obtained by Lopez-Abelairas et al. [20] by applying SSF at flask scale with *I. lacteus* at non-optimised conditions (70.3%), and by Salvachúa et al. [10] (82%), and higher than that reported by García-Torreiro et al. [3] (54.8%).

4 Conclusions

A demo-scale bioreactor was designed for the pretreatment of wheat straw by *I. lacteus* under solid-state fermentation. Passive and forced aeration strategies were evaluated using basket and packed bed configurations, respectively. Analysis of the results indicates that operating the SSF as a packed bed reactor was the most suitable mode. Using this configuration, the cellulose digestibility achieved in the SSF reactor after 21 days of cultivation was similar to that obtained in the flask fermentations with this fungus. The operation of the SSF reactor also allowed a better characterisation of the pretreatment, which allowed correlating some off-line measurements with on-line values, e.g., relating the total final biomass loss (off-line) to the CO_2 composition of the gas leaving the reactor (on-line). In the next experiments, the effect of some operational parameters that can be controlled in the SSF, such as air flow and aeration strategy and temperature, will be studied with the aim of increasing the final digestibility of wheat straw and reducing the pretreatment time.

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Author contribution M. A., M. L., J. M. L., and T. A. L. conceptualisation; M. A. investigation and design of the reactor, performing the experiments; M. L. methodology; S. G. data curation; S. G., G. E. data presentation; M. A., S. G. writing original draft preparation; G. E., M. T. M., T. A. L. writing—review and editing; J. M. L, T. A. L. supervision. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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