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Valorization of *Gelidium corneum* by-product through solid-state fermentation

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ABSTRACT

Red seaweed *Gelidium* is used for agar extraction, generating large quantities of by-products (red seaweed by-product – GBP). Its carbohydrate and protein-rich composition makes it a suitable substrate to produce value-added compounds through solid-state fermentation (SSF). For the first time, GBP was used in SSF with *Aspergillus ibericus* and *Aspergillus niger* for carbohydrases production and protein enrichment. SSF was performed with unsupplemented GBP, supplemented with Mandel salt solution, and mixed at equal dry mass proportion with plant feedstuffs and with the seaweed *Ulva rigida*. *A. niger* CECT 2915 was the best strain under SSF obtaining the maximum xylanase activity (498 ± 49 U g^{-1}), in GBP mixed with sunflower cake and the maximum of cellulase activity (382 ± 37 U g^{-1}) in GBP mixed with rapeseed cake. In these mixtures, protein content increased 1.2-fold by SSF, as well as in GBP mixed with rice bran (1.3-fold). Scale-up of SSF using GBP mixed with sunflower cake from flasks to tray-type bioreactors demonstrated SSF reproducibility, achieving similar xylanase and cellulase activities. In the stirred-drum bioreactor, aeration and low agitation favored both enzymes production. Bioprocessing of GBP with plant feedstuffs using SSF was a cost-effective strategy to produce high-value enzymes, valorizing this seaweed by-product.

1. Introduction

Red seaweeds *Gelidium* and *Gracilaria* are the main raw materials for agar extraction, a phycocolloid utilized as gelling agent in food, biotechnology, and cosmetics industries (Lebbar et al., 2018). The extraction method of agar usually begins with an alkaline pre-treatment, followed by a hot-water and high-pressure extraction at temperatures between 90 °C and 120 °C (Martínez-Sanz et al., 2020). This process generates high quantities of by-products (between 15 % and 40 % of the initial dry biomass), which mostly is directly discarded, and the remaining is used as fodder and fertilizer (Álvarez-Viñas et al., 2019; Ferrera-Lorenzo et al., 2014). Nevertheless, seaweed by-products resulting from agar extraction have an interesting chemical composition, with high carbohydrates (mainly cellulose) and protein contents, around 36 % and 20 % (dry weight), respectively (Mouga and Fernandes, 2022).

Most of the valorization approaches already proposed have addressed the production of different value-added compounds from *Gelidium* by-products (GBP): cellulosic films from *Gelidium* cellulosic fractions (Martínez-Sanz et al., 2020); biochar production through chemical activation and pyrolysis (Tayibi et al., 2019); hydrochar applying hydrothermal carbonization of GBP (Méndez et al., 2019); total phenolic compounds (TPC), sugars and proteins resorting to enzymatic hydrolysis (Trigueros et al., 2021b); protein after subcritical water hydrolysis (Trigueros et al., 2021a).

Despite the valuable components of GBP, its valorization by direct fermentation is still underexplored. The functional and economic value of GBP can be enhanced using eco-saving and biological methods, such as solid-state fermentation (SSF). SSF is a biotechnological process that occurs in the absence or near absence of free water, specially recreating the natural habitat conditions of filamentous fungi (Thomas et al., 2013). During SSF, fungal hyphae grow with reduced water activity,

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covering the surface and penetrating the solid substrate, which serves both as solid and as nutritive support. During this time, fungi produce the necessary enzymes for the hydrolysis of biomass biopolymers, modifying the solid structure and releasing bioactive molecules (Čertík et al., 2012).

Indeed, fungi produce non-starch polysaccharide (NSP)-degrading enzymes that reduce the structural polysaccharides-based matrix of seaweeds, increasing the protein content and improving the overall nutritional characteristics of the final products (Fernandes et al., 2019; Ferreira et al., 2022). Several seaweeds have been used as substrates in SSF aiming to produce and/or release value-added compounds such as xylanase, cellulase, and β-glucosidase (Fernandes et al., 2019; Ferreira et al., 2022; Pellieri et al., 2022) and phenolic compounds with antioxidant potential (Fernandes et al., 2022). In this way, fermented seaweeds are nutritional enhanced feedstuffs that have been proved to be successfully used in aquafeed (Fernandes et al., 2022) and replace other plant feedstuffs, that have more environmental impact.

To the best of our knowledge, *Gelidium* biomass after agar extraction (GBP) has not yet been used as a substrate in SSF. In fact, the fermenation of GBP may allow obtaining functional products with economic and industrial interest, that can be used in feed, food, or other industries.

The microbial enzymes produced through SSF are of the utmost importance for several economic sectors, such as the paper, food, and textile industries, and in biological treatments of agricultural and forest wastes (Rao and Li, 2017). Furthermore, these enzymes can also be used in animal feeding industries, degrading antinutritional constituents present in feeds and improving the nutritional value of feeds, while also added as supplementary enzymes to help the digestive system function of animals (Rao and Li, 2017).

Therefore, this study aims to assess the potential of GBP as a substrate for SSF using two filamentous fungi species for NSP-degrading enzymes production, such as xylanase, cellulase, and β-glucosidase, and to assess SSF impact on crude protein content of the fermented products. The physicochemical composition of GBP was carried out before SSF, including the polysaccharide content and fatty acids profile. Furthermore, in order to improve the SSF of single GBP, supplements were added such as Mandel Weber salt solution. Also, plant feedstuffs that are already used in animal feed, such as rice bran, sunflower cake, corn gluten feed, and rapeseed cake, were also tested in mixtures with GBP, as well as a mixture with green seaweed *Ulva rigida* that was already proved to be well fermented and used in aquafeed.

2. Material and methods

2.1. Raw biomass

Gelidium corneum by-product obtained after agar-agar extraction (GBP) was provided by Iberagar-Sociedade Luso-Espanhola de Colóides Marinhos S.A. (Coina, Portugal). Sunflower cake and corn gluten feed were supplied by Sorgal, S. A. (Aveiro, Portugal), and rapeseed cake was supplied by IBEROL – Sociedade Ibérica de Oleaginosas, SARL (Vila Franca de Xira, Portugal). The green seaweed *Ulva rigida* was provided by Algaplus, an Integrated Multi-Trophic Aquaculture (IMTA) seaweed producer company (Aveiro, Portugal). All plant feedstuffs, GBP, and *U. rigida* were received dried, then milled, stored in hermetic plastic bags, and kept in the dark at room temperature until use.

2.2. Microorganisms

Two fungi species were used in SSF: *Aspergillus ibericus* MUM 03.49 was obtained from Micoteca of the University of Minho (Braga, Portugal) and *Aspergillus niger* CECT 2915 was obtained from Colección Española de Cultivos Tipo (CECT, Valencia, Spain). The strains were maintained in glycerol stocks at -80 °C. During the experimental period, they were revived on potato dextrose agar (PDA), at 25 °C for 6 days.

2.3. Characterization of GBP

The physicochemical characterization of GBP was determined as follows: the ash content by incineration at 550 °C for 2 h in a muffle furnace; the moisture content by drying the samples in an oven at 105 °C for 24 h; the total protein content (N x 5) was assessed by the Kjeldahl method using a Kjeltec system (Foss 8400); the lipids content using the extraction method described by Ferreira *et al.* (Ferreira et al., 2020), the total carbohydrates content by quantitative acid hydrolysis (QAH) in a two-step acid treatment following the method described by Leite *et al.* (Leite et al., 2016).

In order to estimate the soluble protein and free reducing sugars, an extraction with distilled water (dry solid/liquid ratio of 1 g to 8 mL) was performed in GBP. The soluble protein was determined by the Bradford method (Bradford, 1976). Free-reducing sugars were determined using the 3,5-dinitrosalicylic acid method (DNS) (Miller, 1959). The total organic carbon was measured using a Thermo Finnigan Flash Element Analyzer 1112 series (San Jose, CA, USA), and the minerals were determined in the ashes fraction using Flame Atomic Absorption and Atomic Emission Spectrometry (FFAS/FAES; FAAS/FAES).

2.4. Fatty acids composition of GBP

The fatty acid methyl esters (FAMES) production was carried out using the method described by Ferreira *et al.* (Ferreira et al., 2020). The analysis of FAMES was carried out in a gas chromatography (GC) system (VARIAN 3800) equipped with a flame ionization detector (FID). The FAMEs were separated using a TRB-WAX 30 m x 0.25 mm×0.25 μm column (TR140232, Teknokroma, Tr-wax) with helium as the carrier gas at 1.0 mL/min. The airflow was settled at 250 mL/min and the nitrogen and hydrogen flow at 30 mL min^{-1}. The injection port and detector temperatures were 250 and 280 °C, respectively. The initial oven temperature was 40 °C for 2 min, with a 30 °C min^{-1} ramp to 150 °C and a 3 °C min^{-1} final ramp to 250 °C. The relative amount of each fatty acid (%, w/w) was determined as the ratio between its concentration (g of fatty acid per kilogram of dry GBP) and the sum of the concentrations of all fatty acids identified in the sample.

2.5. SSF of GBP

The initial SSF experiments were carried out using GBP as substrate without the addition of nutritional supplements, assessing the growth and enzyme production of two fungi species, *A. ibericus* and *A. niger*. SSF was carried out in 500 mL Erlenmeyer flasks with 10 g of dry GBP. The moisture was adjusted to 75 % (w/w, wet basis) with distilled water and sterilized (121 °C, 15 min). Each flask was inoculated with 2 $x10^5$ spores of *A. ibericus* or *A. niger* per gram of dry substrate, following the method described by Ferreira *et al.* (Ferreira et al., 2022) and incubated at 25 °C for 7 days. At the end of SSF, the fermented GBP was characterized, and the enzymes were extracted with distilled water (dry solid/liquid ratio of 1 g to 8 mL). The crude extract was stored at -20 °C until analysis.

Another SSF was performed using GBP as substrate and with the moisture level adjusted to 75 % (w/w, wet basis) using the Mandel Weber salt medium (Mandels and Weber, 1969). The chemical composition of the medium is: 2 g L^{-1} KH_2PO_4; 1.4 g L^{-1} $(NH_4)_2SO_4$; 0.3 g L^{-1} urea; 0.3 g L^{-1} $CaCl_2$; 0.14 g L^{-1} $MgSO_4{\cdot}7\,H_2O$; 5 mg L^{-1} $FeSO_4{\cdot}7\,H_2O$; 1.56 mg L^{-1} $MnSO_4{\cdot}H_2O$; 1.4 mg L^{-1} $ZnSO_4{\cdot}7\,H_2O$; 2 mg L^{-1} $CoCl_2$. Another experiment was similarly performed but adding 5 g L^{-1} of glucose to the Mandel Weber salt medium.

SSF using a mixture of GBP with plant feedstuffs (rice bran, sunflower cake, rapeseed cake, and corn gluten feed) or with the green seaweed *U. rigida* (1:1, w/w, dry basis) was performed with *A. niger* CECT 2915.

2.6. Scale-up process

The scale-up of SSF was carried out using GBP mixed with sunflower cake as substrate with *A. niger* CECT 2915. SSF was performed in trays with 50 g (16 ×11 x 6 cm) and 200 g (30 ×19 x 9 cm) of dry substrate and the moisture level was adjusted with distilled water to obtain 75 % (after inoculation), followed by sterilization (121 °C, 15 min). Each tray was inoculated with $2x10^5$ spores of *A. niger* CECT 2915 per gram of dry substrate, covered with perforated plastic wrap and incubated at 25 °C for 7 days. The trays were mixed once a day in the first 3 days of SSF.

A horizontal stirred-drum bioreactor was also used for SSF scale-up with *A. niger* CECT 2915 using GBP mixed with sunflower cake as substrate, with and without aeration. The reactor consisted of a horizontal cylindric vessel (made of acrylic plastic) with an interior volume of 4.5 L and a temperature-controlling water jacket, also equipped with paddles coupled to a motor that allowed mixing the substrate at a rate of 2 rotations per minute. A schematic representation of the bioreactor has been already reported (Costa et al., 2023). SSF was performed using 200 g of dry substrate, inoculated as previous described and operated at 25 °C for 7 days. Inlet air at a rate of 0.5 mL min^{-1} g^{-1} (measured at standard conditions of pressure at temperature) was filtered through a 0.22 μm filter before being directed to a humidification chamber filled with sterile water. SSF was also performed without aeration where gas exchange occurred through diffusion using cotton plugs placed at the sampling points (Costa et al., 2023). In both types of SSF, substrate mixing was performed every 6 h for 15 min, during the 7 days of fermentation.

Another SSF was performed with the same aeration rate as above but with agitation only occurring in the first 3 days of SSF, and once a day for 15 min.

Samples were taken daily throughout the fermentation period and the enzymes produced were extracted from the fermented mixture with distilled water (dry solid/liquid ratio of 1 g to 8 mL). The crude extract was stored at -20 °C until analysis.

2.7. Enzymatic activities

The xylanase and cellulase (endoglucanase) activities were determined using xylan from beechwood (1 % in sodium citrate buffer 0.05 N, pH 4.8) and carboxymethyl cellulose (2 % in sodium citrate buffer 0.05 N, pH 4.8) as substrates, respectively. The reactions were performed at 50 °C for 15 min (xylanase) and 30 min (cellulase), and the free sugars released were determined by the DNS method. One unit of xylanase or cellulase activity was defined as the amount of enzyme necessary to release 1 μmol of xylose or glucose per minute, respectively, under the reaction conditions.

The β-glucosidase activity was assessed using 4 mM 4-nitrophenyl b-D-glucopyranoside (pNPG) as substrate. The enzymatic reaction was carried out at 50 °C for 15 min and the absorbance was read at 400 nm. One unit of β-glucosidase activity was defined as the quantity of enzyme required to release 1 μmol of *p*-nitrophenol per minute under the reaction conditions.

2.8. Statistical analysis

All data were analyzed using a t-test to compare the means between two groups, using the probability of 0.05 for rejection of the null hypothesis; or by one-way analysis of variance (ANOVA), to compare the means between three or more groups and, if significant differences were detected ($p < 0.05$), Tukey's multiple range test was applied to discriminate the means. The statistical analyses were performed using Statgraphics Centurion XVI.II software (Statgraphics Technologies Inc., Virginia, United States).

3. Results and discussion

3.1. Characterization of GBP

The physicochemical characterization of GBP after agar extraction is presented in Table 1. GBP protein content is relatively high which may result from the agar extraction, concentrating the protein fraction up to higher amounts than those observed in the unprocessed and wild harvested seaweed, which is around 14 % (dry weight) (Mouga and Fernandes, 2022). The carbohydrate content of GBP was lower than that reported in other studies (Mouga and Fernandes, 2022; Trigueros et al., 2021a; Tůma et al., 2020). Nonetheless, the main carbohydrate fraction of GBP is composed of cellulose, which can be used as carbon source for fungal growth during SSF. The low lipid content of GBP is in accordance with reported values by other authors (Mouga and Fernandes, 2022; Trigueros et al., 2021b, 2021a). Ash content represented up to 20.4 ± 0.01 % (w/w) of GBP dry weight, being Ca, Mg, K, and Na the most abundant macro-elements, while Zn, Mn, and Fe were the micro-elements presented in higher amounts. Some of these elements were also found by Tuma *et al.* (Tůma et al., 2020) and Trigueros *et al.* (Trigueros et al., 2021a) in GBP. Nonetheless, Trigueros *et al.* (Trigueros et al., 2021a) also observed that unprocessed and fresh *Gelidium sesquipedale* has higher inorganic compounds content compared to this seaweed by-product, mainly due to the reduction in Na and K levels during agar extraction of *G. sesquipedale*.

The fatty acid profile of seaweeds contains nutritionally important polyunsaturated fatty acids (PUFAs), despite their low concentration (Kumari et al., 2010). Unsaturated fatty acids account for 73.1 % of the total fatty acids of GBP, including 67.5 % of PUFAs and 5.6 % of monounsaturated fatty acids (MUFAs; Table 2). To the best of the authors' knowledge, there are no studies reporting the fatty acid profile of GBP. Paiva *et al.* (Paiva et al., 2017) studied the fatty acid composition of unprocessed *Gelidium microdon* and observed similar values of SFAs (23.27 % ± 1.41 of total FAMEs), MUFAs (7.50 % ± 0.81) and PUFAs (69.22 % ± 3.30) as observed in the present study. Cavaco *et al.* (Cavaco et al., 2021) observed a seasonal fatty acid profile variation of *G. corneum* and concluded that PUFAs are abundant mostly during summer, being significantly higher than the PUFAs content in the other seasons, particularly in winter.

3.2. SSF of GBP

GBP without any supplementation was used as in SSF with *A. ibericus* MUM 03.49 and *A. niger* CECT 2915, assessing the protein content of fermented biomass and the production of NSP-degrading enzymes.

Table 1
Physicochemical and mineral composition of GBP (% w/w dry solid).

Parameter	GBP
Humidity	12.0 ± 0.1
Ash	20.41 ± 0.01
Crude Protein	23 ± 1
Acid insoluble residue	15 ± 2
N	4.9 ± 0.3
C	35 ± 2
C/N	7.23
Carbohydrate	24.6 ± 0.1
Cellulose	23.07 ± 0.01
Agar	1.6 ± 0.1
Lipids	2.1 ± 0.2
Calcium	6.3 ± 0.3
Magnesium	0.61 ± 0.02
Iron	0.5 ± 0.1
Potassium	0.22 ± 0.02
Sodium	0.18 ± 0.01
Zinc	0.036 ± 0.004
Manganese	0.03 ± 0.01

Table 2
Relative fatty acid content of GBP (%).

	GBP
Caproic (C6:0)	1.13 ± 0.02
Capric (C10:0)	0.31 ± 0.01
Lauric (C12:0)	0.4 ± 0.1
Myristic (C14:0)	2.9 ± 0.9
Palmitic (16:0)	21 ± 4
Palmitoleic (16:1 c6 n9)	2.0 ± 0.4
Margaric (C17:0)	0.6 ± 0.1
Stearic (C18:0)	1.03 ± 0.03
Oleic (C18:1 c9 n9)	3.60 ± 0.02
Linoleic (C18:2 c9 c12 n6)	5.7 ± 0.2
GLA (C18:3 c6 c9 c12 n6)	0.8 ± 0.2
ALA (C18:3 c9 c12 c15 n3)	3.8 ± 0.1
ARA (C20:4 c5 c8 c11 c14 n6)	37.1 ± 0.7
EPA (C20:5 c5 c8 c11 c14 c17 n3)	16.5 ± 0.2
DHA (C22:6 c4 c7 c10 c13 c16 c19 n3)	3.7 ± 0.4
SFAs	27 ± 5
MUFAs	5.6 ± 0.4
PUFAs	67.56 ± 0.4

GLA: γ-linolenic acid; ALA: α-linolenic acid; ARA: arachidonic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids.

Previous results demonstrated that these filamentous fungi species have a high ability to grow using seaweeds as substrates, under SSF conditions, being able to produce NSP-degrading enzymes (Fernandes et al., 2019; Ferreira et al., 2022). In this study, xylanase and cellulase production in GBPwithout supplementation did not differ between SSF experiments with both fungi (Table 3). On the contrary, the maximum β-glucosidase activity was obtained in the SSF of GBP with *A. ibericus* MUM 03.49. The enzyme activities obtained at these conditions were relatively low compared with other studies involving the SSF of *U. rigida* (Fernandes et al., 2019; Ferreira et al., 2022). Nonetheless, similar xylanase and β-glucosidase activities were obtained in the SSF of red seaweeds *Gracilaria gracilis* and *Porphyra dioica* using the same strains as in the present study (Ferreira et al., 2022).

Besides the similar enzyme production by SSF of unsupplemented GBP of both species, also the protein content of unfermented and fermented GBPwas similar (Fig. 1). These results may indicate that the growth of both fungal strains was compromised, at least until a certain extent, using only GBP as substrate. Since *G. corneum* underwent an industrial treatment for agar extraction, it is possible that soluble compounds were equally removed during this process. According to Trigueros *et al.* (Trigueros et al., 2021a), a severe reduction in potassium level occurred in G. *sesquipedale* biomass after agar extraction, which is one element necessary for fungal growth (Walker and White, 2011). In this work, the level of potassium found in GBP was also low (Table 1). Besides, free sugars were also measured in GBP and were not detected. All these substances may boost fungal growth and early proliferation, at least until fungi secret the enzymes that would allow them to degrade the complex structure of the substrate (Khosravi et al., 2015). Therefore, the following experiments consisted in the supplementation of GBP with

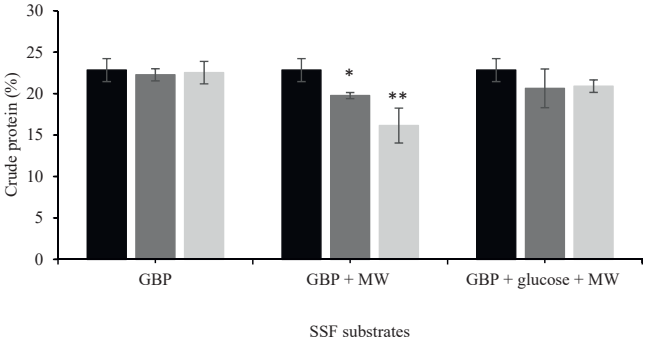

Fig. 1. Crude protein content of unfermented GBP (black bars) and fermented GBP with *A. ibericus* MUM 03.49 (dark gray bars) and *A. niger* CECT 2915 (light gray bars). MW: Mandel Weber salt solution. Results are presented as mean ± SD (n = 3). *, ** Denotes significant differences between unfermented and fermented substrates (one-way ANOVA, $p < 0.05$).

Mandels' salt solution and combining this salt medium with an easily assimilable carbon source, such as glucose.

The supplementation of GBP with Mandels' salt solution had a positive effect on the production of the three enzymes by both fungi (Table 3). Moreover, xylanase activity produced by *A. ibericus* MUM 03.49 increased 14-fold when GBP was mixed with glucose and Mandels' salt solution, while cellulase and β-glucosidase activities increased 62- and 38-fold in SSF with *A. niger* CECT 2915, respectively. Other authors performed SSF of green seaweed *U. rigida* (with *Aspergillus fumigatus* SL1) and brown seaweed *Rugulopteryx okamurae* (with *Aspergillus awamori* 2B.361 U2/1) supplemented with Mandels' salts solution as biological pretreatments to produce biogas and monosaccharides, respectively (Agabo-García et al., 2023; Yahmed et al., 2017). Indeed, in this study, the addition of glucose, an easily assimilable sugar, may have contributed to boost the growth of both fungi, resulting in a major increase of all enzyme production compared to the fermentation of GBP without any supplementation. Thus, the agar extraction process of *G. corneum* reduce the soluble available nutrients for fungi growth and their addition to the GBP substrate improves fungal growth and enzyme production.

The protein content of GBP and GBP supplemented with glucose and Mandels' salt solution was not altered after SSF with both fungal strains (Fig. 1). However, the protein content of GBP supplemented only with Mandel Weber salt solution decreased after SSF by 13 % and 29 % when using *A. ibericus* MUM 03.49 and *A. niger* CECT 2915, respectively. This could indicate that both fungi used the external nitrogen sources added to GBP in the Mandels' salt solution to mycelium growth and the efficiency of other metabolic processes (Šelo et al., 2023). Likewise, the protein content of the red seaweed *Gracilaria* sp. (Ferreira et al., 2022) and different plant feedstuffs such as sunflower, rapeseed, soybean cakes (Sousa et al., 2021), and grape pomace (Šelo et al., 2023) also decreased after SSF.

Table 3
Enzyme production (U g^{-1} dry solid) during solid-state fermentation of unsupplemented GBP, GBP supplemented with Mandel salt solution, and with this salt solution containing glucose.

	Xylanase		Cellulase		β-glucosidase	
	A. ibericus MUM 03.49	*A. niger* CECT 2915	*A. ibericus* MUM 03.49	*A. niger* CECT 2915	*A. ibericus* MUM 03.49	*A. niger* CECT 2915
GBP	8.7 ± 0.5^{a}	10 ± 3^{a}	1.9 ± 0.2^{a}	4 ± 1^{a}	5.2 ± 0.1^{a}	$1.2 \pm 0.2^{a*}$
GBP +MW	13 ± 1^{a}	$44 \pm 1^{b*}$	3 ± 1^{a}	$76 \pm 9^{b*}$	10 ± 1^{a}	$22 \pm 3^{b*}$
GBP + glucose + MW	123 ± 8^{b}	$54 \pm 3^{c*}$	60 ± 8^{b}	$216 \pm 17^{c*}$	32 ± 4^{b}	$46 \pm 4^{c*}$

MW: Mandel salt solution. Results are presented as the mean ± SD (n = 3). * Represent the significant difference from a t-test between the same SSF conditions but with different fungi for each enzyme.
Different letters in the same column indicate significant differences (one-way ANOVA, $p < 0.05$).

Table 4
Lignocellulolytic enzymes production (U g^{-1} dry solid) during solid-state fermentation of GBP mixed with different plant feedstuffs and with the green seaweed *U. rigida*.

	Xylanase	Cellulase	β-glucosidase
GBP	10 ± 3^{a}	4 ± 1^{a}	1.2 ± 0.2^{a}
GBP+ rice bran	136 ± 6^{b}	67 ± 1^{b}	0.10 ± 0.02^{a}
GBP + rapeseed cake	325 ± 18^{c}	382 ± 37^{e}	64 ± 10^{d}
GBP + sunflower cake	498 ± 49^{d}	297 ± 26^{d}	33.4 ± 0.5^{c}
GBP + corn gluten feed	361 ± 1^{c}	133 ± 29^{c}	34 ± 2^{c}
GBP+ *U. rigida*	56 ± 2^{e}	84 ± 7^{bc}	13.7 ± 0.6^{b}

Results are presented as mean ± SD (n = 3). Different letters in the same column indicate significant differences between values (one-way ANOVA, $p < 0.05$).

3.3. SSF of GBP mixed with other substrates

Plant feedstuffs are the main ingredients of animal feed, with a composition rich in simple and complex sugars, minerals, and proteins (Filipe et al., 2023; Naik et al., 2023). In the literature, several studies have successfully demonstrated the use of these plant feedstuffs as a substrate for SSF to produce enzymes and bioactive compounds, including rice bran to produce amylase, protease, cellulase, pectinase (Wang et al., 2021) and pullulanase (Naik et al., 2019); or rapeseed and sunflower cakes to produce lignocellulolytic enzymes, protease, and lipase (Costa et al., 2022; Sousa et al., 2021).

In this sense, GBP was mixed with several plant feedstuffs (rice bran, rapeseed cake, sunflower cake, and corn gluten feed) to a limit of 50% (w/w dry basis) inclusion and used in SSF with *A. niger* CECT 2915 since this fungal species produced the highest values of lignocellulolytic enzymes activities in the previous experiments. Furthermore, since the green seaweed *U. rigida* was a highly suitable substrate in SSF to produce lignocellulolytic enzymes in previous reported works (Fernandes et al., 2019; Ferreira et al., 2022), it was also used in the mixtures with GBP to carry out the SSF with *A. niger* CECT 2915 and assess lignocellulolytic enzymes production. Since mixtures below 50% inclusion of the plant feedstuffs and *U. rigida* did not lead to significant improved results (data not shown), only data with this mixture composition is herein presented. Mixing GBP with eitherplant feedstuffs or *U. rigida* increased the production of xylanase, cellulase and β-glucosidase. Maximum xylanase activity was obtained with the mixture between GBP and sunflower cake, while highest values of cellulase and β-glucosidase activities were observed when GBP was mixed with rapeseed cake (Table 4). Sousa *et al.* (Sousa et al., 2021) used sunflower, rapeseed, and soybean cakes as substrates in SSF with three fungi species (*Rhizopus oryzae* MUM 10.260, *A. ibericus* MUM 03.113 and *A. niger* CECT 2915), obtaining a cellulase activity up to 50 U g^{-1} using sunflower and rapeseed cakes in SSF with *A. niger* CECT 2915, which is lower than the values attained in the present study. Indeed, the present results indicate that RSB mixed with sunflower cake positively influenced cellulase activity without impairing xylanase activity. On the contrary, GBP mixed with rapeseed cake resulted in lower xylanase activity and GBP mixed with sunflower cake led to lower β-glucosidase activity, compared when these plant feedstuffs were individually used in SSF with *A. niger* CECT 2915 (Sousa et al., 2021). The utilization of GBP mixed with *U. rigida* in SSF with *A. niger* CECT 2915 had a positive effect on enzyme production when compared with SSF of unsupplemented GBP, however, did not reach the enzymatic activity values obtained when the GBP was fermented with plant feedstuffs.

Soluble protein content increased after SSF of GBP mixed with the different biomasses, except when GBP was mixed with rice bran (Table 5), ranging between 0.41 (GBP) up to 2.95 mg g^{-1} (GBP + corn gluten feed). Other studies also observed an increase in soluble protein content after SSF of different seaweeds. Indeed, SSF of *U. rigida* with *A. ibericus* MUM 03.49 increased soluble protein from 0.51 mg g^{-1} (DW, crude seaweed) up to 2 mg g^{-1} (Fernandes et al., 2022) while SSF of brewer's spent grain (BSG) with *Rhizopus* sp. (ROR004) increased 6.5-fold soluble protein content of this agro-industrial material (Ibarruri et al., 2019). On the contrary, no clear pattern was observed regarding the reducing sugars content after SSF, since it increased after SSF of GBP, GBP mixed with rapeseed cake, or *U. rigida*, decreased after SSF with GBP mixed with rice bran or corn gluten feed, and maintained after SSF with GBP mixed with sunflower cake (Table 5). Reducing sugars content may vary differently according to the substrate used in SSF and fungal strain used, since a decrease may point out an active consumption of the most available sugars by the fungus, while an increase may indicate that the fungus was able to hydrolyze the substrate structure and release simpler sugars (Zeko-Pivač et al., 2022). Fernandes *et al.* (Fernandes et al., 2022) observed that reducing sugars content of *U. rigida* increased from 4.24 up to 9.28 mg g^{-1} dry biomass after 7 days of SSF with *A. ibericus* MUM 03.49. On the other hand, Costa *et al.* (Costa et al., 2022) used a mixture of olive and sunflower cakes as substrate for SSF with *Yarrowia lipolytica* W29 for 4 days and concluded that in the first two days, the increase in cellular density was accompanied by a decrease in reducing sugars, which remained residual until the end of SSF.

Except for SSF using GBPmixed with corn gluten feed and *U. rigida*, the protein content increased after SSF of GBP mixed with rice bran (1.3-fold), rapeseed cake (1.2-fold), or sunflower cake (1.2-fold) compared to the unfermented mixtures (Fig. 2). The increase of protein content after SSF with different fungal strains has been previously observed using seaweeds (Fernandes et al., 2019; Ferreira et al., 2022) and different agro-industrial by-products, such as BSG (Canedo et al., 2016; Sousa et al., 2018) and vine-shoots trimming, exhausted grape pomace and exhausted olive pomace (Sousa et al., 2018). Also, SSF of a mixture of brown seaweed *Sargassum fulvellum* with palm kernel cake increased the protein content of the mixture from 3.75 % up to 5.23 % (Ilias et al., 2015).

Table 5
Soluble protein (mg g^{-1} dry solid) and reducing sugars (mg g^{-1} dry solid) before and after solid-state fermentation of GBPmixed with plant feedstuffs and with the green seaweed *U. rigida*.

	Soluble protein		Reducing sugars	
	Unfermented	*A. niger* CECT 2915	Unfermented	*A. niger* CECT 2915
GBP	ND	0.41 ± 0.02	ND	1.23 ± 0.06
GBP+ rice bran	4.14 ± 0.11^{a}	0.36 ± 0.04^{b}	14.3 ± 0.07^{a}	4.07 ± 0.83^{b}
GBP + rapeseed cake	0.56 ± 0.02^{a}	2.46 ± 0.25^{b}	3.06 ± 0.39^{a}	$6.43^{a} \pm 0.55^{b}$
GBP + sunflower cake	1.01 ± 0.23^{a}	2.75 ± 0.51^{b}	5.67 ± 1.10^{a}	6.68 ± 0.69^{a}
GBP + corn gluten feed	0.48 ± 0.05^{a}	2.95 ± 0.33^{b}	11.0 ± 1.77^{a}	7.81 ± 0.34^{b}
GBP + *U. rigida*	0.66 ± 0.01^{a}	0.94 ± 0.10^{b}	3.73 ± 0.27^{a}	6.49 ± 0.89^{b}

ND: not detected. Different letters represent significant differences from a t-test (n = 3) between unfermented and solid fermented substrates with *A. niger* CECT 2915 for each parameter.

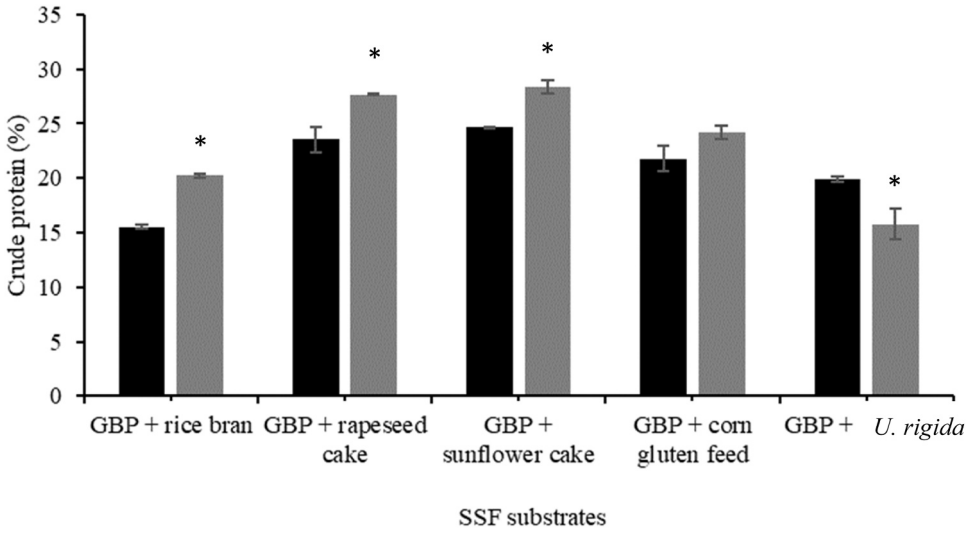

Fig. 2. Crude protein content of unfermented (black bars) and fermented GBP mixed with each plant feedstuff and the green seaweed *U. rigida* with *A. niger* CECT 2915 (gray bars). * Represent the significant difference from a t-test (n=3) between unfermented and fermented substrates.

3.4. Scale-up of SSF of GBP mixed with sunflower cake

As described above, high enzymatic activities were produced in SSF of GBP mixed with rapeseed and sunflower cakes using *A. niger* CECT 2915, thus this last by-product was chosen as a model to carry out a scale-up of 5- and 20-fold of the fermentative process.

Tray bioreactors are simple SSF bioreactors for high substrate quantities, and are constructed from diverse materials such as wood, metal, and plastic, allowing to place multiple individual trays in a single chamber. The trays are static beds in which the substrates are infrequently or not mixed and are placed in a manner that a certain space is left between trays to maximize air availability (Ge et al., 2017). In this study, no differences were observed in xylanase and cellulase activities obtained in SSF carried out with 10 g, 50 g, or 200 g of dry substrate, keeping the same bed height in all the system , which demonstrates the high reproducibility of SSF at different scales (Table 6). Fernandes *et al.* (Fernandes et al., 2019) performed SSF of *U. rigida* with *A. ibericus* MUM 03.49 in tray-type bioreactors and observed that xylanase production was similar using 10 g or 50 g of *U. rigida*, while it decreased using up to 400 g of substrate. These authors also observed that cellulase production using up to 400 g of *U. rigida* was higher than that obtained in flasks with 10 g of substrate. Sousa *et al.* (Sousa et al., 2023) studied the SSF scale-up of two mixtures, one of equal parts of rapeseed cake and soybean cake, and other with one third of each cake, sunflower, rapeseed and soybean, using *A. niger* CECT 2915 with 10 g, 50 g and 400 g of dry substrate mixture using 1 L Erlenmeyer flask or a small tray and did not observed difference in xylanase and cellulase activities according to reactor type or quantity used, while β-glucosidase activity using the mixture of rapeseed and soybean cakes was highest using a small tray bioreactor with 50 g of dry substrate.

Horizontal stirred-drum bioreactors are also utilized in SSF. It consists of a horizontal cylindrical drum partially filled with substrate, in which the air is blown through the headspace and paddles are positioned on a shaft along the bioreactor's central axis to enable mixing, thus preventing bed compaction (Ge et al., 2017). In the present work, SSF was conducted on a stirred-drum bioreactor, with and without aeration, to assess if this parameter would affect xylanase and cellulase

Table 6
Enzyme production (U g^{-1} dry solid) during solid-state fermentation with *A. niger* CECT 2915 of 10 g, 50 g and 200 g of GBP mixed with sunflower cake.

Substrate	Xylanase	Cellulase
10 g	498 ± 49	297 ± 26
50 g	455 ± 11	372 ± 60
200 g	494 ± 44	370 ± 39

Results are presented as mean ± SD (n = 3). The absence of superscript letters indicates no significant differences between the amount of substrate used (one-way ANOVA, p<0.05).

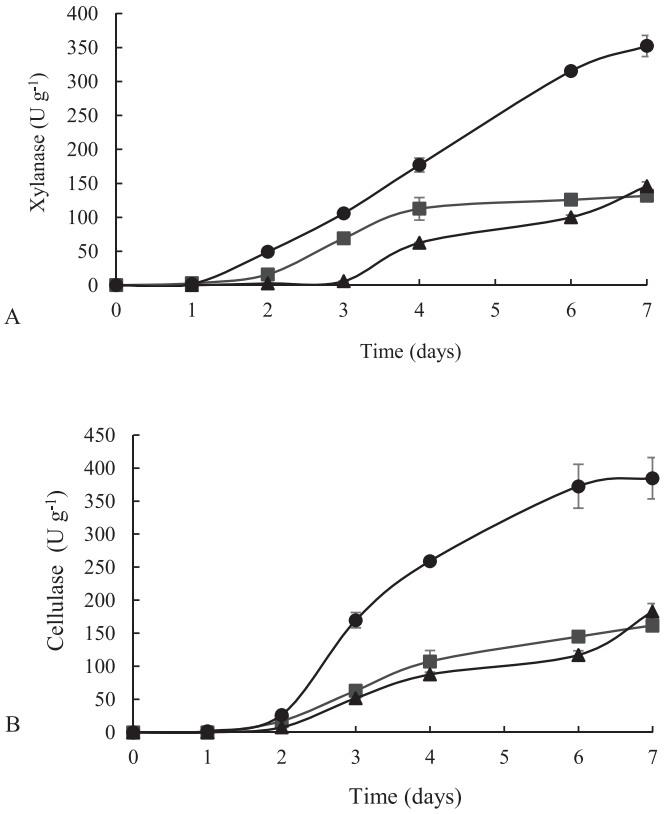

Fig. 3. Xylanase (A) and cellulase (B) production during solid-state fermentation with *A. niger* CECT 2915 for 7 days with 200 g (dry basis) of GBP mixed with sunflower cake using a stirred-drum bioreactor: (●) air flow (0.1 L/min) with low agitation - 2 rpm for 15 min every 24 h in the first 3 days; (■): air flow (0.1 L/min), agitation at 2 rpm for 15 min every 6 h; (▲): diffusion, agitation at 2 rpm for 15 min every 6 h. Results are presented as the mean ± SD (n = 2).

production. When aeration was applied during SSF, xylanase and cellulase production occurred at a faster rate than when the gas exchange was performed only through diffusion (Fig. 3). However, at the end of SSF, similar xylanase and cellulase activities were obtained using both systems. Therefore, applying aeration during SSF may allow to reduce the fermentation period to 4 or 6 days to achieve the maximum xylanase or cellulase production, respectively.

Although the production of both enzymes in a stirred-drum bioreactor is similar at the end of SSF, xylanase and cellulase activities decrease 68 % and 51 %, respectively (Fig. 3), compared to the production in the tray-type bioreactors using 200 g of substrate (Supplementary material: Table S2). In trays, the agitation was carried out once a day in the first 3 days of SSF, while in the stirred-drum bioreactor, the agitation was carried out 4 times a day, during the 7 days of SSF. Therefore, it seems that the higher agitation rate carried out in the stirred-drum bioreactor impaired fungal growth and, consequently, the enzyme production. Mixing is often used to maximize productivity and improve the convective transport by increasing the surface area of the substrate exposed to the moist air but may break fungal mycelia which may impair enzyme production (Arora et al., 2018). Therefore, another assay was performed in the horizontal agitated bioreactor with aeration and only agitating the mixture in the first 3 days of SSF, once a day (Fig. 3). Xylanase production increased starting on the second day of SSF, being always higher than the activity obtained in the other assays carried out in the stirred-drum bioreactor with more agitation. The same pattern was observed for cellulase production but starting on the third day of SSF. At the end of SSF with airflow and low agitation, xylanase, and cellulase activities were 55 % and 52 % higher, respectively, compared to the productivity obtained in the other stirred-drum bioreactor conditions at the same time (7 days).

Comparing the experiments conducted using airflow and low agitation in the stirred-drum bioreactor with the trays-type bioreactor experiments, it is possible to verify that no differences were observed in enzymes productivity, only observing 4 % increase in cellulase activity using the stirred-drum bioreactor. Thus, it is possible to infer that greater agitation conditions did not favor fungal growth and enzymatic production. Costa *et al.* (Costa et al., 2023) also used tray-type (50 and 400 g) and a stirred-drum bioreactor (200 g) for scaling-up SSF of olive cake mixed with sunflower cake (1:1) using the yeast *Y. lipolytica* W29 and observed a significant increase in cellular growth with the stirred-drum bioreactor, especially when using aeration, comparing to tray bioreactors. That may be attributed to the substrate mixing, which may lead to higher metabolic heat dissipation. On the other hand, the same authors observed that SSF performed in trays resulted in higher lipase productivity and specific activity. Also, Rodrígues-Jasso *et al.* (Rodríguez-Jasso et al., 2013) used a rotating drum bioreactor for SSF of *Fucus vesiculosus* with *Mucor* sp. 3 P and concluded that agitated assays were more advantageous in the induction of enzyme production compared to static systems.

4. Conclusions

In this work, the application of GBP for SSF was proven either with unsupplemented GBP, either with supplementation of aqueous solutions of mineral, and glucose. In fact, the removal of minerals and free sugars during the industrial agar extraction may limited fungal development due to the lack of these compounds in GBP, thus the supplementation of GBP with Mandel Weber salt solution and glucose improved carbohydrases production. Moreover, the mixture of GBP with plant feedstuffs increased crude protein content of fermented substrate. Scale-up of SSF for xylanase and cellulase production with *A. niger* using a mixture of 50 % (w/w) of GBP and sunflower cake was successfully achieved in tray-type bioreactors and in a stirred-drum bioreactor with aeration and low agitation. Thus, the utilization of GBP mixed with plant feedstuffs as solid substrates for SSF proved to be a viable and affordable strategy for the production of high-value enzymes, valorizing GBP and improving nutritional value of the final fermented product.

CRediT authorship contribution statement

José Manuel Salgado: Conceptualization, Supervision, Writing – review & editing. **Marta Ferreira:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Isabel Belo:** Conceptualization, Supervision, Writing – review & editing. **Helena Peres:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fbp.2024.05.014.

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