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Potential use of frass from edible insect *Tenebrio molitor* for proteases production by solid-state fermentation

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ABSTRACT

Novel, eco-saving and low-cost food sources are a global priority, such as edible insects. This work sets a valorization pathway for frass and *Tenebrio molitor* adult insects using solid-state fermentation (SSF) to obtain fungal proteases, which are highly used in food, beverages, cosmetics, pharmaceutical and biotechnology industries. Initial screening experiments demonstrated that *Aspergillus uvarum* MUM 08.01 was the most efficient proteases producer. Small-scale SSF with frass resulted in high proteases activity (65 U g^{-1}), which increased 52% when BSG was mixed with frass (1:1, w/w). The proteases activity attained in this study using only frass or mixed with BSG was superior compared to other activities observed when using agroindustry by-products (such as rapeseed, sunflower, soybean, among others) and other species of *Aspergillus* genus. The optimal conditions from full factorial design using frass:BSG mixture resulted in 152 U g^{-1} of proteases activity, which is in full accordance with the value predicted by the model. SSF scale-up with 50 g of frass:BSG increased proteases activity up to 202.2 U g^{-1}. This study presents an innovative utilization of insects' by-products in the field of enzymes production, in full accordance with the circular economy guidelines.

1. Introduction

The use of edible insects for food and feeding purposes is attracting the attention of consumers and the scientific community worldwide, with insects global market expected to reach 1.2 billion dollars (USD) during 2023 (Liceaga, 2021). Insects are an important source of macro and micronutrients, containing between 7–77% lipids (dry weight, DW) and high protein content (40–77% DW) and digestibility (76–98% DW), containing all essential amino acids, including lysine, tryptophan and threonine (Kouřimská and Adámková, 2016; Liu et al., 2022; Mazurek et al., 2023). Insects farming is both economic and environmentally advantageous since has small space and low energy and water requirements compared to traditional protein sources (Madau et al., 2020).

Among the edible insects already used in the food industry, *Tenebrio molitor* (mealworms) is one of the most promising. Mealworms farming results in low greenhouse gas emissions and requires small amounts of water compared to cattle and pigs rearing. Indeed, the water required to rear 1 kg of mealworms (wet weight, WW) is similar to the volume needed to farm chickens and represents almost 25% of that needed for beef cattle (Miglietta et al., 2015), while the global warming potential and land use per kilogram of mealworms protein is lower than that of milk, chicken, pork or beef production (Oonincx and Boer, 2012). Moreover, Thévenot et al. (2018) observed that around 28% of energy and circa 10% of land were required to rear *T. molitor* mealworms. However, since most insects farms are still small-scale, the manual labor is often high and non-mechanized, including the work associated with feeding, harvesting and cleaning (Niyonsaba et al., 2021). The larval stage of *T. molitor* is the most nutritional advantageous since it contains between 14% and 25% (WW) of protein and between 9% to 20% of lipids (WW; Grau et al., 2017). The fast and cost-saving conditions of *T. molitor* farming increases its use in food and feed products at larger scale (Madau et al., 2020). Actually, in 2021, the European Food Safety Agency (EFSA) certified the safety of using mealworms for human consumption (Turck et al., 2021).

Nonetheless, insects farming, including that of *T. molitor*, generates wastes that must be addressed, such as frass and adults. Frass is mainly composed of larvae feces, uneaten feed, and substrate residues during the farming process. Depending on the feed quality and the type of substrate used in the rearing process, frass properties may vary (Poveda

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et al., 2019). Other insects' by-products are the adults, which are maintained to obtain viable eggs that will hatch into new mealworms, producing insects' meal. Similarly to frass, adults composition may vary according to diet type, species, processing method and rearing conditions (Meyer-Rochow et al., 2021). Adults are not commercialized due to their high chitin content which hampers nutrients' bioaccessibility. Therefore, valorization processes are specially important to extract value from these by-products, since it is expected that 1 million tons of insect meal will be produced in Europe by 2030 (IPIFF, 2021).

Frass is an inherent biomass from insects' farming which may be up to 2- or 3-fold higher than the mealworms biomass reared (He et al., 2021). One of the most innovative utilities of frass is using as biochar to remediate soil and in waste pollution treatments (Yang et al., 2019; He et al., 2020). *Tenebrio molitor*́ s frass contains large amounts of trace elements (50% of total organic carbon, 2.5% of nitrogen, 2% of potassium, 1.8% of phosphorous, 0.6% of magnesium and 0.3% of Sulfur) and a small quantity of water (10%; Beesigamukama et al. 2022), increasing the biochar potential to be used as a pesticide absorbent, to boost seedlings resistance to stress factors (Chavez, 2021; Shi et al., 2022), and may widen possible other industrial applications. Indeed, *T. molitor* frass may also be successfully used as substrate in solid-state fermentation (SSF) since carbon and nitrogen contents may boost fungal growth.

The SSF is a low-cost, environmental-friendly, and low labor process which involves the use of solid biomass that acts both as a nutritional and as physical support, in the absence or near absence of "circulating" water (Singhania et al., 2009). Fungi are among the most appropriate microorganisms to use in SSF given the favorable biotic conditions during this process and because they are less prone to contamination compared to bacteria (Verduzco-Oliva and Gutierrez-Uribe, 2020). Fungi belonging to *Aspergillus* genus are among the most used microorganisms in SSF (Teigiserova et al., 2021), resulting in the bioconversion of biological residues from different agrifood industries into high-valued bioactive molecules and/or in upgraded biomasses, with high protein, low fiber and enriched in bioactive compounds (Verduzco-Oliva and Gutierrez-Uribe, 2020).

Proteases are among the most valuable compounds that can be obtained via SSF and may account for up to 60% of global enzymes production market, being the most widely commercialized enzymes and used in many industries, such as beverages, cosmetics, foods and pharmaceutical sectors (Novelli et al., 2016; Raveendran et al., 2018; Ooi and Rasit, 2021). Proteases are used to extract protein from various biomasses, constituting a biological and eco-saving tool compared to the traditional methods involving hazardous chemicals (Hasan et al., 2022). These enzymes hydrolyze proteins' peptide bonds and are obtained from various organisms (fungi, animals, plants, and microorganisms) and processes (submerged or solid-state fermentation) which may entail high production costs (Ooi and Rasit, 2021). Specifically, proteolytic activity may effectively extract and concentrate protein from insects, separating the protein fraction from chitin, the second most abundant complex biopolymer (Mohan et al., 2020). Though, since low-cost and abundantly generated by-products can be used as substrate in SSF, this process may be applied to decrease proteases production costs, increase their cost-effective utilization and lower the disposal of high quantities of hidden-valued residues and extracts.

This study focuses, in a first approach, on proteases production by fungal strains granted the "generally regarded as safe" (GRAS) status. Initial screening experiments were conducted to assess the most promising proteases producers. After, small-scale SSF experiments were carried out with insects' frass and different substrate mixtures, including frass mixed with brewer's spent grain (BSG) and *T. molitor* adults. A full factorial design was applied to assess moisture and temperature levels that maximize proteases production. Furthermore, SSF scale-up was performed with the most suitable substrate, fungal strain, and optimized culture conditions in tray-type bioreactors.

2. Materials and methods

2.1. Raw materials and microorganisms

Frass and adults of yellow mealworm *T. molitor* were obtained from Galinsect S. L. insect farm (Ponteareas, Galicia, Spain). Brewer's spent grain (BSG) was acquired from a soft-drink company (Unicer, Matosinhos, Portugal). Fungi were supplied by Spanish type culture collection (*Aspergillus sojae* CECT 2095, *Aspergillus oryzae* CECT 2094, *Trichoderma reesei* CECT 2414), and by Micoteca culture collection from University of Minho (*Aspergillus uvarum* MUM 08.01). Fungal species were maintained in potato dextrose agar, at 4 °C, until utilization.

2.2. Characterization of raw materials

Frass was characterized according to the standard procedures of Association of Official Analytical Chemists (AOAC International, 2016). Briefly, moisture content was determined by drying samples in an oven at 105 °C until constant weight; total nitrogen and organic carbon were determined by a Thermo Finningan Flash Element Analyzer 1112 series, San Jose, CA (USA) and metals, such as K, Mg, Na, Zn, Cu, Fe and Mn were analyzed in ashes using Flame Atomic Absorption and Atomic Emission Spectrometry (FFAS/FAES); lipids were assessed using a petroleum ether extraction in a Soxhlet extraction system; ash was determined by incineration in a muffle furnace at 450 °C for 16 h; protein content was calculated using the K_p factor of 4.9 for the adult insects and the k_p factor of 6.25 for the frass. The k_p factor of 4.9 used for the protein quantification of adult insects was established by first assessing the chitin content of this biomass and discounting the N of chitin origin. Indeed, Janssen et al. (2017) suggest that a k_p factor of 4.75 should be used for *T. molitor* larvae and indicate the need to increase the factor depending on the chitin content.

Chitin content was quantified using the methods described by Kumari et al. (2015) and Nidheesh and Suresh (2015) with some modifications. The demineralization was performed with an acid treatment using HCl 1 M and a 1:40 ratio (w/v), for 2 h, at room temperature (25 °C). After demineralization, the solid fraction was washed with distilled water until neutral pH and dried at 50 °C for 24 h. The solid was deproteinized using an alkaline solution of NaOH 5 N and a 1:20 ratio (w/v), during 18 h at room temperature (25 °C). After, the solid fraction was washed with distilled water until neutral pH and treated with H_2O_2 solution (1%, w/w) at a 1:10 ratio (w/v), for 10 min at room temperature (25 °C). The solid recovered was washed with distilled water and dried at 50 °C for 24 h and chitin was then quantified.

The lignocellulosic characterization (cellulose, hemicellulose and lignin) was tested according to National Renewable Energy Laboratory (NREL) Technical Report (Sluiter et al., 2008).

2.3. Screening of filamentous fungi for proteases production

An initial screening was carried out to identify the protease producers' fungi. A culture medium was prepared with 10 g L^{-1} powder milk, 20 g L^{-1} agar, 0.1 g L^{-1} Triton X-100 and pH adjusted to 6. The medium was sterilized (121 °C, 15 min) and was placed on each agar plate under aseptic conditions. The plates were inoculated in the center with different fungi. The cultures were maintained at 25 °C for 5 days, and the production of proteases was identified by measuring the halo diameter.

2.4. Solid-state fermentation of frass

The most promising fungi (*A. uvarum* MUM 08.01) with proteolytic potential were selected to carry out a small-scale SSF using frass as substrate. SSF was performed in duplicate in Erlenmeyer flasks (500 mL capacity) with 5 g of dry frass, without nutrients addition. Moisture was adjusted to 75% with distilled water, sterilized (121 °C, 15 min) and

inoculated with a spore's suspension with a microbial load of 2×10^6 spores g^{-1} of dry substrate. The mixture was incubated at 25 °C for 7 days.

Besides frass, BSG (composition in Leite et al., 2019) and *T. molitor* adults were also tested as promising substrates in SSF. To analyze the effect of nutrients addition, the Czapeck medium was used to adjust the moisture. These substrates were individually used or mixed with the insect frass at different proportions. The substrates were previously sterilized (121 °C, 15 min) and the moisture content was adjusted to 75% (w/v). The mixtures were inoculated with a spores' suspension of *A. uvarum* MUM 08.01 with a concentration of 2×10^6 spores g^{-1} of dry substrate and incubated at 25 °C for 7 days.

After SSF, an aqueous extraction was carried out with 10 mL of distilled water per gram of dried fermented biomass for 30 min, at 25 °C, with constant agitation in an orbital shaker at 150 rpm. The liquid fraction was recovered by centrifugation at 3700 *g* for 15 min. Crude enzymatic extract was stored at − 18 °C until analysis of proteolytic activity.

2.5. Determination of proteases activity

Proteases activity was assessed following Sousa et al. (2022). Azo casein (5 g L^{-1}) in sodium acetate buffer 50 mM, pH 5, was used as substrate (0.5% w/v). Briefly, 500 μL of sample was mixed with 500 μL of substrate solution and incubated at 37 °C for 40 min. After, 1 mL of trichloroacetic acid (10% w/v) was added, the mixture was centrifuged (600 *g*, 15 min) and the supernatant was recovered. Then, 1 mL of KOH 5 N was added, and absorbance was read at 428 nm. One unit of enzyme activity was defined as the amount of enzyme that produced an increase of 0.01 of absorbance relatively to the blank, per minute, at 37 °C, and pH 5. Values of protease activity are expressed in units per gram of dry substrate (U g^{-1}).

2.6. Optimization of SSF parameters by full factorial experimental design

A full factorial experimental design (3^2) was conducted to determine optimal conditions that maximize proteases production during SSF. The model was carried out assessing the effect of temperature (°C) and moisture (%) at three levels (−1, 0, +1). The design was performed in a set of 11 experiments, and three central point replicates were used to estimate the experimental error. The factors studied were the moisture and temperature of SSF ranging between 50% and 70% (wet basis) and between 22 and 28 °C, respectively. The range of moisture and temperature were chosen based on reports indicating the suitability of these levels of moisture and temperatures during SSF using *Aspergillus uvarum* MUM 08.01 (Salgado et al., 2015). Proteases activity was the dependent variable studied.

2.7. SSF scale-up

A scale-up of SSF with *A. uvarum* MUM 08.01 using the mixture of BSG and frass as substrate (1:1, w/w) was carried out, increasing substrate quantity from Erlenmeyer flasks with 5 g up to trays with 50 g (30 × 21 x 5 cm), 200 g (38 × 26 x 5 cm) and 500 g (43 × 33 x 7 cm). The trays were inoculated with a spores' suspension with 2×10^6 spores g^{-1} of dry substrate, incubated at 25 °C for 7 days, and proteases activity was assessed. The tray-type bioreactors were chosen given space constraints in the laboratory and since one of the objectives of this research is upscale this process up to quantities that can be included in fish diets. Furthermore, previous studies showed the feasibility of SSF in tray-type bioreactors to produce enzymes (Fernandes et al., 2022).

Assessing of SSF efficiency performed in the conditions found in an insect farm, trays containing 500 g of BSG and frass mixture and with moisture content adjusted to 75% were placed in an oven at 90 °C for 2 h for sterilization. Afterwards, the trays were inoculated with *A. uvarum* MUM 08.01 spores (2×10^6 spores g^{-1} of dry substrate) and incubated at 25 °C for 7 days. A water extraction was carried out (10 mL distilled water per gram of fermented biomass) in the fermented biomass, and the mixture was filtrated with paper filter and a vacuum pump. The liquid extract was used to assess the proteases activity.

2.8. Statistical analysis

Full factorial design data were evaluated using the response surface methodology in the *Statistica 10* software (Informer Technologies Inc., L. A., United States). Data concerning the screening of fungal strains, the SSF with different substrates and the scale-up were analyzed using the *IBM SPSS Statistics* 26 (IBM, NY, United States) software. The data was assessed for homogeneity of variances using Levene's test ($p > 0.05$) and the statistical analyses were conducted using one-way analysis of variance (ANOVA), using the Tukey's multiple range test to detect significant differences among means ($p < 0.05$).

3. Results and discussion

3.1. Characterization of frass and adults of T. molitor

The composition of both frass and adults of *T. molitor* is present in Table 1. Frass has higher moisture content than adult insects, while the inverse was observed for nitrogen content. Accordingly, frass contains approximately half of the protein content found in the *T. molitor* adults, while lipids content was around 84% lower in frass compared to the adults. These results are in accordance to other findings reported in literature (Grau et al., 2017). Similarly, Ravzanaadii et al. (2012) observed that the protein content (46.4%) of *T. molitor* adults was 60% higher than that found in frass (18.5%), while crude lipids were around 83% lower in frass (1.3%) compared to the adults (7.6%). Insects' body composition may be modulated according to the quality of the diet provided during rearing. *Tenebrio molitor* fed either plant wastes (wheat bran, fermented straw and old cabbage leaves) or a conventional diet (wheat bran and cabbage leaves mixture) retained 76% and 68% of body protein, respectively (Li et al., 2013). Adults of *T. molitor* fed with a wheat-based diet contained 44.9% and 1.36% of crude protein and crude lipids, respectively (Khanal et al., 2023). Also, the fatty acids composition of different diets (cookies, bread and BSG) directly affected fatty acids body deposition in mealworms (Mattioli et al., 2021).

Insects interest for human consumption and/or as feed ingredients for animals is exponentially increasing given their promising nutritional composition, easiness of rearing, low energy and water requirements and overall cost-saving production. Although insects consumption is far

Table 1
Proximate and minerals composition of *T. molitor* frass and adults' meal.

g/100 g (dry weight, w/w)	Frass	*Tenebrio molitor* adults
Moisture	9.99 ± 0.2	1.14 ± 0.04
Carbon	44.9 ± 0.18	53.5 ± 0.47
Nitrogen	5.12 ± 0.20	10.1 ± 0.53
Ash	8.15 ± 0.06	4.81 ± 0.2
Protein	24.3 ± 1.37	47.9 ± 2.94
Lipids	2.22 ± 0.10	14.0 ± 0.20
Cellulose	13.6 ± 0.65	ND
Xylan	16.4 ± 0.75	ND
Arabinans	8.58 ± 0.63	ND
Lignin	10.9 ± 0.04	ND
Chitin	7.40 ± 0.37	22.7 ± 0.23
Mineral composition (g/kg, dry weight w/w)		
Potassium	20.98 ± 1.04	10.33 ± 0.022
Magnesium	6.52 ± 0.35	2.38 ± 0.03
Sodium	0.29 ± 0.002	1.30 ± 0.03
Iron	0.19 ± 0.01	0.1038 ± 0.0003
Zinc	0.112 ± 0.003	0.145 ± 0.002
Manganese	0.139 ± 0.006	0.021 ± 0.004
Copper	0.021 ± 0.001	0.028 ± 0.001

ND: non determined.

from being accepted in western regions, more than 200 insect species are already consumed in 113 countries (Kouřimská and Adámková, 2016). However, insect biomass can be used for other industrial purposes. The considerable high nitrogen and potassium content of the frass obtained from *T. molitor* farming makes it a desirable biomass to be used either as a biofertilizer and as a mitigating agent of environmental stress conditions in soils (Chavez, 2021). Also, bio-oils with biological activities obtained from the pyrolysis of frass can also be used as bioinsecticides (Urrutia et al., 2023). The low moisture content of frass facilitates its transport and utilization, apporting important economic savings (Chavez, 2021). Furthermore, insects are also promising ingredients in diets for monogastric animals. Specifically, the utilization of insects may improve the formulation of feeds for aquatic animals, decreasing the reliance on traditional animal (fish meal and fish oil) and plant (such as soybean meal) ingredients, which arise sustainability concerns. However, the use of insects' meal is still limited given their high cost and availability. Nonetheless, the *T. molitor* larvae meals are already being successfully used to replace fish meal in diets for aquaculture fish, such as olive flounder (*Paralichthys olivaceus*) and European seabass (*Dicentrarchus labrax*), with beneficial effects in the immunological state and protein digestibility of fish (Basto et al., 2020; Jeong et al., 2021). On the other hand, frass may upgrade nutrients bioavailability of fish diets since it lacks antinutritional factors that are often present in plant feedstuffs, such as phytic phosphorus, protease inhibitors, cell wall complex polysaccharides, among others (Gasco et al., 2016). Therefore, it would be highly important to assess if *T. molitor* frass would be suitable to be included in aquafeeds, assessing its impact on growth and feed utilization efficiency of commercially advantageous fish species.

3.2. Screening of proteolytic fungi in agar plate experiments

An initial screening experiment was carried out to assess potential proteases producers among *Aspergillus* spp. and *T. reesei* species before SSF. *Aspergillus uvarum* exhibited a larger protease activity halo, of approximately 6 cm, followed by *A. sojae*, *A. oryzae* and *T. reesei* that showed slightly smaller proteases activity halos (around 4 cm; Fig. 1). Furthermore, *A. uvarum* formed spores during incubation which was not observed for the other fungal strains studied. *Aspergillus* spp. are often reported as highly efficient protease producers, either in agar plate experiments or in SSF. A fungal isolate obtained from soil samples and identified as *Aspergillus* spp. exhibited high proteolytic ability, producing an hydrolytic halo of almost 30 mm and a clear zone of 21.3 mm, meaning a relative enzyme activity of 0.74 U g^{-1} (Usman et al., 2021). Zanutto-Elgui et al. (2019) observed that *A. oryzae* produced 6.3 U g^{-1} of protease activity during SSF of soybean bran, but the protease activity increased up to 35 U g^{-1} when using wheat bran as substrate. In this study, *A. brasiliensis* did not exhibited a protease activity halo, indicating that this species lacked the capability to produce active proteases (Fig. 1).

Screening experiments are useful to assess at a first stage which microbial strains may be more suitable to produce value-added compounds. Initial screening assessments using a complex culture medium (1.5% soybean extract, 0.5% wheat bran, 0.44% K_2HPO_4, 0.06% $MgSO_4.7\ H_2O$ and 0.25% glucose) also permitted Gomes et al. (2022) to discern five protease producing fungi, including *A. viride-nutans* URM 6629, among twelve strains. Furthermore, *A. niger* Aa20, *A. oryzae* and *A. fumigatus* were among the fungal strains that exhibited halos of extracellular proteases production when cultured in skim milk media agar (10% w/v of commercial Nestle® skim milk; Martínez-Medina et al., 2023). Likewise, among six fungal species, two *Aspergillus* strains (*A. flavus* and *A. niger*) isolated from paddy crop fields showed higher protease production during initial screening experiments using a medium with 1% gelatin, 5 g peptone, 3 g beef extract, 5 g NaCl, and 15 g agar (Chandrasekaran et al., 2015).

3.3. Solid-state fermentation using frass as substrate

The SSF was performed using the fungal strains that showed higher proteases activity halos during the screening experiments, namely *A. oryzae*, *A. sojae*, *T. reesei* and *A. uvarum*. SSF with *A. uvarum* resulted in higher proteases activity (65 U g^{-1}), followed by SSF of *A. sojae* (45.3 U g^{-1}), *A. oryzae* (42.7 U g^{-1}) and *T. reesei* (42.5 U g^{-1}; Fig. 2). The high enzymatic capacity of filamentous fungi is widely documented since they may grow in a broader range of substrates, frequently result in high enzymes productivity, are often used in cost-effective processes (such as SSF) and the compounds are easily separated from the culture media (Souza et al., 2015). Among the most suitable fungal strains used in the present study, three belong to the *Aspergillus* genus. Other authors focused on proteases production using chitin- (shrimp shell, other chitin-based substrates) and/or protein-rich substrates (such as high protein plant and cereals feedstuffs) using *Aspergillus* spp. Ooi and Rasit (2021) obtained 35 U g^{-1} of protease activity during SSF of shrimp shells with *A. niger*, at 30 °C for 5 days. Different by-products from the vegetable oil industry were also used as substrates in SSF (sunflower, rapeseed, and soybean meal cakes) and higher proteases activity was achieved during SSF of sunflower cake using *A. niger* CECT 2915 (circa 150 U g^{-1}) compared to *Rhyzopus oryzae* MUM 10.260 (circa 110 U g^{-1}) and *A. ibericus* MUM 03.113 (100 U g^{-1}; Sousa et al., 2022). Also, the co-cultivation of *A. niger* NRRL3 and *A. oryzae* NRRL695 in a substrate mixture of wheat bran:soybean husk (75%:25% w/w) led to a protease production of 2.02 U g^{-1} (Morilla et al., 2023).

The high production volume, low cost and the high-quality

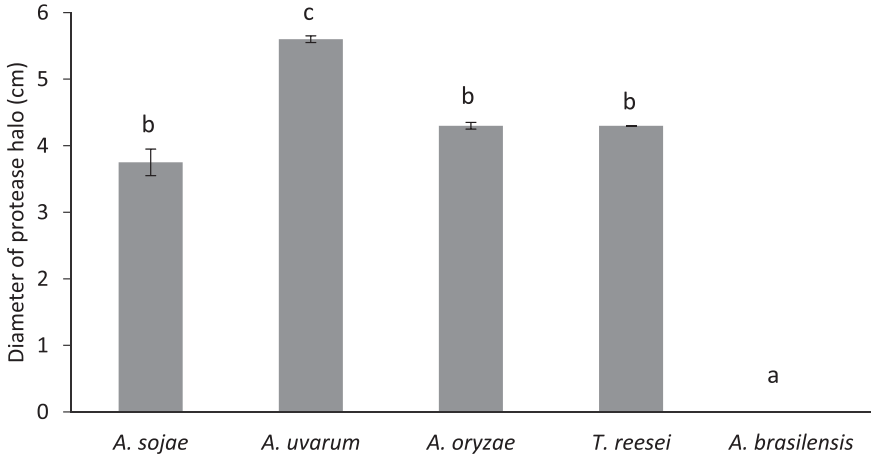

Fig. 1. Screening experiments in plates of filamentous fungi with proteolytic activity.

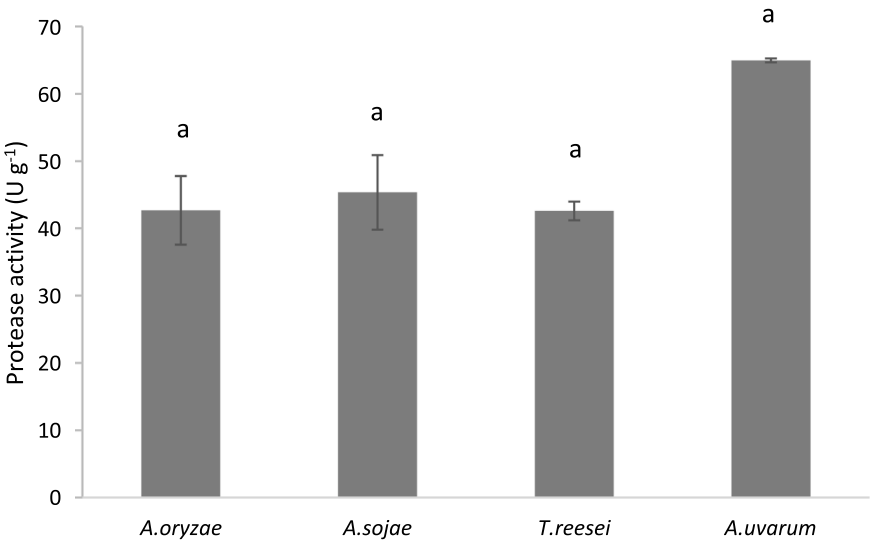

Fig. 2. Production of proteases during SSF with different fungal strains using frass as substrate.

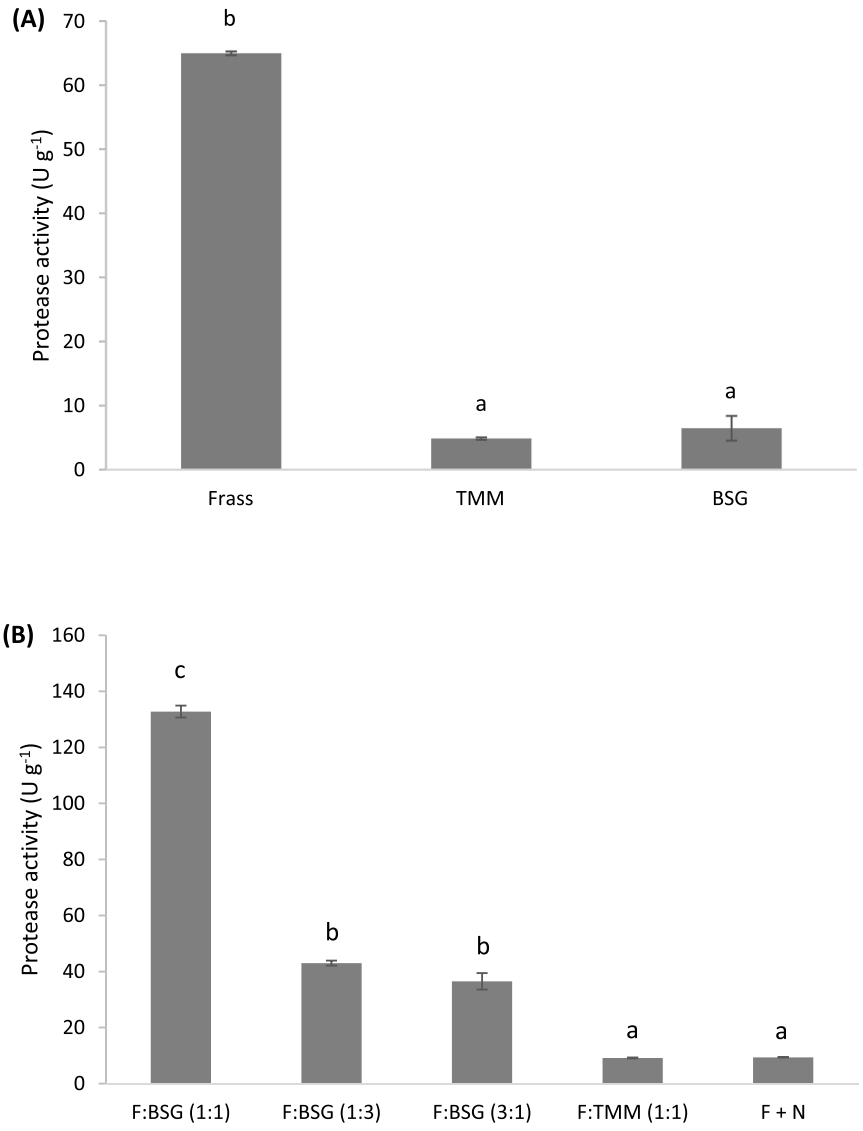

Fig. 3. Proteases production during SSF using: (A) different substrates individually; and (B) a mixture of frass (F) with other by-products (BSG; *T. molitor* adults' meal, TMM) and frass mixed with nutrients (Czapeck medium, F+ N).

composition of frass, this by-product is still underutilized despite having promising utilities. Indeed, research has been carried out to assess the effectiveness of using frass as an alternative fertilizer for plants, to mitigate stress factors in soils, and as an ingredient in fish diets (Yildirim-Aksoy et al., 2020; Chavez, 2021). Nonetheless, using frass to obtain highly functional compounds, such as proteases, seems to be a fully innovative field. To the best of our knowledge, there are no publications regarding the use of *T. molitor* frass as substrate in SSF to produce proteases. Nonetheless, carbon is essential in SSF to promote fungal growth and synthesis of important molecules, such as protein, lipids, nucleic acids, among others (Manan and Webb, 2017). Therefore, the high carbon content of frass positively contributed for the growth of *A. uvarum* MUM 08.01 during SSF and thereof the production of proteases.

3.4. Supplementation of frass substrate with different by-products

Besides frass, *T. molitor* adults' meal and BSG were also individually tested as substrates in SSF with *A. uvarum* MUM 08.01 since this strain showed higher proteases activity in the screening experiments. Higher proteases activity was observed in SSF using frass as single substrate compared to using *T. molitor* or BSG as substrates (Fig. 3A). It would be expected that the high protein content of *T. molitor* adults (47.9% w/w, Table 1) and BSG (up to 18.9% w/w; Leite et al., 2019) would potentiate proteases production. However, high C/N ratios often positively impact enzymes production (Souza et al., 2015) which may explain the higher proteolytic activity of *A. uvarum* MUM 08.01 when frass was used as substrate in the SSF rather than when the adult' insects or BSG were used. One other explaination may be the structure of BSG and the *T. molitor* adults, that is, the composition in complex polysaccharides and/or chitin, respectively, may hamper the access of fungi to the protein fraction, limiting proteases production.

Since frass was the most suitable substrate to enhance proteases production by *A. uvarum* MUM 08.01 and considering the high protein content of BSG and *T. molitor* adults' meal, further experiments were conducted to assess if mixing frass with these substrates could enhance proteases production. Mixing frass with BSG in equal parts (1:1, w/w) increased 52% the proteases activity (up to 133 U g^{-1}) compared to using frass as single substrate (Fig. 3B). Contrarily, increasing either the proportion of frass or BSG in the mixture up to 66% (1:3 or 3:1, w/w) decreased 72% the proteases activity.

The production of microbial proteases is positively impacted by the presence of metal ions and simple sugars (as glucose), high C/N ratios, and also abiotic factors such as aeration, incubation time, pH, among others (Souza et al., 2015). Indeed, carbon and nitrogen contents strongly influence enzymes production during SSF, providing the energy required for microbial growth and stimulating conidia formation, respectively (Sousa et al., 2022). de Castro et al. (2014) observed that a high protein content present in soybean meal (49.2%) favored proteases production in the first 48 h (152.4 U g^{-1}), while high C/N ratio showed a negative impact in the first 24 h of SSF with *A. niger* using wheat bran (13.4 U g^{-1}), soybean meal (20.7 U g^{-1}) or cottonseed meal (16.6 U g^{-1}) as substrates. BSG is a lignocellulosic by-product from the brewery industry which has been used in SSF to obtain value-added compounds, including proteases, containing a protein content between 19% and 30% (w/w; Fărcaș et al., 2021). In fact, contrarily to what was observed in the present study, Castro et al. (2015a) assessed enzymes activity during SSF of babassu cake with *A. awamori* and obtained higher proteases yield when the substrate was mixed with BSG (up to circa 18 U g^{-1} after 120 h), sunflower cake (up to circa 24 U g^{-1}), canola cake (up to 22 U g^{-1}) and castor bean cake (up to circa 21 U g^{-1}) rather than was mixed with corn starch (15 U g^{-1}) or babassu meal (15 U g^{-1}). However, it is also important to point out that the proteases activity decreased when either BSG or frass proportion increased in the substrate mixture. The low enzymatic activity observed in the mixtures of frass:BSG may be due to the low quantity of frass content, which is insufficient to trigger proteases production that will then hydrolyze the BSG (1:3 w/w); or be due to the higher quantity of BSG hampering the proteases production since the fungi is not able to disrupt its complex structure (3:1 w/w), as similarly observed when BSG was used as single substrate. Indeed changes in the substrate can strongly impact enzymatic activities since adding more BSG changes the C/N ratio, affecting the synthesis of other enzymes, such as cellulases or xylanases, and consequently reducing the proteases in the extract (Moran-Aguilar et al., 2021). Other factors may also affect proteases production, such as oxygen dissolution in the medium during SSF that increases along with the porosity of substrate when the particles size is higher. Consequently, high particle sizes decrease fungi anchoring, while microscopical particles difficult oxygen transfer and microbial growth. Therefore, changes in the structure, nutrients access and oxygenation may also have occurred and negatively influenced the proteases activity.

3.5. Optimization of SSF parameters

Moisture and temperature conditions were evaluated using a full factorial experimental design (3^2) to optimize the proteases production during SSF of frass mixed with BSG (1:1, w/w; Table 2). Multivariate response models are utilized to optimize production systems, simultaneously reducing the number of experiments and resources used. Optimization strategies are key procedures to increase production systems profitability which is specifically important in proteases production as well, since medium composition may account up to 40% of total costs in enzymes industry (Siala et al., 2012). Therefore, SSF of low-cost substrates to produce highly functional enzymes is a cost-effective and sustainable approach to increase the productivity of enzymes industry. In the present study, the model showed a good fit for both studied variables with an adjusted R^2 above 0.99, confirming that more than 99% of the variability of proteases activity is explained by the model (Table 3). Both variables significantly impacted proteases activity since *p* values were much lower than 0.05. Furthermore, the response surface analysis evidences that middle conditions of moisture and temperature result in higher proteases activity. The water content during SSF strongly influences the growth and metabolism of the microorganisms (Buenrostro-Figueroa et al., 2014). Indeed, a higher moisture level reduces the porosity of substrate and reduces the oxygen transfer, while a low moisture content reduces the nutrients solubility, decreases the degree of swelling, and increases the water tension (Benabda et al., 2019).

Therefore, a balance between temperature and moisture positively affects the proteases synthesis. The model predicted that 24.7 °C and 61.8% maximized the proteases activity during SSF of frass:BSG mixture (1:1, w/w) with *A. uvarum* (Fig. 4). Indeed, the predicted optimal conditions were tested and it was possible to obtained 152 U g^{-1} of proteases activity, which is in accordance with the theoretical value determined

Table 2
Matrix of experiments of the full factorial experimental design to optimize proteases production during solid-state fermentation of frass with *A. uvarum* MUM 08.01.

Runs	Temperature (°C)	Moisture (%)	Protease activity (U g^{-1})	
			Observed	*Predicted*
1	22	50	106.10	106.10
2	22	60	123.11	123.11
3	22	70	119.63	119.63
4	25	50	110.57	110.00
5	25	60	150.85	150.38
6	25	70	131.44	131.44
7	28	50	100.75	100.00
8	28	60	106.72	106.72
9	28	70	110.09	110.09
10	25	60	151.82	150.38
11	25	60	148.48	150.38

Table 3
Statistical parameters of the full factorial experimental design.

Variables	Regression coefficients	*P-value*
Intercept	150.38	0.00004
Temp. (L)	-8.1952	0.02122
Temp. (Q)	-35.465	0.00190
Humid. (L)	10.719	0.01325
Humid. (Q)	-29.664	0.00283
Temp. (L) x Humid. (L)	-0.86000	0.34644
Temp. (L) x Humid. (Q)	4.2834	0.09496
Temp. (Q) x Humid. (L)	-4.8153	0.08650
Temp. (Q) y Humid. (Q)	23.701	0.00826
Model parameters		
R^2	0.9984	
R^2 adjust	0.9921	

L: linear; Q: quadratic; R^2: determination coefficient; Temp.: temperature; Humid.: humidity

by the model. Temperature and initial moisture content strongly influence microbial enzymes production in SSF. Proteases production was maximum (1550 U g^{-1}) with initial moisture of 65% and temperature of 30 °C during SSF of bread wastes with *R. oryzae*, decreasing with 45% moisture level (Benabda et al., 2019). Optimum conditions for proteases production by *A. oryzae* during SSF were determined using wheat bran supplemented with casein (1% w/w) and glucose (0.5% w/w) as substrate, 55% of moisture, pH of 6, spores concentration of 0.5×10^6 spores mL^{-1}, and incubation at 30 °C for 5 days (Mamo et al., 2020). Likewise, SSF of potato pulp powder with a mutant strain of *A. oryzae* RIB 40 (ATCC 42149) yielded 31 U g^{-1} of protease activity when moisture, temperature and fermentation time conditions were optimized for 50%, 30 °C and 120 h, respectively (Murthy and Kusumoto, 2015).

3.6. Scale-up of SSF

A scale-up of the SSF was also carried out using up to 500 g of substrate (frass mixed with BSG), assessing potential differences in proteases activity according to the utilization of tray or column-type bioreactors. The proteases activity increased when up to 50 g of solid was used compared to using 5 g, while no significant differences were observed between the SSF carried out in tray bioreactors with different solid loads (50, 200, and 500 g of dry solid; Fig. 5). The higher proteases activity observed in the scale-up may be related to the better maintenance of aeration and moisture levels in trays compared to the flasks. In addition, a slight increase in the height of the solid substrate may also improve fungal growth and positively impact enzymes production. Nonetheless, the bed height may have not impacted the proteases production in this study since it was similar (between 2 and 3 cm) in the Erlenmeyer flasks with 5 g of frass and in the trays containing 50 g or 400 g. Furthermore, aeration improves the oxygen transfer and the removal of the carbon dioxide generated during fermentation (Melikoglu et al., 2015), enhancing the microbial growth and metabolism, increasing enzymes' production. However, when high solid loads are used, the heat generated during the SSF can also increase the substrate layer temperature, negatively affecting the microbial growth and the enzymes' production (Castro et al., 2015b). Therefore, an efficient aeration can help dissipate the metabolic heat generated by the microbial metabolism during SSF, promoting the production of enzymes, which may explain the current results.

The effects of SSF scale-up to produce different industrial enzymes has been reported by other authors. Increasing the BSG quantity from 5 g to 50 g in SSF with *A. ibericus* MUM 03.49 increased xylanase and cellulase activities in 25% and 63%, respectively, while increasing the solid load up to 400 g decreased enzymes activity (14% and 79% in xylanase and cellulase activities, respectively; Fernandes et al., 2022). Castro et al. (2015b) observed that SSF with *A. awamori* IOC-3914 using a cylindric fixed-bed bioreactor (1.8 L working volume) with 400 g of babassu cake resulted in 31.8 U g^{-1} of proteases activity, also observing the production of exo-amylases (73.4 U g^{-1}), endo-amylases (55.7 U g^{-1}), xylanases (23.8 U g^{-1}) and cellulases (6.2 U g^{-1}). The production of alkaline proteases during SSF of soy fiber inoculated with a

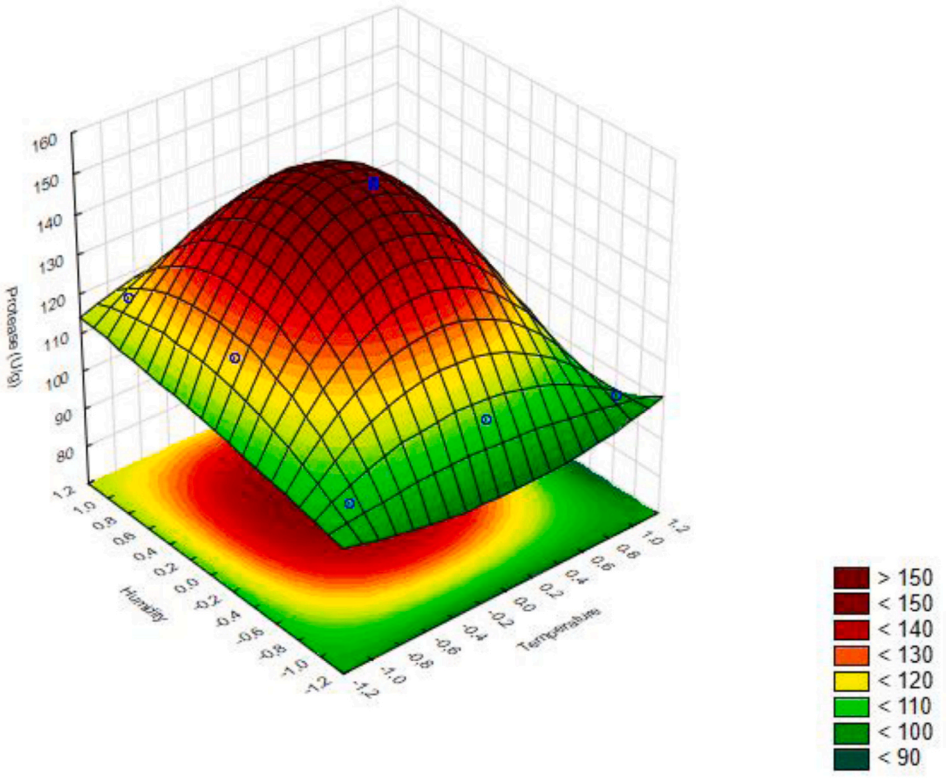

Fig. 4. Surface response for the proteases' activity according to humidity (%) and temperature (°C) conditions.

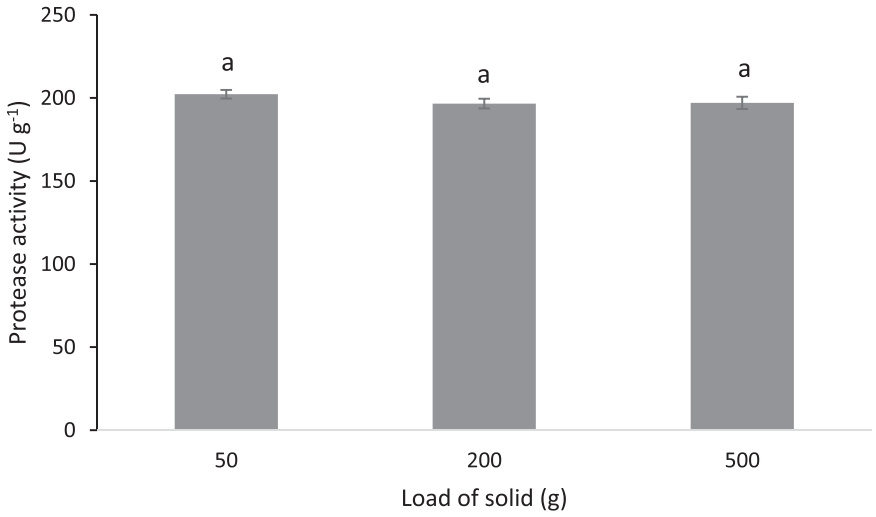

Fig. 5. Proteases production during SSF with different loads of the frass:BSG mixture (50, 200 and 500 g).

plant compost was scaled-up from 100 g up to 1.25 kg (4.5 L airtight reactors) and enzymes activity increased from 45,000 U g^{-1} up to 47, 331 U g^{-1}, respectively (Abraham et al., 2013). A pilot scale SSF was carried out in 10 L and 50 L-capacity bioreactors, recovering 91% and 121% of protease activity when using soy fiber (up to 17.5 kg) or hair and sludge mixture (up to 20 kg), respectively (Marín et al., 2018). A large scale SSF with *Rhizopus oligosporus* using 1.5 kg (wet weight) of *Chenopodium formosanum* sprouts as substrate resulted in 1.6, 1.63 and 1.49 times higher α-amylase, β-glucosidase and proteases activities compared to when 15 g (wet weight) of substrate was used in traditional plate bioreactors (Hsieh et al., 2023).

4. Conclusions

This study shows the innovative utilizations of insects' frass to produce industrial proteases. *A. uvarum* MUM 08.01 showed higher proteolytic activity using frass as substrate. Mixing frass with BSG further increased the proteases activity, foreseeing a successful reutilization of both insects and agro-by-products. Optimal moisture and temperature maximized proteases production and the scale-up allowed to increase the protease activity. These results confirm the hidden value of insects' by-products, constituting a step towards the exploitation of the suitability of frass to produce functional compounds. This study is in line with circular economy, improving the sustainability and cost-effectiveness of insects' and enzymes production.

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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