Universidade do Minho
Escola de Engenharia

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Biotechnological approaches to boost the application of insect meal as a source of feed protein

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Trabalho efetuado sob a orientação da

Professora Doutora Isabel Maria Pires Belo

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DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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Um grande obrigado a todos.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ABORDAGEM BIOTECNOLÓGICA PARA POTENCIAR A UTILIZAÇÃO DE FARINHA DE INSETO COMO FONTE DE PROTEÍNA EM ALIMENTAÇÃO ANIMAL

RESUMO

O crescimento da população mundial resulta no aumento da procura por proteína animal para alimentação humana. A produção de ração animal, depende de fontes de proteína como as de origem vegetal, que levam à redução de terra e recursos hídricos disponíveis. Assim, a procura de ingredientes alternativos é de grande importância. Os insetos são consumidos naturalmente por animais monogástricos, como espécies aquáticas, e a produção em larga escala de insetos para rações (ou alimentos) está a começar a ser explorada, uma vez que os insetos podem crescer em resíduos, sendo uma produção sustentável. No entanto, a incorporação de farinha de insetos (FI) para rações ainda é limitada devido ao seu alto teor em quitina, que é de difícil digestão para os animais. O objetivo principal deste trabalho foi o desenvolvimento de estratégias biotecnológicas para o tratamento de FI de modo a reduzir o seu conteúdo em quitina.

O trabalho envolveu a aplicação de duas abordagens: a primeira baseou-se na produção de quitinase por fermentação em estado sólido (FES) utilizando subprodutos, seguida da utilização das quitinases para tratar as FI; a segunda centrou-se no tratamento direto de FI por SSF. Os resultados obtidos permitiram selecionar *Aspergillus niger* CECT 2088 como o fungo melhor produtor de quitinase entre espécies testadas de *Aspergillus* e *Trichoderma*. A produção de quitinase por FES usando cascas de camarão (CC) foi 12 vezes mais elevada quando CC foi misturada com dreche (D). A produção de quitinase em CC+D foi otimizada em termos de tempo de fermentação e humidade usando um desenho experimental fatorial. A enzima obtida na fermentação foi usada no tratamento enzimático de FI de moscas-soldado-negro (*Hermentia illucens*), verificando-se que 2 h de tratamento foram suficientes para hidrolisar este material nas condições otimizadas de pH e temperatura.

Na segunda abordagem foi utilizado um planeamento centroide simplex para determinar o impacto da mistura de FI com farelo de trigo (FT), observando-se que a mistura de 50% (p/p) de FT com a FI foi a que produziu melhores resultados. O tratamento de FI por FES aumentou 1.2 vezes a proteína, diminuiu o teor de lipídios em 55% e diminuiu 27% a fibra total, demostrando o potencial de FES como estratégia de bio processamento para melhorar as propriedades nutricionais de FI, com vista a potenciar a sua aplicação na alimentação animal, particularmente na aquicultura.

PALAVRAS CHAVE: FARINHA DE INSETO, QUITINASE, FERMENTAÇÃO EM ESTADO SÓLIDO

BIOTECHNOLOGICAL APPROACHES TO BOOST THE APPLICATION OF INSECT MEAL AS A SOURCE OF FEED PROTEIN

ABSTRACT

The growth of human population has greatly increased the demand for animal proteins for human food. The provision of animal feed, depends on protein sources, such as of vegetable origin, that lead to land and water resources occupation. Thus, search for new alternatives of feed protein to animal production is of most importance. Insects are naturally consumed by monogastric and aquatic species, and the large-scale production of insects for feed (or food) is starting to be exploited, since insects can grow in wastes, so having a sustainable production. However, Insect meal (ISM) incorporation in animal feed is still limited due to the high content of chitin that are difficult to digest by some animals.

The main goal of this work was to develop biotechnological strategies of insect meal treatment to reduce the levels of chitin. The work was developed in two approaches: one was based on the production of chitinase by solid-sate fermentation (SSF) using as substrates by-products, followed by the use of chitinases to treat ISM; the other was the direct treatment of ISM by SSF. The results allowed to select *Aspergillus niger* CECT 2088 as the best chitinase producer among the species tested of *Aspergillus* and *Trichoderma*. The production of chitinase by SSF of shrimp shells (SS) was 12-fold improved by the mixture of SS with brewer spent grain (BSG). The production of chitinase in SS+BSG was optimized by a factorial design of experiments in terms of time and moisture. The enzyme obtained was tested in the enzymatic treatment of ISM derived from black soldier flies (*Hermentia illucens*) and it was found that 2 h of treatment led to the maximum hydrolysis under the optimized conditions tested of pH and temperature.

The second approach studied in this work was the feasibility of direct ISM treatment by SSF. A simplex centroid design of experiments was applied to find the impact of mixing ISM (two ISMs were tested differing in the fat content) with wheat bran (WB) and it was found an optimum with a mixture of 50% (w/w) of WB with the ISM not defatted. ISM treatment by SSF led to an increase of protein of 1.2-fold, a decrease of lipids content of 55% and a decrease of total fiber of 27%, showing the great potential of SSF as a bioprocessing strategy to improve nutritional properties of ISM to boost its application in animal feed, particularly in aquafeed.

KEY WORDS: INSECT MEAL, CHITINASE, SOLID-STATE FERMENTATION

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List of Abbreviations

ADF	Acid detergent fiber
ANOVA	Analysis of variance
BSF	Black soldier fly
BSG	Brewers spent grain
CP	Crude protein
DM	Dry matter
DNS	3,5-dinitrosalicylic acid
GHS	Greenhouse gases
ISM	Insect meals
ISM-A	Insect meal A
ISM-B	Insect meal B
LOD	Limit of detection
LSD	Least significant difference
NAG	N-Acetyl-D-glucosamine
NDF	Neutral detergent fiber
P	*p-value*
PDA	Potato extract, dextrose agar medium
Rpm	Rotations per minute
RS	Reducing sugars ($g \cdot L^{-1}$)
SD	Standard deviation
SmF	Submerged fermentation
SS	Shrimp shell
SSF	Solid state fermentation
WB	Wheat bran
w/v	weight/volume
w/w	weight/weight
4-NP-NAG	4-Nitrophenyl N-acetyl-β-D-glucosamine
4-NP	4-Nitrophenyl

1. INTRODUCTION

In this chapter, a general review of current status of food and feed industries, as well their challenges and alternative solutions that may increase these sectors profitability and sustainability. In this sense, a revision on some of the major environmental impacts of traditional feed is introduced. Then a survey of the characteristics, both positive and negative of insects, is presented. Finally, potential biotechnological solutions that may potentially improve insects' nutritional characteristics as a feed source are described.

1.1. Current status of food and feed industries

Agricultural practices have been drastically changing in the last decades, in which increasing mechanization of this sector along with increasing income levels of societies worldwide resulted in higher food demand, while boosting changes in food habits and human consumption patterns. Indeed, this 'nutrition transition' is based on a general substitution of consumed foodstuffs from carbohydrate-rich staples (cereals, roots, tubers) to vegetable oils, sugar-rich foods and animal-based products (Kearney, 2010). For example, it is estimated that meat consumption in Asia will increase 50% until 2030 compared to 2000 consumption levels. Therefore, higher food and feed demand caused by increasing world population along with the stagnation of current production systems and the mentioned income levels increase will led to rising prices and menace food sources sustainability (Msangi and Rosegrant, 2011).

1.1.1. Traditional food and feed sources

The main issues by the feed industry lie with the origins of the feed material utilized. Though human inedible byproducts of vegetable and animal origin are utilized as feeds, such as hay, brewers spent grains, and meat and bone meal, the need for high throughput animals rearing led to a demand for nutritionally richer feed sources (Sapkota et al., 2007), some of which also sharing uses as a human food source, such as soybeans and fishmeal. An overreliance on which can result in environmental and food security treats.

Soybeans are a great source of high-quality protein for humans but is also commonly used for feeds for monogastric animals, as it happens with other crops, such as wheat, corn, and barley (Sapkota et al., 2007). Since soybeans are directly consumed by humans, estimated at only one fifth of 350 million annual tones produced (Ritchie and Roser, 2021), and their cultivation requires existing arable land, the utilization of soybean meal settles a competitive scenario between feed and food production, increasing demand and costs (Dei, 2011; Mottet et al., 2017). Leading to higher food prices and market supply fluctuations. Environmental problems are also linked to soybean cultivation, since may be responsible for deforestation, requires high freshwater quantity, and greenhouse gas emissions (GHG) associated to cultivation parameters (utilization of pesticides and fertilizers; Sánchez-Muros et al., 2016) and from the transportation of industrial amounts of soybean (Herrero et al., 2016; Wiedemann et al., 2016).

Fish is one of the food sources that receive an increase of demand in the last decades, with a 2020 estimate of a 6-fold increase in fish consumption in the last 60 years (FAO, 2022), with aquaculture being responsible for 49% of total worldwide fish production, while also experiencing a 6-fold production

extension in the last 30 years. Traditionally, fishmeal is one of the main ingredients used in feeds for aquaculture fish, and is derived from whole or processed fish leftovers from the seafood industry (Fry et al., 2016). Currently, 12% of all produced fish (captures plus aquaculture) is used to create fishmeal and fish oil. However, the stagnation of wild fish stocks, due to poor management, overfishing, and climatic change, and increasing world population have led to a rise in fish and fish-derived products prices, risking the growth of societies food insecurity (van Huis et al., 2013; Cao et al., 2015), while also endangering aquatic habitats.

Although research is being made to mitigate the impacts of both agriculture and fish production practices and increase the productivity of these sectors (Herrero et al., 2016), alternative ingredients and implementation of sustainable rearing and human consumption practices are being developed towards innovative food and feed production systems. Such alternatives comprise the study of a wide range of potential nutritional sources along with background technologies, for example artificially cultured meat (Bhat and Fayaz, 2011), seaweeds (Fleurence, 1999), fungi (Asgar et al., 2010) and insects.

1.1.2. Insects as alternative food and feed sources

Insects (derived from *insectum*, Latin for "with a notched or divided body") are a highly diverse and widespread class of invertebrates, belonging to the Arthropod phylum and are found in practically all habitats. Insects are constituted by a chitinous exoskeleton, their body in divided in three parts, have three pairs of jointed legs, compound eyes and one pair of antennae (Delong, 1960). Insects are also cold-blooded and reproduce rapidly, originating a large amount of individuals without needing parental care, developing in stages from a larval or nymph form to a mature adult via either complete of incomplete metamorphosis, depending on the species live cycle (van Huis et al., 2013). Insects have also been reported to present suitable amounts of dietary energy and protein, as well as high fatty acid content (Rumpold and Schlüter, 2013) presenting themselves as potential source of animal feed.

Although human consumption of insects (entomophagy) is practiced mainly in Asia, Africa, Mexico and South America, in western cultures they are often disapproved and not accepted by the consumers (Gahukar, 2011; Yen, 2015). The occident repulsion towards insect consumption is linked to historical socioeconomical changes that based societies on utilizing domesticated mammals for work, food, transport and to obtain other products (van Huis et al., 2013). Although attempts are being made to increase entomophagy acceptance among western societies, insects may also be used as ingredient in animals' feeds, since its nutritional quality may help decrease the use of traditional, expensive and less

sustainable feed ingredients commonly used. Indeed, several insect species have already been recognized as potential feed sources, such as black soldier flies (BSF; *Hermetia illucens*), common housefly (*Musca domestica*) larvae, house cricket (*Acheta domesticus*), and yellow mealworms (*Tenebrio molitor*) (Makkar et al., 2014; Oonincx et al., 2015). The increasing interest is associated to several advantages presented by insects compared to the traditional feedstuffs:

(1) Fast growth rates and small space requirements, allowing simpler and space-saving infrastructures compared to traditional livestock production (van Huis et al., 2013);
(2) Efficient feed conversion ratios, requiring less feed to gain 1 kg of weight. Insects may achieve feed convention rates similar or better than those observed for farmed animals, depending of species and diet used for their production (Oonincx et al., 2015). Indeed, Oonincx et al. (2015) observed a the ration in yellow mealworm can vary of 4.9 to 19.1, depending on the diet. Whilst van Huis (2013) presented values of 4.5, 9.1 and 25 for poultry, pork and beef, respectively;
(3) Insects can be reared using organic side streams from several industries, reducing ecological contamination that they may cause and allowing a circular use of materials (van Huis, 2013; Oonincx et al., 2015);
(4) Insects rearing cause low GHGs (CH_4 and N_2O) and ammonia emissions compared to traditional livestock, such as pigs and cows (Oonincx et al., 2011). It is estimated that up to 14.5 % of global anthropogenic GHG emissions are caused by the animal farming, such as CH_4 that is originated from enteric fermentation of ruminants and N_2O from crop fertilizer/waste management (Gerber et al., 2013). Furthermore, up to two thirds of global ammonia emissions are also associated to livestock waste management (Steinfeld et al., 2006).
(5) Insects present low risk of zoonotic infections transmission (van Huis et al., 2013). Nevertheless, research for proper handling of reared insects is ongoing and must be carefully made, especially given insects nature as pathogen vectors.

1.2. Insect meal utilization – advantages and current obstacles

Despite the issues associated with the traditional feed sources utilization and the potential advantages that insects may provide, there are still some challenges that must be addressed and mitigated to allow a cost-effective utilization of this biomass. For example, it is important to assess the presence of any potential antinutritional components in insects' composition as to allow its control or removal in formulated feeds in the production and post production systems. The scale-up of insects

farming must be carefully made to ensure the same developmental conditions and cost benefits as used in small-scale production. The legislation of insects for feed/food production is also important, settling safety rearing practices that will also help in gaining consumers acceptance their utilization in the livestock sector.

1.2.1. Characterization of insect meal

Insects are mainly constituted by protein, lipids and fiber, with concentrations varying between species (Table I). The commonly used insect species generally have a higher crude protein (CP) content than soybean meal (46 ± 2 % dry matter; DM; Dei, 2011), although still lower than fishmeal protein content (75 ± 2 % DM; Heuzé et al., 2015c). Lipids are the second most abundant nutrient of insects, being higher than lipid content of both soybean (4 ± 2 % DM; Dei, 2011) and fishmeal (11 ± 2 % DM; Heuzé et al., 2015c). Low fiber content is present in insects, varying according to their life stage (Finke, 2002). Among the most used feedstuffs, fishmeal is often not associated to any fiber content (Heuzé et al., 2015c) while soybean fiber content levels are similar to those of *Musca domestica* larvae (Dei, 2011).

Table I – Protein, lipids, and fiber contents of different insect species promising for use in animal feeds (% DM).

Insect species	CP	Lipid	Crude fiber	References
Hermetia illucens **(larvae)**	42 ± 1	26 ± 8	7 ± 2	(Makkar et al., 2014) (Adebayo et al., 2021)
Musca domestica **(larvae)**	50 ± 5	19 ± 6	6 ± 2	(Makkar et al., 2014) (Heuzé et al., 2015a)
Tenebrio molitor **(larvae)**	53 ± 4	36 ± 4	8 ± 4	(Makkar et al., 2014) (Rumpold and Schlüter, 2013)
Acheta domesticus **(adult)**	63 ± 6	17 ± 6	19 ± 2	(Makkar et al., 2014) (Rumpold and Schlüter, 2013)

Besides insects high protein and lipids content their nutritional suitability for feed formulation must be assessed in feeding trials, since amino acids and fatty acids profile variations may influence animals growth performance and health status (Rumpold and Schlüter, 2013). Indeed, negative impacts of insect-based diets have been reported in pig, poultry and fish due to unbalanced amino acid profiles (Newton et al., 1977; Bondari & Sheppard, 1987; Nakagaki et al., 1987; Makkar et al., 2014). Nonetheless, these nutritional limitations may be overcome by assessing insects composition before feeds formulation, which is dependent of species, life stage, feeding habits during rearing, and post-harvesting processing conditions (van Huis et al., 2013). Also, insect meal may be used in combination

with other feed ingredients, improving the nutritional quality of feeds while decreasing the impact of the sole use of traditional feedstuffs.

Insect and plants fibers are long-chain sugars polymers, serving as a structural support in exoskeletons and rigidity of plants cellular walls, respectively (Holtzapple, 2003a; Brzezinska et al., 2014). However, plant fiber is manly comprised of cellulose, hemicellulose and lignin (Holtzapple, 2003a), while the main fiber of insets is chitin (Brzezinska et al., 2014). Thus, the dietary incorporation of plant or insect meals have to be specifically considered, since it is expected that animal species will respond differently according to which feedstuff is used.

1.2.2. Chitin

Chitin is a β-(1→4) linked N-Acetyl-D-glucosamine (NAG) polymer and is one of the most abundant fibers in nature, and is typically found in arthropods exoskeletons, fungi cell walls, and in crustaceans outer shells (Brzezinska et al., 2014). The degradation of this fiber results in numerous products, such as chitin and chitosan oligomers, as well as chitin monomers, NAG, and their hydrolysis byproducts, acetic acid and glucosamine (Shahidi et al., 1999).

Chitin may cause a decrease in feed consumption since some animals do not have the capability to break down chitin bounds, including most fish species (Alfiko et al., 2022), with the accumulation of this material causing gut and health problems and impacting nutrients digestibility (Longvah et al., 2011; Kroeckel et al., 2012). However, some benefits may also be observed by chitin inclusion in animal feeds. For example, feeding yellow mealworm (*Tenebrio molitor*) to broiler chicks reduced cecal *E. coli* and *Salmonella* spp. amplifying IgG and IgA levels (Islam and Yang, 2017). Though some advantages of chitinous aquafeeds are have been reported (Ringø et al., 2012), chitin presence in aquafeeds is generally recognized as a constrictive factor for the application of insect meals in aquaculture (Kroeckel et al., 2012; Sánchez-Muros et al., 2014).

Nonetheless, chitin is also a valuable compound from which different substances may be obtained, such as chitin derivatives (Shahidi et al., 1999) and chitinases (Thakur et al., 2022). Chitin and its derivatives may have several applications, such as anti-tumor agents (Park and Kim, 2010), food preservative (Fernández-de Castro et al., 2016) and immunostimulant (Zhang et al., 2014). Chitinases, a class of enzymes capable hydrolyzing chitin, can be obtained using microbial fermentation in chitin-rich wastes, in which chitin may induce microbial chitinases production (Binod et al., 2007; López-Mondéjar et al. 2012). Chitinases may be used as bioinsecticides, antifungal agent, and for biofertilizer production (Singh et al., 2021).

1.2.3. Insect meal production

Insect meal may be obtained in three ways: (1) wild harvesting of insects; (2) insects semi domestication (natural habitat manipulation to increase production); or (3) farming practices (ranging from small rural settings up to factory production; Yen, 2015). Insects farming may contribute for reintegration of organic streams of several industries with negative environmental impact, such as from livestock production (van Huis, 2013) and non-human edible scraps derived from agriculture (Oonincx et al., 2015).

Currently, large-scale rearing of insects aiming commercialization remains limited due to barriers in production, such as lack of guidelines and scaling difficulties, and market barriers gaps of supply-demand. Though many European companies already operate large scale insect farms aiming to increase the economic viability of the insect market by overcoming existing barriers alongside with European Union funded projects (Veldkamp et al., 2022). However, insect farming is still economic and social advantageous at small scales. Indeed, the relatively simple infrastructures needed for small insect rearing operations can be cost-effective for both urban and rural communities, being specially economic advantageous in poorer societies (Wang & Shelomi, 2017).

1.2.4. Legislation

European legislation regarding the feed and food formulation focus on the prevention of diseases transmission , ruling out the utilization of catering waste, manure, or former foods containing meat or fish as feed for farmed animals (Regulation (EC) No 999/2001, 2022), i.e., animals reared aiming the production of food, feed or other products. Since the taxonomic distance from humans and insects would reduce the risk of zoonotic infections, research is ongoing trying to understand if there is a need to keep having these strict regulations also in insects utilization (van Huis et al., 2013). Indeed, recent research allowed some amendments to these restrictions. Regulation No 2001/999 amended by Regulation 2017/893 authorizes the use of seven insect species for aquaculture feeds, including *H. illucens*, *M. domestica*, and *T. Molitor* (Regulation 2017/893/EC, 2017). Later, another amendment authorized the utilization of processed insect proteins in poultry and swine feeds (Regulation 2021/1372, 2021).

1.3. Insect meal processing

Insect processing methods can include physical, chemical, and biological treatments. The great knowledge already being developed in the food industry concerning to numerous ingredients allows that some traditional any processing methodologies may easily be employed in insects (Azagoh et al., 2016; Dossey et al., 2016; Kim et al., 2019). Nonetheless, it is important to maintain protein functionalities and lipids structures, since negative alterations may occur during processing and impact the chemical/physical properties of the final product (Mutungi et al., 2019). However, several studies showed that the diets given to insects during rearing have greater influence on insects digestibility when included in monogastric feeds (Longvah et al., 2011; Poelaert et al., 2017; Traksele et al., 2021; Alfiko et al., 2022).

1.3.1. Physical and chemical processing of insect meal

Insects can be processed and consumed in three forms: the whole insect, in powder or paste forms, or as a protein, fat or fibrous extracts (van Huis et al., 2013). Drying and milling insects into a powder is considered as the easiest application of this biomass. Indeed, it presents numerous advantages: (1) reduced water content increases shelf life, (2) powders can be easily combined with other ingredients (blending insect products with traditional animal feed ingredients), and (3) can be manipulated by most food processing equipment used without needing specific equipment (Dossey et al., 2016).

The removal of insects protein, lipid or chitin may be required to facilitate physical processing of the insect matter (Kim et al., 2019), exploiting insects as a source of these compound for other purposes, such as converting lipids to biofuels (Zheng et al., 2013), or applying protein as a food additive (Vadivelu Amarender, 2019), or modulating insects nutritional profile to meet specific nutritional needs of animals or humans. These alterations can be achieved with established techniques in the food industry, such as lipid recovery via supercritical CO_2 or organic solvents (Kim et al., 2019; Laroche et al., 2019), and chitin or protein removal via acid or alkaline extraction (Percot et al., 2003; Azagoh et al., 2016). However, some of these chemical procedures, such as acid or alkaline extraction, are associated with toxic pollution of water and undesirable changes in extracted material properties (Caligiani et al., 2018; Pojić et al., 2018; Mohan et al., 2020).

1.3.2. Biological processing

Given the negative impacts often associated to chemical treatments, interest in cleaner and eco-friendly extraction methods of protein or chitin is increasing, such as biological-based extraction, such

as enzymatic hydrolysis (De Holanda & Netto, 2006; Caligiani et al., 2018) or microbial fermentations (Sedaghat et al., 2016; Yadav et al., 2019). Biological processing requires the careful selection of microorganism according to their capacity to use the substrates and their enzyme producing capabilities. For example, Jung et al. (2007) applied sequential submerged fermentations using *L. paracasei* KCTC-3074 and *S. marcescens* FS-3 to obtain chitin from crab shell waste, causing the demineralization and deproteinization of this material, respectively.

Biological approaches also improve the functionality of insects protein (Purschke et al., 2018) and may balance their amino acid and fatty acid profiles (Luparelli et al., 2022).

1.4. Solid-state fermentation (SSF)

Microorganisms are used for enzyme production since they grow easily using nutritionally simple substrates on large scale submerged or solid-state fermentations. They also may have varied metabolic pathways that allow the production of a wider diversity of enzymes (Illanes, 2008).

Solid-state fermentation is an biotechnological technique characterized by the coexistence of solid, liquid, and gaseous phases, and occurs in the absence or near absence of free water in a solid and non-soluble organic substrate (Yafetto, 2022). SSF may be used to obtain diverse biologically active metabolites with wide ranges of applications, such as production of organic acids (Barrington et al., 2009), enzymes (Thomas et al., 2013), antibiotics (Ano et al., 2009) and bioethanol (Liu et al., 2015). It may also be used to enhance the nutritional quality of low-valued materials, bioprocessing these substrates into value added products, such as animal feed (Vitina et al., 2007), and biofertilizers (Chen et al., 2011). The substrate acts as a solid support (Couto, 2008) and nutrients source (Zepf and Jin, 2013) for microbial anchoring, growth, and compounds synthesis. The substrate composition is a very important factor that must be assessed since a pretreatment or supplementation of fermentation medium may be necessary for microbial growth, and may results in different impacts for each microorganism. Agro-industrial wastes are organic residues from agricultural production and processing methods that may be disposed of in landfill, destroyed using environmentally harmful means, or can be directly utilized as feed for ruminants given their high fiber content (Graminha et al., 2008). However, these materials characteristics may be ideal for use in SSF, their availability and abundance resulting in low cost and plentiful substrates for this process (Bharathiraja et al., 2017; Manan & Webb, 2017b), their fiber content, linked to a porous structure, are considered ideal for microbial anchoring (Cooray and Chen, 2018), and the residual presence of lipids and protein can provide a rich growth medium for microorganisms (Ravindran et al., 2018). Indeed, various works use these wastes in SSF, producing

enzymes, biofuel and biofertilizers (Dahiya et al., 2005; Panagiotou et al., 2006; Vitina et al., 2007; Aliyu & Bala, 2011; Chen et al., 2011).

Currently submerged fermentation (SmF) remains as the most widespread biotechnological technique for microbial metabolite production (Soccol et al., 2017), however SSF presents itself as a prospective alternative to Smf tanks to several benefits the former holds. SSF differs from SmF regarding microbial development, productivity, and resource requirements. Generally, when compared to SmF, SSF requires less energy and time, achieving higher productivity, products yield, increase enzyme stability, have lower risk of contamination and produces less wastewater volumes, simultaneously using low-cost substrates derived solid wastes from food or agroindustry (Pandey, 2003; Couto and Sanromán, 2006; Manan & Webb, 2017b; Soccol et al., 2017). Several studies compared both fermentation types. For instance, *Verticillium lecanii* was used for β-N-acetyl-hexosaminidase production under SmF and SSF using shrimp waste silage, attaining 1.88 times more productivity using in SSF than SmF (Matsumoto et al., 2004). Suprabha et al. (2008) studied xylanases and cellulases production using *Aspergillus* sp. and *Penicillium* sp. with wheat bran and birch wood xylan as carbon sources, and observed that enzymes production was 26 times higher in SSF than in SmF, while many species were unable to produce cellulases in SmF.

Besides controlling the fermentation conditions, such as temperature, pH, moisture, aeration, and nutrients availability, the choice of the microorganism is very important to achieve the desired product (Pandey, 2003). Generally, the most suited microorganisms for SSF are filamentous fungi, followed by yeasts and some bacteria species (Yafetto, 2022). Fungi may easily penetrate into the solid particles of the substrate (Mienda et al., 2011), and the conditions used in SSF often mimic fungi and yeasts s natural habitats (Yazid et al., 2017) given their generally lower water activity requirements (0.5 to 0.6 aw) opposed to bacteria (0.8 to 0.9 aw; Thomas et al., 2013).

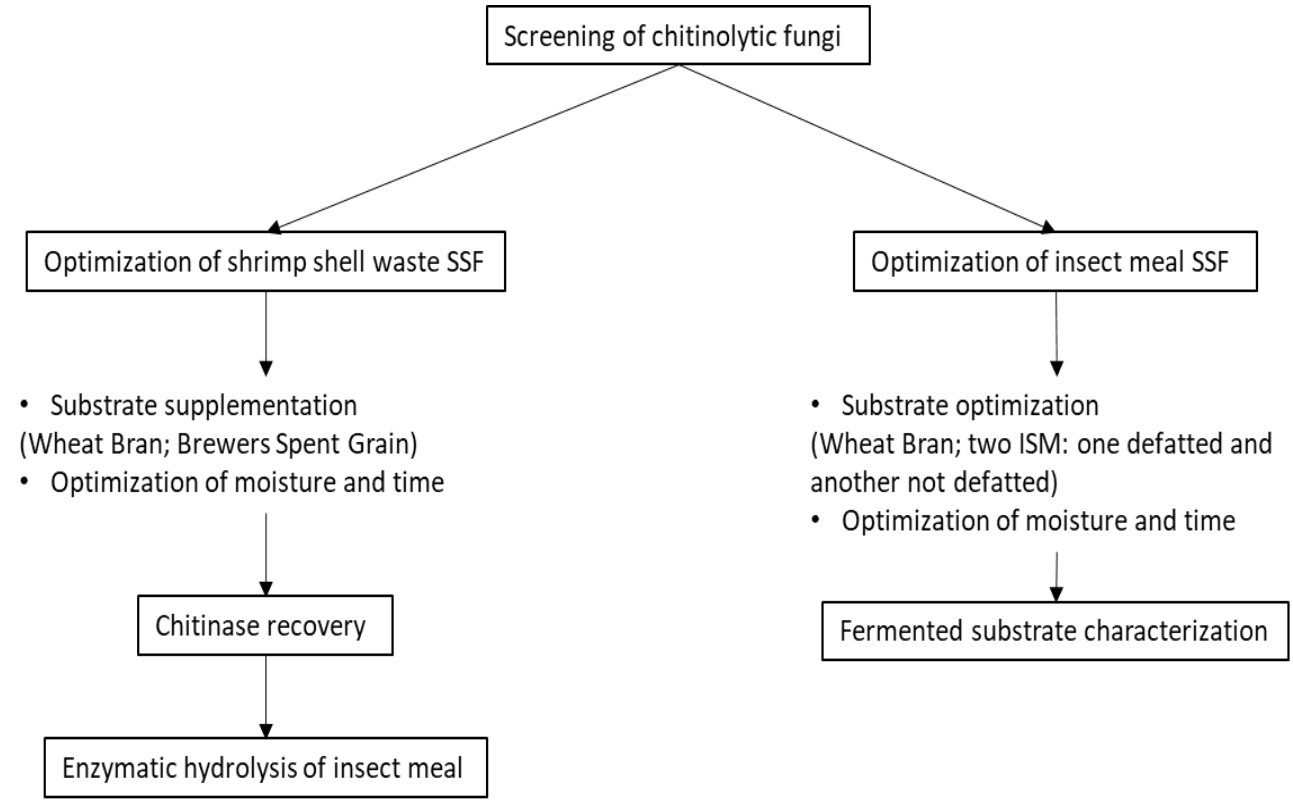
Besides the advantages, some drawbacks are also associated to SSF, such as the uneven distribution of fermentation conditions, challenges in scaling up and difficulty of controlling and monitoring certain process parameters, such as temperature, moisture, dissolved oxygen, and pH (Couto and Sanromán, 2006; Manan and Webb, 2017b). Nonetheless, some measures may be applied to mitigate these constraints, such as use forced aeriation and weight reliant automated moisture control, although their application depends of the reactor type and control scheme for the fermentation process (Mitchell et al., 2000; Farinas, 2015). Therefore, research is ongoing aiming further improvements.

2. GOALS

The main goal of this work is to study possible biotechnological solutions to decrease the chitin level of ISM that hinder the incorporation of this protein source in animal's diet. SSF technology is proposed to be applied for the biomodification of insect meal, via fermentation of this material by chitinolytic microorganisms and enzymatic hydrolyses with recovered chitinase. Therefore, the specific objectives were:

- Screening of chitinolytic fungi in agar plates;
- Optimization of chitinase production by SSF of shrimp shell, followed by enzymatic treatment of ISM with extracted chitinases;
- Optimization of insect meals SSF;
- Recovery and analysis of fermented ISM and products of chitin hydrolysis.

ISM-A



3. MATERIALS AND METHODS

Description of procedures, equipment and operation conditions used in the screening of chitinolytic fungi and in the SSF experiments. Alongside with the analytical methods used to determine chitinase activity and evaluate enzymatic products, as well as to chemically characterize solid substrates used in this work.

3.1. Fungi species and preservation

Aspergillus carbonarius MUM 04.41, *Aspergillus ibericus* MUM 04.86, *Aspergillus niger* CECT 2088, *Trichoderma harzianum* CECT 2413 and *Trichoderma atroviride* CECT 20720 were maintained in Potato Dextrose Agar (PDA) medium (4 g·L^{-1} potato extract, 20 g·L^{-1} dextrose, 15 g·L^{-1} agar) at 4 °C until utilization.

3.2. Screening of chitinolytic fungi

The fungi mentioned in Section 3.1. were evaluated for their chitinolytic capabilities in chitin-rich agar plates. The basal medium ($(NH4)_2SO_4$, 2 g·L^{-1}; K_2HPO_4, 0.7 g·L^{-1}; Na_2HPO_4, 1.1 g·L^{-1}; $MgSO_4.7H_20$, 1.8 g·L^{-1}; $CaCl_2$, 0.02 g·L^{-1}; yeast extract, 0.5 g·L^{-1}; agar, 15 g·L^{-1}) was adapted from Baldoni (2016). Basal media were supplemented with different carbon sources: 10 g·L^{-1} of glucose, grounded shrimp shell or powdered chitin. Each medium was sterilized (121 °C, 15 min) and distributed in 5 Petri dishes. Each Petri dish was inoculated with fungi and incubated at 25 °C for 21 days.

3.3. Chitinase production during SSF

To determine the substrate composition that induces the highest chitinase production, SS or a mixture of SS with WB or BSG (Table II) were studied. Both BSG and SS were supplied by Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), while WB was commercially sourced. SSF was carried out with 5 g of substrate in 250 mL capacity Erlenmeyer flasks, distilled water was added to moist the substrates and allow the adjustment to 75 % (w/w) of moisture after sterilization (121 °C, 15 min) and inoculation with 2 mL of a spore's solution (1 x 10^7 spores·mL^{-1}). SSF was carried out by incubation at 25 °C for 14 days.

Table II – Composition of the substrate or mixture of substrates used in SSF (g, w/w).

Run	**SS**	**WB**	**BSG**
/	1	0	0
//	0.5	0.5	0
///	0.5	0	0.5

3.3.1. Factorial experimental design

A complete 3^2 factorial design was used to assess the optimal conditions within the experimental levels for each variable studied that maximizes the chitinase production during SSF. The variables studied were the moisture (%, w/w wet basis) and fermentation time (days). Each variable was studied at three levels (-1, 0, and +1; Table II) and the design was carried out in one block of 11 runs (Table VI) and two central points were used to estimate the experimental error of the model. The substrate used in these experiments was the mixture of SS & BSG substrate with sterilization and inoculation as previously described.

From the results (response) obtained in each experiment, a multiple non-linear regression analysis was carried out to acquire the coefficients of a quadratic model equation as follows (Eq. 1):

$$Y = \beta 0 + \sum \beta i * Xi + \sum \beta ij * Xi * Xj + \sum \beta ii * Xi^2 \quad \text{(Eq. 1)}$$

, where Y is the predicted response (chitinase activity); Xi and Xj are the independent variables (moisture and time); β0 is a constant; βi is the linear coefficient; βij is the interaction coefficient; and βii is the square coefficient.

Table III – Coded levels used in the complete 3^2 factorial experimental design.

Coded levels	**-1**	**0**	**1**
Moisture, X_1 (%)	70	75	80
Time, X_2 (days)	7	14	21

At the end of each SSF, the recovery of chitinases, and reducing sugars derived from their activity, was performed by the addition of a $NaH_2PO_4 \cdot H_2O$/ Na_2HPO_4 buffer (pH 6, 0.2 M) in a 3 ml :1 g of liquid volume to solid mass ratio. Following a 30 min mechanical agitation (150 rpm) at room temperature, the separation of the supernatant from the majority of the solid material was achieved by a nylon net filtration and centrifugation (7000 rpm at 4 ^oC, 10 min), and a vacuum filtration (paper filter with 10-20 um particle retention). The resulting extracts were stored at -20 ^oC for further analysis.

3.4. SSF using insect meal as substrate

Two types of insect meals and wheat bran were studied as substrates for SSF with *A. niger* CECT 2088 aiming to maximize chitinase production (U·g^{-1} dry substrate). Insect meals are derived from black soldier flies (*Hermentia illucens*), having been donated by CIIMAR, while WB was obtained from commercial sources. One of the insect meals was defatted by previous chemical treatment (insect meal A; ISM-A), while the other has higher fat content (insect meal B; ISM-B), since was not processed by the defatting treatment.

3.4.1. Simplex centroid design

A simplex centroid mixture design was used to optimize the substrate composition using both insect meals and wheat bran, studied separately or in different mixtures. Accordingly, three factors were considered (WB, ISM-A and ISM-B) in the design matrix at four coded levels (0, 0.333, 0.5, and 1; Table IV) and one block of 9 runs was carried out (Table VII). Each coded level indicates the dry mass fraction of each insect meal and wheat bran constituting each substrate mixture. The experiments were carried out in 250 mL Erlenmeyer flasks, using *A. niger* CECT 2088 following the procedure above described for SSF.

The results obtained (chitinase activity per dry mass of substrate) of each experiment carried out in the different conditions were fitted to a multiple non-linear regression analysis to acquire the coefficients of the special cubic model equation (Eq. 2).

$$Y = \sum \beta i * Xi + \sum \beta ij * Xi * Xj + \beta 123 * X1 * X2 * X3 \quad \text{(Eq. 2)}$$

, where Y is the predicted response (chitinase activity); βi is the linear coefficient; βij is the interaction coefficients between two factors and β123 is the interaction among the 3 factors; Xi and Xj are the independent variables, where X1, X2 and X3 are the factors of mass composition of WB, ISM-A and ISM-B, respectively.

Table IV – Coded levels used in the simplex centroid experimental design.

Coded levels	**0**	**0.333**	**0.5**	**1**
WB, X_1 (g)	0	3.33	5	10
ISM-A, X_2 (g)	0	3.33	5	10
ISM-B, X_3 (g	0	3.33	5	10

3.4.2. SSF kinetics and substrate moisture

The effect of time and moisture on chitinase production during SSF of a substrate mixture constituted by 50% (w/w) WB and 50% (w/w) ISM-B with *A. niger* CECT 2088, at 25 °C.

First, different fermentation times (5, 10, 17, and 21 days) were studied.

The second set of fermentation, carried out for 5 days, studied the effect of different moisture levels (70%, 75% and 80%, w/w) on chitinase production.

At the end of each SSF, the recovery of chitinases, and reducing sugars derived from their activity, was performed by the addition of $NaH_2PO_4*H_2O$/ Na_2HPO_4 buffer (pH 6, 0.2 M) in a 3 mL of buffer to 1 g of dry solid. Following a 30-minute mechanical agitation (150 rpm) at room temperature, the separation of the supernatant and the solid material was carried out with a nylon net filtration. The supernatant was then centrifuged (7000 rpm at 4 ^{o}C for 10 min), and vacuum filtrated (paper filter with 10-20 um particle retention). The resulting extract and solid substrates were then stored at -20 ^{o}C.

3.5. Enzymatic treatment of powdered chitin and ISM-B

An enzymatic treatment was carried out in the chitin-rich materials of powdered chitin and ISM-B, using the enzymatic extract produced during SSF of a mixture of SS and BSG. Different levels of pH, temperature and reaction time were tested to determine the optimal conditions that maximize the enzymatic hydrolysis of chitin-rich substrates, which was assessed by quantification of the released reducing sugars.

Values of pH of 4, 5, 6 ,6.5, 7 and 7.5 and temperatures conditions of 32, 37 and 42 °C were applied in the enzymatic treatment. The tested pH values were adjusted using citric acid (0.1 M) and Na_2HPO_4 (0.2M) buffers and were studied for each temperature. Fixed quantities of enzymatic extract (50 %, v/v) and concentrated powdered chitin (1 %, w/v) were used in all experiments. The mixtures were incubated for 2 hours, 200 rpm of agitation.

The kinetics of the hydrolysis of 1% (w/v) powdered chitin was also studied. For that, the hydrolysis was carried out for 3 hours with a sample of the solution taken every 30 minutes. The hydrolysis occurred at adjusted pH of 7.5, at 37 °C, and at 200 rpm of agitation. The kinetics was also studied regarding a potential reduction from the previously applied quantity of enzymatic extract (50 %, v/v).

Three trials were carried out, as described above, testing the enzymatic extract quantities of 30, 20 or 10 % (v/v).

Enzymatic treatments of chitin and ISM-B were tested using 1% (w/v) of solid in a solution of undiluted extract. The mixtures were incubated for 6 hours, at 37 °C, under constant agitation (200 rpm), and samples were taken every 2 hours.

3.6. Analytical methods

3.6.1. Substrates characterization

WB, ISM-B and mixtures were characterized before and after SSF. The substrates were characterized as follows: crude protein by the *Kjedahl* method, after digestion with sulfuric acid (>95 %) using a Kjetelc system (Foss 8400), applying a factor of 6.25 to convert nitrogen into protein; crude lipids, using petroleum ether as solvent in a Soxtec HT System (Tecator, Hoganas, Sweden); neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using a FIWE 6 Fiber Analyser (VELP Scientifica, Usmate, Italy) using the method adapted from (Goering, 1970).

3.6.2. Spores count

To adjust the spores' concentration to $1x10^7$ spores·mL^{-1} for SSF, a solution with peptone (0.1% w/v) and Tween 80 (0.01% w/v) was used to recovery the spores from an agar slant. A Neubauer counting chamber (Paul Marienfeld GmbH & Co, Lauda-Königshofen, Germany) was used in a binocular bright-field microscope.

3.6.3. Reducing Sugars

Free reducing sugars (RS) were determined in the extracts obtained in SSF, and in the samples of the enzymatic treatment, using the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959). Briefly, 100 µL of enzymatic extract was added to 100 µL of DNS and the test tubes immersed at 100 °C for 5 minutes. Distilled water (1 mL) was added to each test tube after cooling in a cold-water bath. The mixture was agitated in a vortex, and 300 µL were transferred into a 96-well microplate. Absorbance was read at 540 nm read using a microtiter plate reader (Multiskan Sky, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Blanks were used as control by replacing the samples with distilled water.

Different concentrations (0 g·L^{-1} – 2.5 g·L^{-1}) of NAG were used as standard. The absorbance was converted to reducing sugars concentration (g·L^{-1}).

3.6.4. NAG determination

The released NAG was estimated by reducing sugars when no other sources of RS was present and also quantified by high-performance liquid chromatography (HPLC Shimadzu LC 2060C) using a Aminex HPX-87H column (300x7.8 mm). The column was eluted with H_2SO_4 5 mM, and the flux rate was settled at f 0.7 mL·min^{-1} at 60 °C.

3.6.5. Acetic acid determination

The released acetic acid was quantified by high-performance liquid chromatography (HPLC Shimadzu LC 2060C) using a Aminex HPX-87H column (300x7.8 mm). The column was eluted with H_2SO_4 5 mM, and the flux rate was settled at f 0.7 mL·min^{-1} at 60 °C. The presence of acetic acid was linked to the degradation of chitin, via the action of chitin deacetylase (Bonin et al., 2021), and NAG deacetylase (Roseman, 1957).

3.6.6. Chitinase quantification

Chitinase activity was quantified using 4-Nitrophenyl N-acetyl-β-D-glucosamine (4-NP-NAG; 1 mM) dissolved in Na_2HPO_4/NaH_2PO_4 0.2 mM buffer (pH 6) as substrate. Briefly, 100 µL of sample was mixed to 100 µL of 4-NP-NAG solution and incubated for 15 minutes at 37 °C. After, 1 mL of NaOH 1M was added to each tube to stop the reaction. The mixture was vortexed and 300 µL were transferred into a 96-well microplate. The absorbance was read at 405 nm and values were adjusted with a blank of the process (samples were replaced by an extract devoid of functioning chitinases).

One unit of chitinase activity was defined as the amount of enzyme required to release 1 µmol of 4-NP, from the hydrolysis of a 4-NP-NAG molecule, per minute under the assay conditions.

3.6.7. Statistical analysis

All results were presented as the mean ± standard deviation (SD). These statistical analyses were carried out by one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) test was used to detect significant differences among means ($p<0.05$). The analyses were carried out using the STATGRAPHICS Centurion software (version 16.2.04).

Likewise, the response data from the Box-Behnken and Simplex centroid design were proceeded by STATGRAPHICS Centurion software in order to construct the intended models and subjected to ANOVA statistical analyses to determine the relevancy of the model and comprising individual variables.

4. RESULTS AND DISCUSSION

In this chapter, results are described and discussed on SSF experiments carried out to determine the ability of different fungi species to produce chitinases and the nutritional changes of substrates after SSF. Factors affecting the chitinase production were studied, such as substrate composition, moisture and time of fermentation. *A. niger* CECT 2088 was the best chitinolytic activity producer, leading to 2.82 $U \cdot mL^{-1}$ in the optimized substrate mixture. These enzymes were then successfully applied in an enzymatic treatment of chitin-rich material, such as ISM using powder chitin as comparison. SSF by *A. niger* CECT 2088 also improved the nutritional composition of ISM under optimized conditions, reducing fiber content and increasing protein/lipid ratio.

4.1. Screening of chitinolytic fungi

Different fungi species were inoculated in agar medium containing glucose (control), powdered chitin or SS (Table V) and the fungal growth was evaluated.

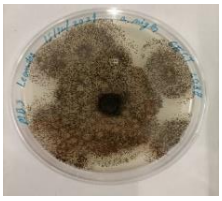
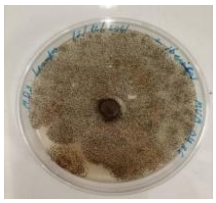
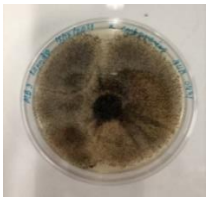
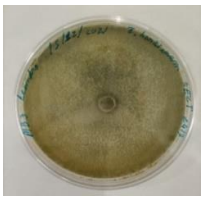
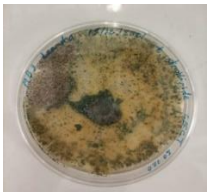
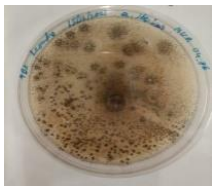
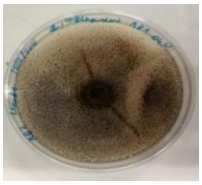
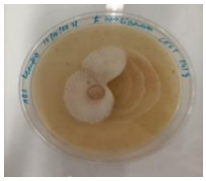
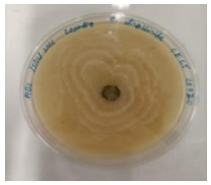
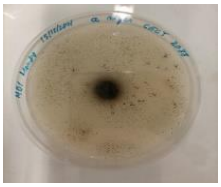
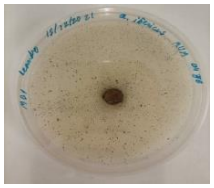
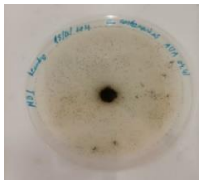
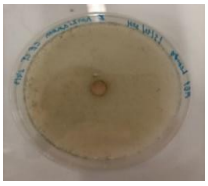
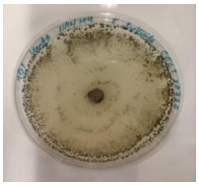
All fungi species grew both in glucose or, to an extent, in a chitin-rich media. Among fungi studied, *A. carbonarius* MUM 04.41, *A. niger* CECT 2088 and *A. ibericus* MUM 04.86 grew at highest extent in shrimp shell medium, indicating that these species have the capability to produce chitinolytic enzymes and use this substrate. Nonetheless, when these three species were inoculated in chitin powder agar, a noticeable growth reduction was observed. Rattanakit et al. (2002) reported the chitinolytic potential of *Aspergillus* sp. S 1 – 13 via a successful growth in agar plates containing shellfish waste (0.5% w/v). Xia et al. (2009) reported the chitinolytic capabilities of *A. fumigatus* CS-01 in an agar plate containing colloidal chitin (1% w/v) via successful growth and formation of a clear zone. As it was observed in the present work, along with other authors findings on the chitinolytic properties of *Aspergillus* spp. (Brzezinska and Jankiewicz, 2012; Rattanakit et al., 2002; Xia et al., 2009), these fungi were deemed to be able of utilizing chitin-rich substrates to grow.

T. harzianum CECT 2413 was not able to grow in the powdered chitin medium, with limited development in the SS agar plate. López-Mondéjar et al. (2012) studied the influence of chitosan, SS and mushroom wastes as carbon sources in SSF of *T. harzianum* CECT 20714, observing its ability to grow and produce chitinases in mushroom wastes medium, while the presence of chitosan and SS did not benefit chitinases production. One explanation presented was that chitosan, a material present in SS wastes and derived from chitin hydrolysis, holds antifungal characteristics that inhibit fungi growth (Bautista-Baños et al., 2006). Therefore, *T. harzianum* CECT 2413 was not utilized in the next experiments of the present study.

Similarly, *T. atroviride* CECT 20720 was inhibited in the SS medium, with a weak growth in the powdered chitin medium. López-Mondéjar et al. (2009) reported the chitinolytic capabilities of *T. atroviride*, observing mycelium growth in agar plate supplemented with colloidal chitin (1% w/v). As observed in this study, along with the findings of López-Mondéjar et al. (2009), *T. atroviride* was capable to use chitinous substrates to grow. However, due to the limited growth in this study, when compared to *Aspergillus* spp. Thus, *T. atroviride* CECT 20720 was not further utilized in subsequent experiments.

Since the different *Aspergillus* species were able to grow in the chitin rich SS agar media, chitinase production was evaluated in SSF experiments using each *Aspergillus* species.

Table V – Growth evaluation of chitinolytic fungi in agar plates.

	A. niger **CECT 2088**	***A.ibericus*** **MUM 04.86**	***A. carbonarius*** **MUM 04.41**	***T. harzianum*** **CECT 2413**	***T. atroviride*** **CECT 20720**
Glucose					
Shrimp shell					
Chitin powder					

4.2. Chitinase production during SSF

4.2.1. Substrate composition during SSF

SSF was carried out to determine with the optimal substrate composition that maximizes chitinase production. For that, *A. niger* CECT 2088 was used in SSF with SS or a mixture of SS with WB or BSG (Figure 1). SS was used as substrate for the optimization of SSF conditions to maximize chitinase production since it is an abundant and accessible material (FAO, 2022), and presents a high chitin content (Wang and Chang, 1997), thus acting as an inducer of microbial chitinase production (Binod et al., 2007; López-Mondéjar et al. 2012). The agro-industrial wastes used in SSF, namely WB and BSG, were chosen since they are low cost and highly accessible (Bharathiraja et al., 2017), and possess a number of positive features for use in a SSF carried out by fungi, such as complex nutritional profiles, rich in nitrogen and polysaccharides, (Fernandes et al., 2021; Javed et al., 2012), and a fiber rich content (Cooray and Chen, 2018).

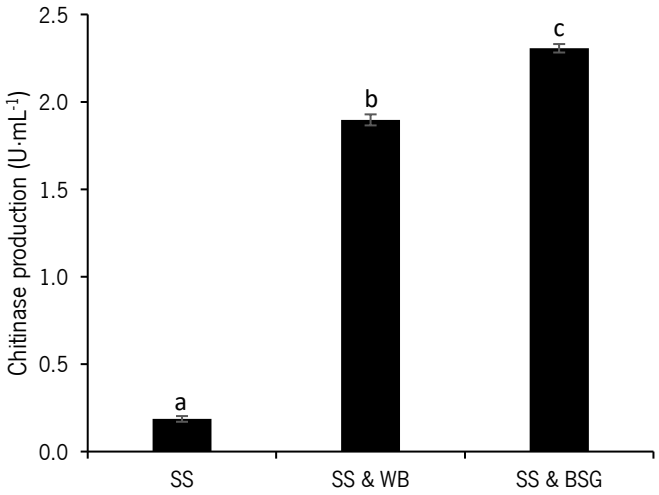


Figure 1 – Chitinase production during SSF with A. niger CECT 2088 using SS or SS mixed with BSG or WB. The results are the average of two independent replicates and error bars represent SD. Bars with the same letter are not significantly different ($p \leq 0.05$).

In this work, *A. niger* CECT 2088 produced chitinases during SSF using SS as substrate, and the addition of WB or BSG significantly increased chitinase production (Figure 1). The highest chitinase production was observed in SSF of SS and BSG mixture (8.1 ± 0.5 $U \cdot g^{-1}$).

Furthermore, in this study, mixing BSG to SS increased 12-fold the chitinase production in comparison to the use of single SS as substrate. Therefore, this result indicates that the nutritional value of BSG, such as the considerable protein content and free reducing sugars (Fernandes et al., 2021), as well as a nitrogen rich content and a fibrous structure (Cooray and Chen, 2018) benefited fungi growth and the utilization of SS, inducing chitinase production. Rattanakit et al. (2002) assessed chitinase production during SSF of SS with *Aspergillus* sp. S1-13 and observed that when the substrate was supplemented with 0.1% (w/v) of ammonium sulfate, chitinase production increased 1.4-fold.

The chitinase production by the fungi may be also related with the free sugars available in the substrates for fungal growth. As can be seen from Figure 2, the reducing sugars concentration in extracts of the controls (without inoculation) were higher in both mixtures than in single SS. This allowed better growth of fungi and consequently increased chitinases production in SSF with mixtures than in single SS. In fact, BSG and WB are described as having high polysaccharide content (Javed et al., 2012; Fernandes et al., 2021).

Reducing sugars quantification may provide valuable information regarding chitin hydrolysis and the fungi ability to grow in SSF substrates, since reducing sugars may be released during SSF by the action

of different enzymes, including chitinase, or physical transformations, but may also be consumed by the fungi (Wu et al., 2013). Indeed, the larger decrease of reducing sugars in the experiments with substrates mixtures, compared to single SS, are an indication of the use of those sugars by the fungi. Final reducing sugars concentration are in fact the balance between sugars released from polysaccharides hydrolysis and the consumed by the fungi during SSF.

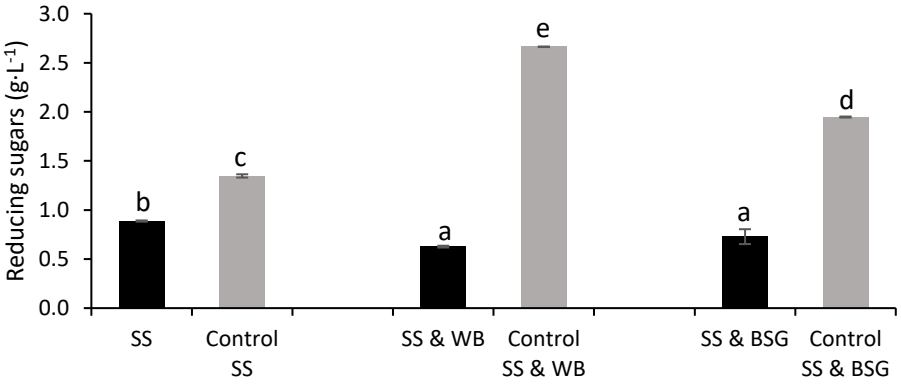


Figure 2 – Reducing sugars released during SSF (black) of SS or a mixture of SS with WB or BSG, and respective controls (grey). The results are the average of two independent replicates and error bars represent SD. Bars with the same letter are not significantly different ($p \leq 0.05$).

Based on above results, the mixture of SS and BSG was chosen as substrate for the next experiments focusing on the optimization of chitinase production by SSF.

A. ibericus MUM 04.86 and *A. carbonarius* MUM 04.41 were also used in SSF of SS mixed with BSG. However, no chitinase activity was detected in extracts carried out in the fermented solids. Therefore, *A. niger* CECT 2088 was selected for the experiments focusing on maximization of chitinase production.

4.2.2. Factorial experimental design of SSF with SS & BSG

The influence of different moisture contents and time of fermentation were studied in a complete 3^2 factorial design to optimize chitinase production by SSF of SS and BSG with *A. niger* CECT 2088 (Table VI).

Table VI – Chitinase activity ($U \cdot mL^{-1}$) obtained in each run of the complete 3^2 factorial experimental design.

Run	**Independent variables**		**Response variable**	
	Moisture (X_1)	Time (X_2)	Chitinase activity ($U \cdot mL^{-1}$)	Reducing sugars ($g \cdot L^{-1}$)
1	-1	-1	-	3.03
2	-1	0	1.9	1.02
3	-1	1	2.7	0.93
4	0	-1	-	3.45
5	0	0	2.3	1.02
6	0	1	2.6	0.94
7	1	-1	1.7	1.58
8	1	0	2.1	0.93
9	1	1	2.5	0.88
10	0	0	2.3	0.69
11	0	0	2.2	0.80

The chitinase activity obtained in the experimental domain was highest in SSF occurring during 21 days, the highest level tested for time, and for the lowest level of moisture, ie, 70%, while at the lowest level of time no chitinase activity was detected in SSF with 70% and 75% of moisture.

The quadratic model equation (Eq. 3) obtained from the multiple regression analysis of the data fitting to the model, accurately represented 92.9% of the experimental variance of chitinase production, according to the analysis of the determination coefficient (R^2).

$$\text{Chitinase production } (U \cdot mL^{-1}) = 2.121 + 0.283*X_1 + 1.017*X_2 + 0.097*X_1^2 - 0.475*X_1*X_2 - 0.603*X_2^2 \quad \text{(Eq. 3)}$$

The low enzymatic activity observed at lowest time of fermentation are in accordance with the highest quantity of reducing sugars obtained (Table VI), indicating, as previously discussed, the lowest fungal activity.

The significance level of each studied variable is represented in the Pareto chart (Figure 3). Time of fermentation was a key factor in chitinase production since longer fermentation periods increase

chitinase activity. The interaction between time and moisture was slightly significant and indicates that those factors have antagonistic effects on chitinase production by SSF.

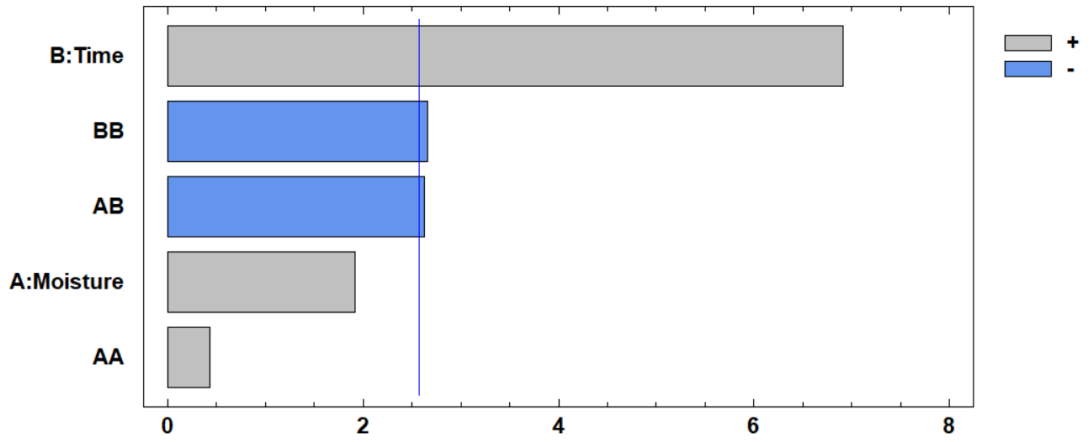


Figure 3 – Pareto diagram of the normalized effects of moisture and time on chitinase production during SSF of SS & BSG with A. niger CETC 2088.

The response surface curve (Figure 4) indicates that, within the tested values for each variable, increasing fermentation time and decreasing moisture contents enhance chitinase production, resulting in the highest predicted values. Longer fermentation times (21 days) combined with higher moisture content (80%) results in low chitinase production. Nampoothiri et al. (2004) studied the effects of moisture level and fermentation time in chitinase production during SSF of WB supplemented by colloidal chitin 1% (w/w) with *T. harzianum* TUBF 781, and observed that maximum enzyme production (3.18 $U \cdot g^{-1}$) was obtained with 65.7 % moisture and when SSF was carried out for 96 h, decreasing the enzyme production when the moisture and time were higher than the optimal level due to lower oxygen transfer among substrate particles, and a reduction in nutrient availability.

Although the shape of the surface response curve did not allow the determination of an optimal level of moisture and fermentation time within the studied ranges for each variable an optimization analysis indicate that maximum chitinase production (2.82 $U \cdot mL^{-1}$) is achieved when SSF occurs during 21 days with a moisture content of 70%.

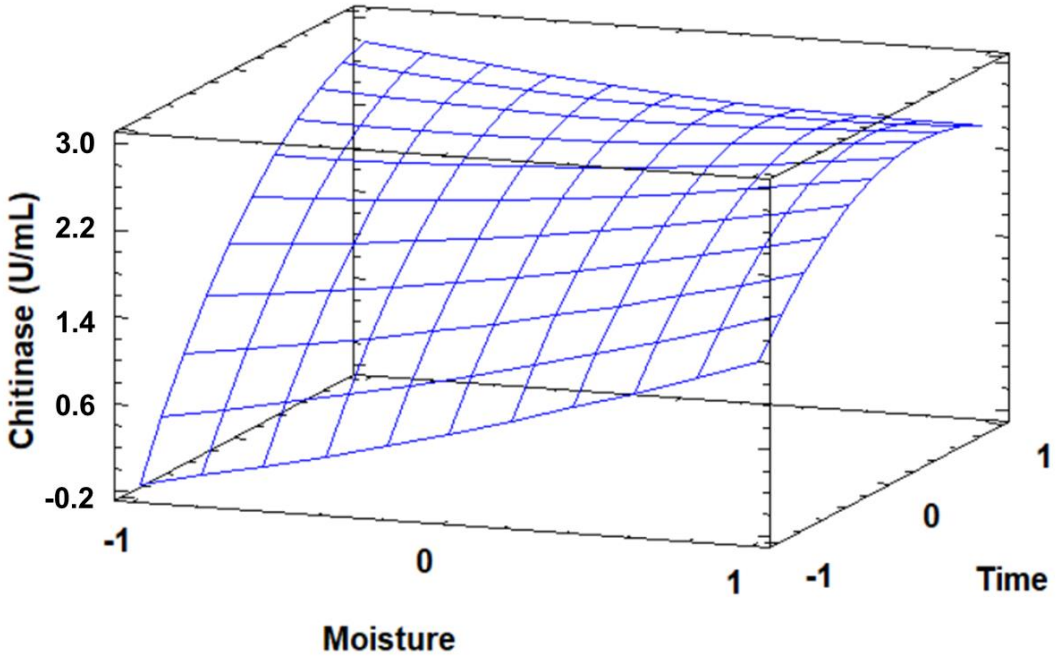


Figure 4 – Response surface for chitinase production obtained from the different levels of moisture and time of SSF assessed in the experimental design.

4.3. SSF using insect meal as substrate

4.3.1. Simplex centroid design

SSF was applied aiming to study the potential of using direct microorganisms to reduce chitin content of insect meal. For that, SSF optimization was studied to maximize chitinase production using two types of black soldier flies' meals (one defatted and other non-defatted) and supplementation of these substrates with wheat bran. WB was used in these SSF experiments since this is an usual ingredient in feed, contrarily to BSG. Moreover, insect meal composition before and after SSF was evaluated.

Chitinase activity was detected after all experiments carried out with *A. niger* CECT 2088 in WB, each insect meal and the mixtures of WB and the insect meals (Table VII), with the highest levels occurring in the WB and ISM-B fermented mixture, while the lowest was observed in the fermented wheat bran.

Table VII – Chitinase activity ($U \cdot g^{-1}$) obtained in each run of the complete simplex centroid experimental design.

Run	**Independent variables**			**Response variable**
	WB (X_1)	ISM-A (X_2)	ISM-B (X_3)	Chitinase activity ($U \cdot g^{-1}$)
1	1	0	0	8.7
2	0	1	0	11.9
3	0	0	1	10.0
4	0.5	0.5	0	12.7
5	0.5	0	0.5	13.7
6	0	0.5	0.5	13.2
7	0.333	0.333	0.333	10.0
8	0.333	0.333	0.333	11.4
9	0.333	0.333	0.333	9.3

The cubic model equation (Eq. 4) obtained from the multiple regression analysis of the experimental values for chitinase activity, accurately represents 91.1% of chitinase production variability according to analysis of the coefficient of determination (R^2).

$$\text{Chitinase production } (U \cdot g^{-1}) = 8.7*X_1 + 11.9*X_2 + 10.0*X_3 + 9.6*X_1*X_2 + 17.4*X_1*X_3 + 9.0*X_2*X_3 - 107.1*X_1*X_2*X_3 \quad \text{(Eq. 4)}$$

The model predicted that the interaction between WB and ISM-B (X_1*X_3; $X_1*X_2*X_3$) significantly maximize chitinase production ($p<0.1$). Whereas the model predicted that the interaction between WB with ISM-A (X_1*X_2) and between both ISM (X_2*X_3) did not significantly influence chitinase production during SSF ($p>0.2$).

The chitinase production according to the substrate or different proportions of substrates mixture used in SSF is represented in a surface response analysis (Figure 5). The optimum mixture predicted by the model to maximize the chitinase production is constituted by nearly 50% of WB and 50% ISM-B. The optimization analysis estimated that maximum chitinase activity (13.7 $U \cdot g^{-1}$) would be achieved in a SSF using a substrate mixture composed by 46% WB and 54% ISM-B, which is in accordance with the surface response estimation.

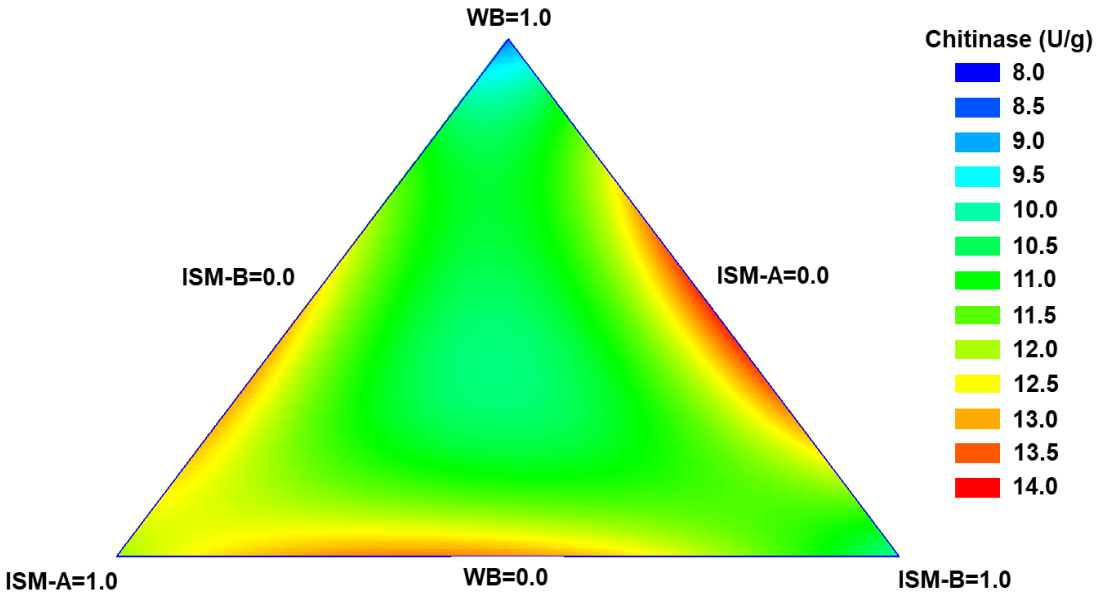


Figure 5 – Surface response analysis of chitinase production for the SSF experiments of the simplex centroid design. SSF was performed with A. niger CECT 2088 for 14 days with 75% of moisture content.

Furthermore, all SSF carried out during the simplex centroid design resulted in lower quantities of reducing sugars released in comparison to each control experiment (Figure 6), indicating, as previously discussed, a facilitated fungal growth and thus a generally high enzyme activity.

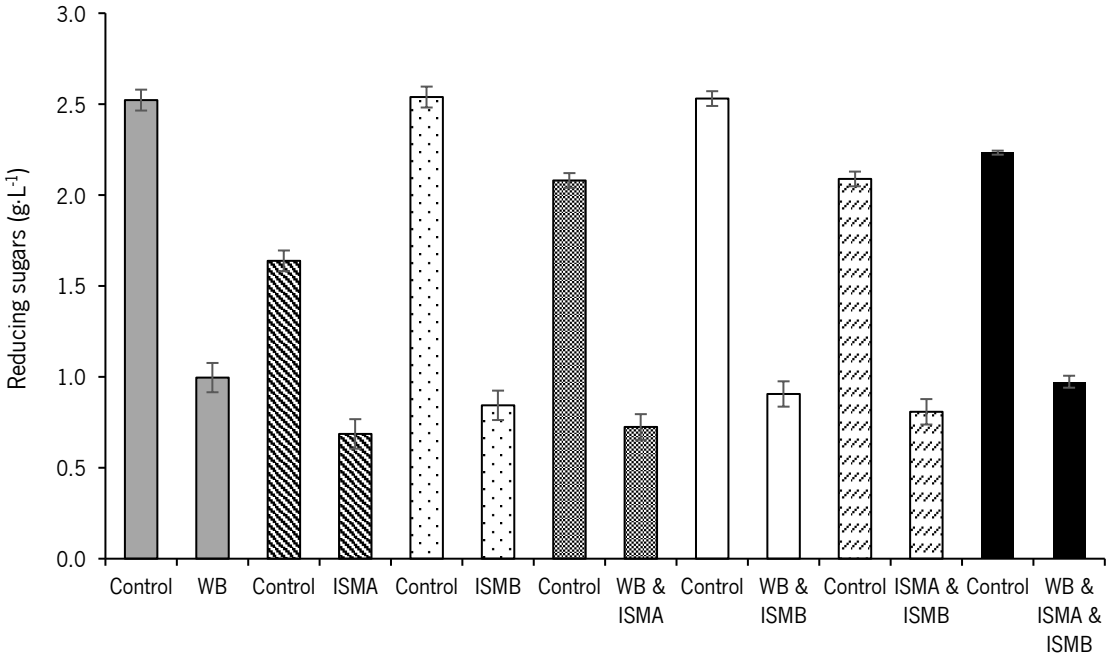


Figure 6 – Reducing sugars released during optimization experiments for maximum chitinase production using a simplex centroid design in SSF of wheat bran and the insect meals.

The optimal substrate mixtures of WB, ISM-B and ISM-A predicted by the model were tested. Chitinase production was highest (13.7 $U \cdot g^{-1}$) in SSF of 50% (%, w/w) WB and 50% ISM-B, while the for mixtures with ISM-A the highest value was obtained with of 33% WB to ISM-A resulted in 13.1 $U \cdot g^{-1}$ chitinase activity (Figure 7). The results obtained with 33% WB and 50% WB are not statistically different ($p<0.05$) indicating that the amount of WB can be reduced without significant loss of chitinase activity produced.

Nevertheless, and based on these results, a mixture of 50 % (w/w) WB and 50 % (w/w) ISM-B was selected as substrate for the following SSF optimization studies.

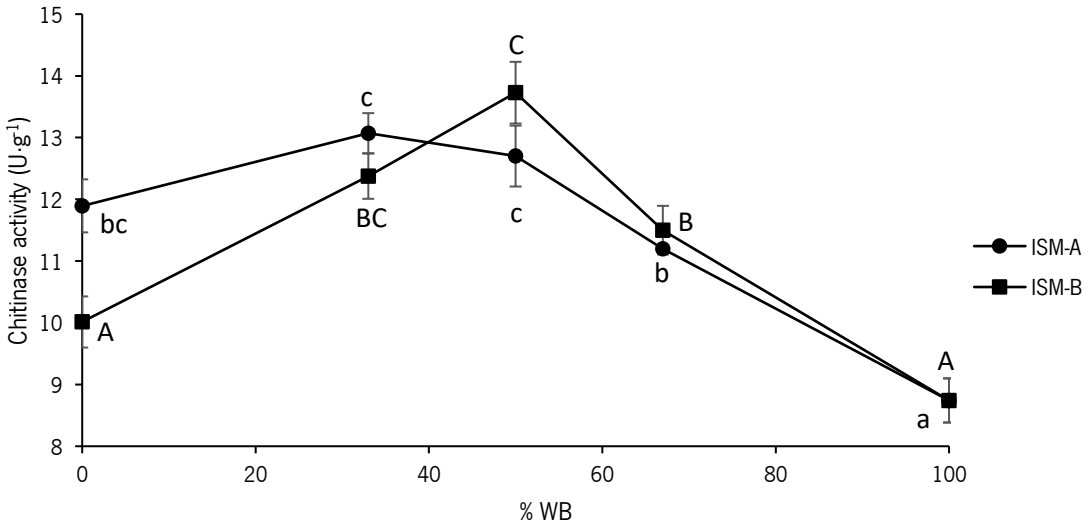


Figure 7 – Chitinase production in SSF experiments using substrate mixtures of WB, ISM-A and ISM-B (%, w/w). Results are the average of two independent replicates and error bars represent the SD. Values sharing letters for each ISM are not significantly different ($p \leq 0.05$).

4.3.2. SSF kinetics and substrate moisture

From the results obtained in the simplex centroid design, additional SSF experiments were conducted to determine the effect of fermentation time (5, 10, 17, and 21 days) and substrate moisture (70%, 75% and 80%) on chitinase production using a substrate mixture composed by 50% (w/w) WB and 50% (w/w) ISM-B.

Chitinase production was not affected by the fermentation time and ranged between 12.7 and 13.7 $U \cdot g^{-1}$ (Table VIII). Reducing sugar levels decreased until the 10th day of SSF, remaining practically stable

until 21 days of fermentation (Table VIII), indicating, as previously discussed, a facilitated fungal growth, and consequently high enzyme production, which remained generally constant.

The stabilization of chitinase production indicated that the composition of the SSF substrate allowed a stable fungal growth, and thus enzyme production, for long time periods. Indeed, Nampoothiri et al. (2004) and Patil et al. (2013) reported the connection of fermentation time and chitinase production in wheat bran substrates by fungi, relating a decrease in production after 4 days due to a decrease in nutrient resources availability hindering steady growth.

Table VIII – Chitinase production at the end of each fermentation period. Data is present as average ± SD of two independent replicates. Values with the same letter are not significantly different ($p \leq 0.05$).

Time (days)	Chitinase production ($U \cdot g^{-1}$)	Reducing sugars ($g \cdot L^{-1}$)
5	12.7 ± 0.4 ^{a}	1.35 ± 0.04 ^{c}
10	12.9 ± 1.1 ^{a}	1.03 ± 0.01 ^{ab}
14	13.3 ± 0.2 ^{a}	0.91 ± 0.01 ^{a}
17	12.9 ± 0.3 ^{a}	1.16 ± 0.06 ^{bc}
21	13.7 ± 0.7 ^{a}	1.01 ± 0.00 ^{ab}

Regarding the moisture of SSF, chitinase activity was highest when SSF was conducted with 75% and 80 % moisture for 5 days (Table IX). These results were in disagreement with works by Binod et al. 2007, Patil et al., 2013, and Nampoothiri et al. 2004 having reported optimal moisture of 65% to 70% for colloidal chitin supplemented wheat bran substrates in SSF by fungi. Also, in the experiments with SS and BSG it was shown that for long time SSF the reduction of moisture content improves chitinase production, while for short time SSF best results are obtained with the moisture increase.

Released reducing sugars in SSF with moisture of 80% were higher than those in SSF with lower moisture levels (70 and 75%), that can indicate a higher chitin hydrolysis at the highest moisture than in the other conditions. Nonetheless, reducing sugars quantity was still higher in the control experiment than in the SSF experiments, that can be due to the up-take by the fungi of the initial free sugars and of the sugars liberated trough SSF (Table IX).

Therefore, the results indicate that the SSF of a mixture of 50% WB and 50% IMS-B during 5 days with a moisture of 80 % (w/w) are the best conditions, among the SSF conditions tested in this work, to maximize chitinase production in substrates with ISM. In the experiments with SS and BSG the maximal chitinase production (8.74 $U \cdot g^{-1}$) was obtained during an optimal 21 day fermentation with a 70% moisture, illustrating the claim by Soccol et al. (2017) of how microbial species can respond in very distinct ways to different substrates.

Table IX – Chitinase production during SSF with different substrate moisture. Data is present as average ± SD of two independent replicates. Values with the same letter are not significantly different ($p \leq 0.05$).

Moisture (%)	Chitinase production ($U \cdot g^{-1}$)	Reducing sugars ($g \cdot L^{-1}$)
70	10.2 ± 0.1 [a]	1.9 ± 0.1 [a]
75	13.3 ± 0.2 [b]	1.1 ± 0.0 [a]
80	13.9 ± 0.1 [b]	2.9 ± 0.5 [b]

4.3.3. Wheat bran and insect meal characterization

Crude protein, lipids, and fiber composition of ISM-B (*Hermetia illucens*) and WB are depicted in (Table X). The results obtained in the present work are in accordance to other authors findings.

Table X – Crude protein (CP), lipid, ADF, and NDF contents of wheat bran and insect meal (Hermetia illucens) observed in the present study and reported in other works (% dry mass).

	ISM-B (*Hermetia illucens*)			**Wheat bran**		
	Observed	**Reported**	**References**	**Observed**	**Reported**	**References**
CP	41.1 ± 0.9	[41; 59]	(Makkar et al., 2014) (Marono et al., 2015)	15.7 ± 0.0	[14;21]	(Heuzé et al., 2015b)
Lipids	26.0 ± 2.0	[11; 35]	(Makkar et al., 2014) (Marono et al., 2015)	3.6 ± 0.2	[2; 6]	(Hell et al., 2014) (Heuzé et al., 2015b)
ADF	10.2 ± 0.9	[5; 29]	(Marono et al., 2015) (Nafisah et al., 2019)	14.3 ± 0.1	[8;18]	(Heuzé et al., 2015b)
NDF	23.0 ± 1.0	[6; 34]	(Marono et al., 2015)	46.0 ± 1.0	[32;57]	(Heuzé et al., 2015b)

Nutritional composition of WB, ISM-B, and mixture of WB & ISM-B was evaluated before and after SSF with *A. niger* CECT 2088, also assessing the effect of enzymes extraction.

The identical CP levels between the extracted and non-extracted controls (Figure 8) were associated to the generally low solubility of the protein content of WB (Idris et al., 2003) and *H. illucens* (Bußler et al., 2016). Protein content of fermented mixture without extraction increased 5.6% (1.19-fold) relatively to the respective control, while the opposite occurred when the fermented mixture was extracted,

decreasing protein content compared to the control, indicating that a solubilization of protein occurred during the SSF, thus increasing protein availability in the treated mixture. Silveira and Badiale-Furlong (2009) and Joseph et al. (2008) observed a protein content increase in wheat bran after SSF (for 72 h with *Rhizopus* sp. and 8 days with *A. niger* S_i4, respectively), that was mainly attributed to the fungal growth and mycoprotein production and also to the synthesis of various enzymes, which are proteinaceous in nature. Silveira and Badiale-Furlong (2009) also noted a clear impact in the protein content solubility, increasing by 11% in that same time frame. Nafisah et al. (2019) and Luparelli et al. (2022) reported that the solid state fermentation of BSF by lactic acid bacteria does not influence the crude protein levels in the insect material, however Luparelli et al. (2022) and Saadoun et al. (2020) noticed that significant redistribution of amino acids profile took place.

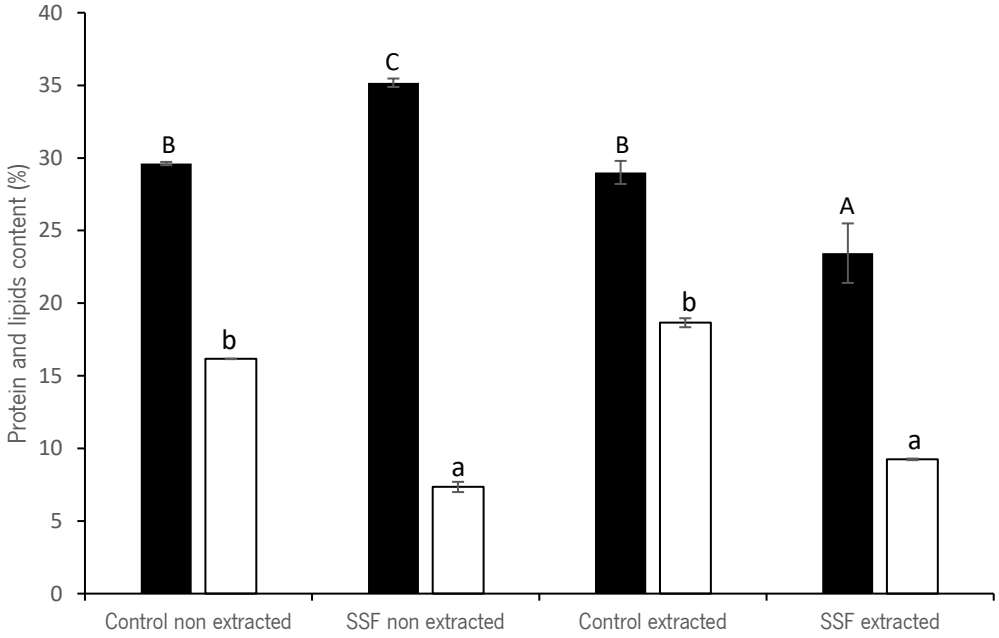


Figure 8 – Protein (black) and lipid (white) content of WB, ISM-B, and mixture of WB & ISM-B before and after SSF with or without enzymes extraction. Data are present as average and standard deviation of two independent replicates. Bars with the same letter are not significantly different ($p \leq 0.05$).

A significant reduction in lipid content was obtained after SSF either with or without extraction displaying fungal consumption of lipids (Figure 8), since the extraction was performed with an aqueous buffer solution. This, indicates that SSF may be a biotechnological approach of fat-ISM treatment, preventing the need of defatting process using organic solvents. This feature was also observed by Oliveira et al. (2011) in SSF of rice bran by *R. oryzae* CCT 7560. Similarly, Saadoun et al. (2020) linked

the reduction of BSF substates lipid content after SSF by *Lacticaseibacillus* sp. to microbial consumption, whilst also inducing a significant redistribution of fatty acid profiles.

ADF content allows an estimate of cellulose and lignin content in animal feed (Holtzapple, 2003a), though its implementation in insect based materials also permits an estimation of chitin content (Han, 2018). NDF content allows an estimate of cellulose, hemicellulose and lignin levels in animal feed (Holtzapple, 2003b). ADF and NDF contents were greater after extraction both in the control and SSF mixture when compared to the unextracted mixtures (Figure 9). Nonetheless, higher ADF and lower NDF contents were observed in the fermented mixture after extraction when compared to the respective control. ADF content remained identical in the control and in the fermented mixture when no extraction was performed, while the NDF content decreased. Compared to the unfermented mixture, no alteration was detected in ADF content when the fermented material was not extracted, increasing both in the extracted control and fermented mixtures. Furthermore, NDF content of WB and IMS-B mixture decreased after SSF with and without extraction, compared to the respective controls. The increased ADF content of control and fermented mixtures after enzymes extraction may be due to solubilization effects of the extraction, concentrating the remaining fiber. In fact, Zhao et al. (2017) and Mao et al. (2020) observed an increase of WB soluble fiber after biological treatments (SSF with *S. cerevisae* and *E. faecalis*, respectively). Likewise, Nafisah et al. (2019) also observed that a SSF by *B. subtilis* ATCC 19659 can decrease fiber fraction (NDF and ADF) of BSF substates, leading to a reduction of chitin content.

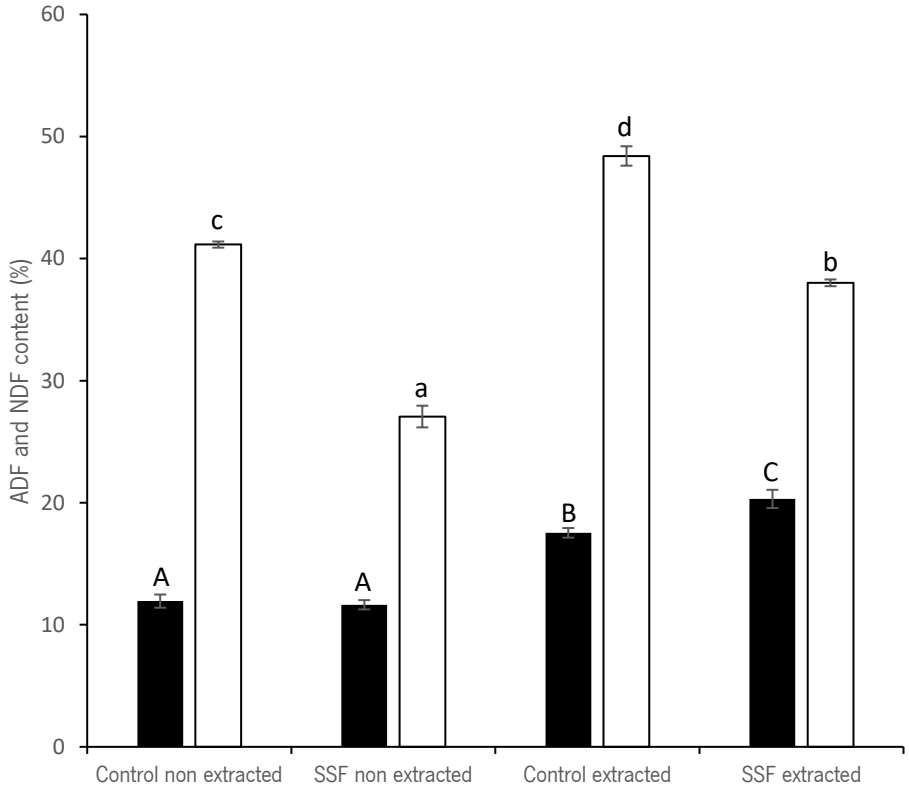


Figure 9 – Acid (black) and neutral (white) detergent fiber content of WB, ISM-B, and mixture of WB and ISM-B before and after SSF with or without enzymes extraction. Data are present as average and standard deviation of two independent replicates. Bars with the same letter are not significantly different ($p \leq 0.05$).

The fermented WB and ISM-B mixture without extraction seems to be the most promising material to inclusion in animal feeds, given the increased protein and reduction of fiber and lipid contents. Indeed, Lall and Dumas (2015) reported that protein levels in the ranges achieved after SSF with *A. niger* CECT 2088 of the WB and ISM-B mixture, can be accepted by most fish species. Partial removal of lipid content, into the obtained ranges in the fermented WB & ISM-B mixture, may be beneficial for a fish feed, both for feed intake (Jin et al., 2013) and processing (Kim et al., 2019). Chaves et al. (2015) and Prabu et al. (2017) indicated that reducing the insoluble fiber content is beneficial for fish feed, since these materials generally do not play an important role in nutrition and, as is the case of chitin, can interfere with the digestibility of other nutrients (Alfiko et al., 2022; Sánchez-Muros et al., 2014).

4.4. Enzymatic treatment of powdered chitin and ISM-B

Besides the SSF of ISM another approach of treatment can be the enzymatic hydrolysis by chitinase. Thus, in order to access the applicability of the enzymatic extract obtained in SSF of SS and BSG, enzymatic treatments were tested in ISM-B. Firstly, powdered chitin was used as the substrate to access best conditions of pH, temperature and reaction time on chitinase activity.

For that, liberation of reducing sugars as an indirect measurement of N-acetyl-glucosamine was monitored in a treatment of 2 hours (Figure 10). The largest release of reducing sugars took place at pH 7.5 and temperatures between 37 and 42 °C, indicating the highest chitin hydrolysis efficiency in the studied ranges. Indeed, chitinases functionality may be ensured at temperatures up to 40 °C and pH ranging from 5 to 9 (Stoykov et al., 2015; Chen et al., 2020; Patil and Jadhav, 2014).

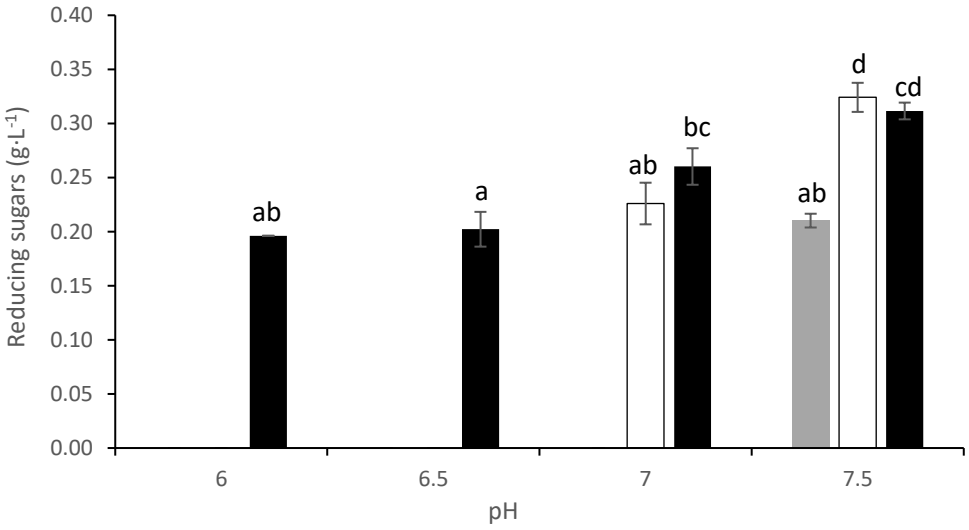


Figure 10 – RS released during enzymatic hydrolysis of powdered chitin under varying pH and temperature levels (grey – 32 °C, white 37 °C, black - 42 °C) during a 2 h reaction with a 50 % (v/v) enzyme concentration. The results are the average of two independent replicates and error bars represent SD. Bars with the same letter are not significantly different ($p \leq 0.05$).

During the trial using 30, 20 and 10 % (v/v) of enzymatic extract, the reducing sugars release remained practically stable from the beginning and until the 3 h hydrolysis mark (data not shown). Suggesting that the reduction in enzyme concentration, compared to the previous experiment at 50 %(v/v), may have hindered the hydrolytic capabilities of the chitinases during the reaction time. Therefore, the reaction time for ideal chitinase activity was tested in both ISM-B and powdered chitin, utilizing a 100 %(v/v) enzyme concentration during a 6 hours reaction at 37 ºC.

A larger NAG release occurred after 2 h of hydrolysis of both powdered chitin and ISM-B (Figure 11A), indicating that the greatest degree of chitin hydrolysis took place in the first 2 hours of hydrolysis. Cardozo et al. (2019) also observed a fast increase in NAG production in the first 12 hours of colloidal chitin (5%) hydrolysis by *A. caviae* CHZ306 chitinases (46ºC, pH 6.0), and a stabilization after this time.

A stabilization which may have occurred since an amorphous (non-crystallized) portion of chitin is present at the first stages of reaction, while highly crystallized particles are constantly accumulating in the medium with the hydrolysis progression, limiting chitinases access to the β-glycosidic bonds. The same suggestion has been reported by other researchers seeking to explain the decrease in NAG release after certain reaction periods (Jamialahmadi et al., 2011; Chen et al., 2010).

Acetic acid release increased in the first two hours of hydrolysis of ISM-B, while no differences were observed during the powdered chitin hydrolysis (Figure 11B). Win and Stevens (2001) observed a rapid chitin deacetylation in the first hour of a 4 h enzymatic deacetylation of purified shrimp chitin (5%, w/v; 50 °C; pH 8.5) by *C. lindemuthianum* produced chitin deacetylases followed by a noticeable slow down into a stable plateau, attributed to the crystalline structure of chitin present in late stages of hydrolysis limiting the access to acetyl groups.

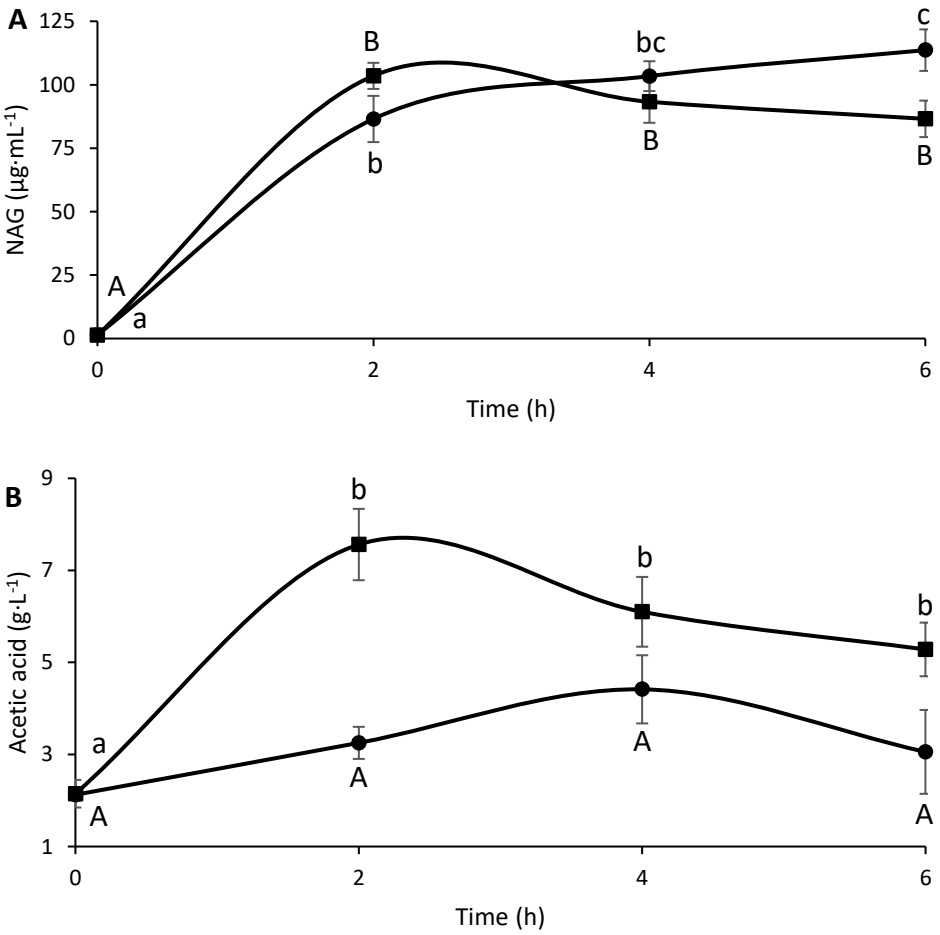


Figure 11 – NAG (A) and acetic acid (B) released by the enzymatic hydrolysis of powdered chitin (•) and ISM-B (■), during a 6 hour by a 100% (v/v) enzyme concentration at 37 °C. Data is present as average ± standard deviation of two independent replicates. Values sharing letter for each material are not significantly different ($p \leq 0.05$).

5. CONCLUSIONS

Solid-state fermentation of chitin rich substrates was successfully applied to produce chitinases by *Aspergillus niger* CECT 2088. Moreover, it was shown that the enzyme production by SSF was greatly improved by the mixture of chitin sources with other substrates.

BSG was found to be a suitable supplement of shrimp shell for SSF by *A. niger* CECT 2088, achieving maximum chitinase production when SSF was performed with 50%(w/w) of each substrate, during 21 days at moisture content of 70% (w/w). Recovered enzyme produced under these conditions was successfully tested to treat ISM, and a 2 h treatment was sufficient for maximum hydrolysis attained under the optimized pH and temperature conditions.

SSF was also proven to be an effective approach to be applied as a treatment of ISM. SSF by *A. niger* CECT 2088 led to highest chitinase production in a mixture of WB and non-defatted ISM of *Hermentia illucens* when the fermentation was carried out for 5 days with a moisture content of 80% (w/w). This biological treatment resulted in a decrease of total fiber of 27%, as well as an increase in protein of 1.2-fold and a decrease of lipids content of 55%. Thus, this treatment proves to be an alternative to defatting processing of ISM using organic solvents.

The results reported in this study demonstrated the potential application of *Aspergillus niger* CECT 2088 and SSF as bioprocessing strategy to improve the nutritional properties of ISM, boosting its application in animal feed.

6. FUTURE WORK PERSPECTIVES

The presence of chitin in feeds is linked to various digestibility problems by animals, such as fish from aquaculture.

In this work, *A. niger* CECT 2088 demonstrated to be able to reduce the levels of overall fiber in insect material, and simultaneously improve the nutritional balance of protein and lipids in ISM based feed. However, the estimation of chitin was performed via methodologies that also detect the presence other fibers. For these reasons, future work should focus on applying chitin specific analysis in order to more accurately access the elimination of this fiber. An investigation on the influences of other parameters, such as temperature of culture medium or aeration, on chitinase production during SSF of ISM could also be performed, alongside with *in vitro* and *in vivo* digestibility trials of the fermented material, in order to continue the nutritional improvement of ISM and their applicability as a feed ingredient.

Some authors have also observed the potential of lactic bacteria to reduce the presence of chitin in ISM. Thus, it could be interesting to study the differences between fungal and bacterial chitin reduction and of potential sequential fermentation for the improvement of this material.

It would be also interesting to scale-up the fermentation process, in an attempt to identify potential challenges on the large-scale production of ISM feed. Enzymatic treatment of fermented ISM could be implemented in order to observe if the hydrolysis of chitin could be further extended without a direct fermentation of ISM.

Lastly, the extraction, purification and characterization of enzymes and of by products derived from the fermentation, can be performed in future work, as a means to find if byproducts of ISM feed production can be values for other industries, creating further economic incentives for the utilization of insects in biotechnological applications.

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