Ana Catarina Sá Mota

Bioprocessing by solid-state fermentation of underexplored agro-Industrial by-products for their valorisation

Universidade do Minho
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outubro de 2023

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UMinho | 2023

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Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação da
Professora doutora Isabel Maria Pires Belo

outubro de 2023

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ACKNOLEDGMENTS

Estes meses foram uma longa caminhada recheada de desafios e incertezas, mas também muita aprendizagem, concretização e crescimento. Assim, gostaria de agradecer a todos os que de alguma forma contribuíram para a realização deste trabalho.

Em primeiro lugar, gostaria de agradecer à Professora Isabel Belo por toda a ajuda, orientação, disponibilidade e ensinamentos ao longo desta caminhada.

Um agradecimento também a todas as meninas do Laboratório de Bioprocessos e Biossistemas por toda a ajuda, disponibilidade e bom ambiente de trabalho. Um agradecimento especial à Ana Guimarães, apesar de ter o seu trabalho, sempre se mostrou disponível para me ajudar em tudo o que precisasse.

Aos meus amigos, em especial à Francisca Silva e Inês Tinoco, e ao meu namorado pela motivação diária, encorajamento, amizade e especialmente pelos seus conselhos e sinceridade nos momentos mais difíceis.

Aos meus pais, pelo apoio diário, motivação e esforço para que tudo isto fosse possível.

Este estudo foi apoiado pelo projeto integrado be@t – Bioeconomia Têxtil (TC-C12-i01, Sustainable Bioeconomy No. 02/C12-i01/2022), promovido pelo Plano de Recuperação e Resiliência (PRR), Next Generation EU, para o período 2021 – 2026; pela Fundação para a Ciência e a Tecnologia (FCT) no âmbito do financiamento estratégico da unidade UIDB/04469/2020; pelo Laboratório Associado LABBELS em Biotecnologia, Bioengenharia e Sistemas Microeletromecânicos (LA/P/0029/2020).

A todos, o meu grande obrigada!

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Bioprocessamento por fermentação em estado sólido de subprodutos agroindustriais pouco explorados para a sua valorização

RESUMO

Todos os anos são produzidas grandes quantidades de subprodutos agroindustriais que acabam por ser descartados ou utilizados em aplicações de baixo valor. Contudo, estes podem ser valorizados por fermentação em estado sólido (FES) para a produção de enzimas, com várias aplicações industriais, como a alimentar, na produção de biocombustíveis, em aplicações farmacêuticas, em rações animais e na indústria têxtil são utilizadas nas etapas de desengomagem e lavagem dos tecidos e acabamento das calças de ganga.

Neste trabalho foram utilizados os subprodutos dreche, casca de arroz e poda de videira para a produção de enzimas (xilanase, celulase, β-glucosidase e amílase) por FES com *Aspergillus niger* CECT 2088. Foi observado um efeito positivo da suplementação do meio com 2 % (p/p) $(NH_4)_2SO_4$ e 1 % (p/p) K_2HPO_4, que conduziu a um aumento de 2 a 10 vezes da produção de enzimas utilizando casca de arroz e dreche. Nas fermentações realizadas com diferentes tamanhos de partículas (1, 4 e 10 mm) de cada subproduto, foram obtidas atividades enzimáticas mais elevadas com o tamanho de partícula de 10 mm de dreche e casca de arroz, e com 4 mm de poda de videira.

Através de um planeamento experimental simplex – centroide procedeu-se à otimização de misturas dos substratos para a produção de enzimas. Foi possível verificar que a atividade enzimática ótima para a xilanase (651 U/g), β-glucosidase (363 U/g), celulase (189 U/g) foi obtida usando 100 % (p/p) de dreche, e que a atividade ótima para a amilase (263 U/g) foi obtida usando uma mistura 72 % (p/p) de dreche com 28 % (p/p) de casca de arroz. A monitorização da cinética de produção das enzimas por FES durante 14 dias, permitiu observar que a atividade enzimática máxima da xilanase foi conseguida ao 5º dia, e ao fim ao 10º dia para a celulase, amilase, e β-glucosidase. Os resultados deste trabalho indicam que o bioprocessamento de dreche, casca de arroz e poda de videira por FES é uma abordagem eficaz e sustentável para a produção de enzimas de interesse para a indústria têxtil, contribuindo para impulsionar a economia circular neste setor.

Palavras-chave: *Aspergillus niger*, enzimas, fermentação em estado sólido, subprodutos

Bioprocessing by solid-state fermentation of underexplored agro-Industrial by-products for their valorisation

ABSTRACT

Large quantities of agro-industrial by-products are produced every year and end up being discarded, or used in low-value applications. However, lignocellulosic by-products can be valorised by solid state fermentation (SSF) to produce enzymes with various industrial applications, such as in the food industry, biofuel production, pharmaceutical applications, animal feed and in the textile industry, where they are used across various stages of textile processing.

In this work, the by-products brewer's spent grain (BSG), rice husk (RH), vine shoot trimmings (VST), were used to produce the enzymes xylanase, cellulase, β-glucosidase, and amylase, by SSF with *Aspergillus niger* CECT 2088.

The granulometry of the by-product, initial humidity content, and supplementation with nitrogen and phosphorus sources were factors studied with impact on SSF. A positive effect was observed of adding to the medium 2 % (w/w) $(NH_4)_2SO_4$ and 1 % (w/w) K_2HPO_4, which led to a 2 to 10-fold increase in enzyme production using RH and BSG. In SSF carried out with different particle sizes of 1, 4 and 10 mm of each by-product, supplemented with 2 % (w/w) $(NH_4)_2SO_4$ and 1 % (w/w) K_2HPO_4, the highest enzymatic activities were obtained with 10 mm of BSG and RH, and with 4 mm of VST.

A simplex-centroid mixture design was used to optimise the substrate mixtures to produce enzymes. The optimum enzymatic activity for xylanase (651 U/g), β-glucosidase (363 U/g), and cellulase (189 U/g) was obtained using 100 % (w/w) BSG and the optimum activity for amylase (263 U/g) was obtained using a mixture of 72 % (w/w) BSG and 28 % (w/w) RH. The kinetics of enzyme production by SSF was monitored for 14 days and It was found that the maximum enzymatic activity of xylanase was obtained on day 5, while maximum activity of cellulase, amylase, and β-glucosidase was reached after 10 days of SSF.

The results obtained indicate that the bioprocessing of BSG, RH, and VST by SSF is an effective and sustainable approach for the production of enzymes of interest to the textile industry, helping to boost the circular economy in this sector.

Keywords: *Aspergillus niger*, by-products, enzymes, solid state fermentation

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LIST OF ABBREVIATIONS

BSG	Brewer's spent grain
CMC	Carboxymethylcellulose
CO_2	Carbon dioxide
DNS	Dinitrosalicylic acid
EC	Enzyme comission
HPLC	High performance liquid chromatography
KOH	Potassium hydroxide
PDA	Potato dextrose agar
pNPG	p-Nitrophenylglucopyrano
RH	Rice husk
RHA	Rice husk ash
Rpm	Rotations per minute
SD	Standard deviation
Smf	Submerged fermentation
SSF	Solid state fermentation
TCA	Trichloroacetic acid
VST	Vine trimming shoots
XOS	Xylooligosaccharides

GOALS AND MOTIVATION

The main objective of this work is to study the sustainable production of enzymes used in the textile industry for the pre-treatment and finishing processes of natural fibres. Target enzymes were, specifically, cellulases and xylanases, although other enzymes of interest to the textile industry, such as amylase, and others, were also monitored.

Solid-state fermentation (SSF) was the technology selected to study and optimise the production of enzymes by *Aspergillus niger* CECT 2088, using agricultural and food industry by-products. Thus, the specific objectives of this work were:

- Characterisation of the selected by-products (BSG, RH and VST) with potential for SSF;
- Study the effect of operational parameters: granulometry of solids, humidity, and medium supplementation, on enzyme production by SSF;
- Identification and quantification of enzymes with higher activity and with greater interest in the textile industry;
- Study substrate mixtures to optimise SSF to maximize enzyme production;
- Study of enzyme production kinetics by SSF.

1 INTRODUCTION

1.1 Agro-industrial by-products

The world's population is constantly increasing. It is estimated that by 2050 the world's population will reach 9.8 billions by 2050 and up to 11.2 billion by 2100 (FAO, 2023). As the population increases, so does the need for agricultural production. Currently, agriculture is one of the industrial sectors responsible for the production of the highest volume of residues (Dahiya et al., 2018), This is because to the fact that a high quantity of food products does not reach the final consumer in its original state, but in a processed form, which, generally, results in the creation of by-products. (Tapia-Quirós et al., 2022). Nowadays, it is estimated that the annual world production of agro-industrial by-products is around 1 billion tones (Moayedi et al., 2019).

According to the European Commission (2011) the term "by-product" refers to any material or object generated as a result of a production processes which is not its primary aim. These by-products generated in agro-industrial activities are a major problem (Maicas and Mateo, 2020). The untreated disposal of these products has serious environmental, social, and health consequences, and proper disposal can have a significant impact on the economy of the food industry. The most used methods for managing food waste include disposal in landfills, incineration, composting, and animal feeding, which are not regarded as sustainable or environmentally responsible practices (Gómez-García et al., 2021).

Presently, there is a great interest in the valorisation of these by-products, with the goal of free environmental pollution and generate value-added resources, which can lead to new markets and income sources (Routray and Orsat, 2019). The re-use and valorisation of by-products are an important part of establishing a circular economy (Aschemann-Witzel and Stangherlin, 2021). This way, the discarded materials can be put back into the supply chain, increasing economic growth, free waste, and ending the term "end of life" of products (Haque et al., 2023; James et al., 2023).

One of the ways of adding value to agro-industrial by-products is to use them in the production of enzymes through solid state fermentation (SSF). Most of these by products are lignocellulosic by-products, which benefits the growth of microorganisms that produces lignocellulosic enzymes (Ozcirak Ergun and Ozturk Urek, 2017). The composition of the substrates chosen for SSF influences the production of enzymes, since the concentration of each lignocellulosic fraction can act as an inducer or repressor of the production of a given enzyme (Leite, Sousa, et al., 2021).

1.1.1 Rice husk

Rice is the most important food crop worldwide. It is an important source of energy, vitamins and essential elements, and it is an important staple food for over half of the world's population, particularly in Asia, Africa, and Europe (Kukusamude et al., 2021).

Rice is the second most widely planted cereal in the world (Fraga et al., 2019) and is grown on every continent except Antarctica (Prasad et al., 2017). There are two cultivated species of rice, *Oryza sativa* and *Oryza glaberrima*, the former being the most widely cultivated and the latter only present on the African continent (Muthayya et al., 2014).

Rice production extends to more than 100 countries, with Asian countries accounting for the majority of production (around 95 % of world production) (Rathna Priya et al., 2019), with China and India producing around half of the world production, the equivalent of 300 million tonnes per year (Fraga et al., 2019). Italy and Spain, the largest European rice producers, are responsible for 80 % of all rice produced in Europe (a total of 300 million tonnes per year) (Pinto et al., 2016). Portugal is the fourth largest rice producer in Europe, producing around 180 000 tonnes a year (6 % of European production). However, Portugal is the largest per capita consumer in Europe, consuming 16 kg of rice/year (Fraga et al., 2019).

To be consumed rice grains have to go through several processing steps. Rice processing involves cleaning, hulling, and post-hulling, which result in several rice by-products. The most abundant by-product of this processing is rice husk (RH) (Fasuyi and Olumuyiwa, 2013). This is the outermost layer of the grain and represents around 20-25 % of the total weight of the rice grain and it is separated from the grain during milling (Dada et al., 2022). Approximately 120 million tonnes of RH are generated every year and the disposal of these by-products are a current problem (Mohiuddin et al., 2016; Wu et al., 2018). A solution often used by rice producers is to burn the RH, which results in a loss of organic raw material and greenhouse gas emissions into the atmosphere (Asadi et al., 2021).

However, this by-product can be used in several applications with added value. RH can be used to produce bioenergy by burning it in a closed reactor. This combustion reaction also produces rice husk ash (RHA). There are various techniques for producing electricity from RH, some of which are direct combustion heating, direct combustion power generation, gasification, and power generation. It is estimated that producing energy by gasification using RH emits 1 tone less CO_2 than the currently used methodologies (Pode, 2016). Although the calorific value of RH is 41 % lower than coal, RH is also 36 % cheaper (Mohiuddin et al., 2016). Pode et al (2016) reported the production of 800 kWh of electricity with one tone of RH.

RHA, in turn, can be used as an adsorbent to decontaminate water containing heavy metals (Pode, 2016). RHA is insoluble in water and chemically stable, making it a good alternative to currently used materials, which are very expensive (Daifullah et al., 2003). This technology appears to be a good solution not only for valorising RH as well as to solve the problem of access to cheap raw materials for the treatment of contaminated water (Kiran and Prasad, 2019). A maximum metal adsorption of around 88 % has been reported (Ahmaruzzaman and Gupta, 2011). The use of silica from RHA as an adsorbent can also be extended to biodiesel purification, making the process easier and faster (Pode, 2016).

Due to RHA high amount of amorphous silica in its composition (approximately 95 %), this mineral can also be used as a raw material for building materials such as bricks and cement (Pode, 2016). Additionally, the biochar produced through the incomplete combustion of RH is rich in silicon, an important mineral for the healthy growth of rice plants and can be used as a complement to fertilisers. It helps plant resistance and does not damage the plant when used in excess (Pode, 2016). In addition to improving the composition and fertility of the soil, it increases nutrient retention (Asadi et al., 2021; Win et al., 2019).

1.1.2 Brewer's spent grain

One of the most popular and widely consumed alcoholic beverages in the world is beer. It is the third most consumed drink in the world, with an average annual consumption of beer is 23 litres per capita (Oliveira et al., 2018; Villacreces et al., 2022).

The main ingredients for its production are water, yeast, hops and fermentable carbohydrates such as barley (Villacreces et al., 2022). Beer is produced through 9 stages: malting, milling, mashing, boiling, cooling, fermentation, maturation, filtration and carbonation (Salanţă et al., 2020). The yeasts most commonly used in the production of this alcoholic drink belong to the *Saccharomyces* genus (Capece et al., 2018).

Brewing practices produce several by-products, such as brewer's spent grain (BSG), residual yeast and hops (Kerby and Vriesekoop, 2017). With the world's beer production reaching 1.89 million hectolitres in 2022, and it is estimated that for every 100 litres of beer produced, 20 kg of BSG are formed (Bachmann et al., 2022).

BSG consists of the husks of the barley grain and other residual solids formed after the wort formation stage and once removed from the brewing process, has several possible applications (Mussatto, 2014). BSG has a high fibre and protein content (Bachmann et al., 2022). Thus, it has been used in animal feed with satisfactory results. Its application in cows' diets has increased milk production

and decreased milk fat content. When applied to the diet of pigs, chickens, and other poultry, good weight gain results were obtained. However, the use of BSG in animal feed must be accompanied by enzymatic treatment to degrade the fibres in the cell wall (Mussatto, 2014).

The interest in BSG extends beyond animal feed, it is also used in human food due to its nutritional value. For instance, BSG can be ground into flour that is then used in cakes, breads, and biscuits (Mussatto, 2014).

The extraction of phenolic compounds is another application for BSG. To do this, a solid-liquid extraction must be carried out (Mussatto, 2014). After being extracted, phenolic compounds can be applied to some foods, increasing their shelf life and avoiding the use of synthetic antioxidants (Bachmann et al., 2022). Moreover, these antioxidants can also be used in the pharmaceutical industry (Mussatto, 2014).

BSG can also be used to produce charcoal bricks. The production of charcoal consists of drying, pressing and carbonising it in a low-oxygen environment (Mussatto, 2014). This by-product can also be used in biorefineries, as it stands out from the rest due to its high cellulose and hemicellulose content (Mussatto, 2014). BSG is also used to produce paper, which is then turned into cups, plates, and coasters (Mussatto, 2014).

Similar to RH, BSG also has a good adsorption capacity. It is therefore used in water decontamination processes. It is able to adsorb organic compounds such as dyes and heavy metals (Bachmann et al., 2022; Mussatto, 2014).

1.1.3 Vine shoot trimmings

Wine production is a technique that is more than a thousand years old (Harb et al., 2021) and the vine is considered one of the oldest plants (Rodrigues et al., 2020). The most cultivated species for wine production is *Vitis vinifera* (Tapia-Quirós et al., 2022), and it is present on all continents except Antarctica (Soceanu et al., 2021).

The wine industry is one of the most important socio-economic activities in the world, every year 60 million tonnes of grapes are produced, 80 % of which are used for winemaking (Perra et al., 2021). Europe is responsible for 39 % of all grape production worldwide, followed by Asia (34 %) and America (14 %) (Ahmad et al., 2020). In Europe, the countries that produce the most wine are Italy, Spain, France, Germany, and Portugal. In 2018, 300 million litres of wine were produced worldwide, of which Portugal produced 6.1 million litres (OIV, 2019). Portugal is the second largest wine consumer in Europe (Ferreira et al., 2019).

Wine production generates a huge amount of by-products, such as pomace, seeds, stems, prunings and yeast lees, among others (Ferrer-Gallego and Silva, 2022). As a result, around 20 million tonnes of by-products are generated annually as a result of winemaking (Maicas and Mateo, 2020).

Recent studies have concluded that around 40 % of wine by-products are burnt. Burning not only contributes to increased pollution but is also a waste of biomass, phenolic compounds and other biomolecules that are present in the by-products (M. Jesus et al., 2022). Recent studies have verified the potencial of the wine industry's solid residues for the production of antioxidant compounds, tartaric acid, bio-oil, fertilizers and biosurfactants among others Bharathiraja et al (2020).

The use of vine shoot trimmings (VST) in biorefineries involves chemical treatment, through hydrolysis (Garita-Cambronero et al., 2021) or an enzymatic treatment for the production of sugar monomers (Bustos et al., 2007).

VST are rich in polyphenolic compounds, which are of great interest due to their preventive action in cardiovascular problems and inflammatory processes, thus another way of valorising VST is to extract its phenolic compounds (M. Jesus et al., 2022; M. S. Jesus et al., 2019).

VST can also be used in the production of biosurfactants. These biosurfactants are biodegradable and their production does not have as much environmental impact as synthetic surfactants (Cortés-Camargo et al., 2016; Vecino et al., 2013). The production of biosurfactants using VST has been studied by several authors. Vecino et al (2017) concluded that the carbon source used for biosurfactant is related to its composition and that the behaviour of a biosurfactant produced from VST is similar to that of a synthetic surfactant.

1.2 Solid - state fermentation

Solid – state fermentation (SSF) is a biotechnological process in which microorganisms grow on solid, organic material (the substrate) in the absence or near absence of free water (Aita et al., 2019; M. Zhao et al., 2015), however, the solid must contain sufficient humidity to support the growth and metabolism of the microorganism (Singhania et al., 2009). SSF has the advantages of having low energy requirements, producing less wastewater and being environmentally safe. This process is especially useful in the processing of agro-industrial residues, contributing to resolving the problem of solid waste disposal by valorising these low-cost by-products.

Selection of a suitable substrate is a crucial aspect of SSF. The solid material should be non-soluble and act as physical support and as a source of nutrients. However, a nutrient solution can be

added if the material cannot alone support alone the growth of the microorganism (Singhania et al., 2009).

Similarly important is the selection of microorganism. The most suitable microorganisms to be used in SSF are filamentous fungi since the fermentation medium simulates their natural habitat. The hyphal mode of fungal growth that allows them to grow and penetrate the spaces between particles, and their tolerance to lower water activities, than bacteria, and high osmotic pressure conditions are major advantages in the colonization of the substrate and obtention of available nutrients (Graminha et al., 2008; Krishna, 1999; Muller dos Santos et al., 2004). Moreover, filamentous fungi are efficient enzyme producers, and can also produce aroma compounds and other substances of interest to the food industry. However, yeasts and some bacteria can be used in SSF, such as *Bacillus subtilis*, *Bacillus thuringiensis* and *Lactobacillus* sp., (Verduzco-Oliva and Gutierrez-Uribe, 2020). Mycelial bacteria such as *Streptomyces spp* have also be used in SSF (Yafetto, 2022).

Besides substrate and microorganism, there are several important factors that affect SSF. These include a wide range of physicochemical and biochemical factors, mainly: inoculum age and size, humidity and water activity, pH, temperature, particle size of the substrate, pre-treatment of the substrate, aeration and agitation, nutritional factors (such as nitrogen, phosphorus and trace elements), use of additional carbon sources and oxygen and carbon dioxide levels. Depending on the type, extent, and intended application of the experiments, methods for optimizing single or multiple variable parameters may be employed (Krishna, 1999; Pandey, 2003).

SSF process is defined as a four-phase system: the solid phase consists of a water-insoluble support; the liquid phase that is adsorbed in the solid matrix and that surrounds the cells; the gaseous phase, in the space between the particles; and the microorganism that grows on the surface and inside the solid support (Graminha et al., 2008).

Although submerged fermentation (SmF) is currently still the process most used by the industry to produce compounds of interest, SSF has several advantages over SmF. SSF can use raw materials directly as substrates, use a wide range of matrices, has lower costs, lower energy consumption, is more cost-effective in the downstream processing, uses less water minimizing wastewater output, has higher product concentration, and requires less fermentation space. Additionally, presents a higher productivity and higher final concentration of the molecules of interest, as well as a more stable final product (Krishna, 1999; Singhania et al., 2009; Teles et al., 2019). Enzymes produced by SSF show greater stability when subjected to high temperatures and extreme pH (Hölker et al., 2004). Low water availability also reduces the risk of contamination (Singhania et al., 2009). However, SSF has some disadvantages: it is difficulty

to homogenise the substrate bed, due to difficult agitation; difficulty in fermentation control (problems in the accumulation of gradients in temperature, pH, humidity, and aeration levels), difficulty in determination of microbial growth, and limitations in the type of microorganism that can be used (Hölker et al., 2004; Krishna, 1999). There are also reports of numerous challenges in the scale-up of SSF processes. The costs of purifying the compounds produced may also be high, since the solids used may contain impurities (Couto and Sanromán, 2006).

SSF is widely used in the valorisation of agro-industrial by-products with low economic value (Singhania et al., 2009). The price of the raw material represents around a third of the final cost of bioproducts thus, the use of cheaper raw materials in SSF can considerably reduce the investment and product cost, therefore increasing profitability (Brijwani et al., 2010; Leite, Belo, et al., 2021). An economic analysis for the production of lipase in both SmF and SSF verified that for the same production scale, the investment needed for SmF was 78 % higher than for SSF (Castilho et al., 2000).

In the past, SSF was used to produce foods such as alcoholic beverages, vinegar, soy sauce and some flavoured spices. However, currently, it has been used to produce microbial biomolecules, such as enzymes, organic acids, nucleotides, lipids, and amino acids and aromatic compounds (Vandenberghe et al., 2021; M. Zhao et al., 2015). It is also used to produce biofuels and, in agriculture, to produce fermented feeds rich in nutrients, biofertilizers and spores to control agricultural pests (Aita et al., 2019; Vandenberghe et al., 2018; Yafetto et al., 2023).

1.3 Enzymes

Enzymes are biocatalysts with high specificity for their substrate. They have a complex molecular structure, consisting of two parts, a protein part and a lipid or carbohydrate part (Denti, 2021).

Its primary structure is related to the sequence of the amino acids in the chain. The secondary structure depends on the interaction of each amino acid with the adjacent amino acid and may form β -sheets or α-helices. Regarding the tertiary structure, folds are formed according to the interactions between amino acids that are not close together. Finally, the quaternary structure is related to the interaction between the different polypeptide chains that constitute the protein (Denti, 2021). The integrity of all these structures is fundamental to the catalytic activity of the enzyme (Lehninger et al., 2013).

Enzyme production is currently a fast-growing area in biotechnology. Currently, there is a great interest in using SSF for the production of enzymes to be used in several industries. Besides the well-established applications in food and fermentation industries, microbial enzymes have been employed in textile, paper, leather, feed, pharmaceutical, and biofuel industries. Most relevant enzymes in industrial

applications, such as amylase, glucoamylase, cellulases, xylanase and pectinase, have already been produced through SSF.

1.3.1 Xylanase

The endo – 1,4 – β - xylanases (EC 3.2.1.8) are glycosidases that catalyse the hydrolysis of the 1,4 – β – D – xylosidic bonds of xylan. The activity of this enzyme results in the production of xylose, a primary carbon source for cellular metabolism, and xylooligosaccharides (XOS) (Collins et al., 2005; Goulart et al., 2005). Xylan is an important structural polysaccharide in plant cell walls and is the second most abundant renewable polysaccharide in nature, accounting for approximately one third of all renewable organic carbon on earth (Rabha et al., 2023).

These enzymes are produced by several microorganisms, in particular fungi and bacteria (Mendonça et al., 2023), in particular by *Trichoderma* spp. and *Aspergillus* spp. (Archana and Satyanarayana, 1997; Haltrich et al., 1996).

Recently, the industrial interest of xylanases has increased considerably due to its multiple applications (figure 1) in the food industry. This enzyme can be used in fruit juices to improve food safety and the shelf life (Y. Zhao et al., 2023). These enzymes can also be used in the aggregation of polymeric carbohydrates, such as starch, pectin and cellulose, to reduce the viscous and cloudy appearance of fruit juices. Thus, improving the appearance of the juices to the consumer (Rosmine et al., 2017). Xylanases can also act in bread fermentation (Fernandes de Souza et al., 2022), improving the quality of the dough and subsequently of the bread (A. D. Harris and Ramalingam, 2010). The breakdown of the hemicellulose in wheat flour also makes the bread dough softer (A. D. Harris and Ramalingam, 2010). Xylanase also breaks down arabinoxylan, present in the cell wall of wheat and rye, and produces oligosaccharides (prebiotics) (Damen et al., 2012).

In animal feed, xylanases are used in conjunction with other enzymes to increase the bioavailability of nutrients in the feed. They hydrolyse the indigestible fibres present in cell walls (Bello et al., 2023), thus free intestinal viscosity, increasing nutrient digestibility (Zhang et al., 2014) and consequently less feed is needed for the animals to gain weight (Bajaj and Mahajan, 2019).

In the textile industry, xylanases are used in the desizing process (S. Kumar et al., 2011), where xylanase, together with other enzymes, degrades the fibres that for gum (Polizeli et al., 2005).

Xylanases can also be used in the production of biofuels from lignocellulosic material, by taking part in biomass hydrolysis (Dodd and Cann, 2009).

In the paper industry, the use of xylanase can reduce the use of health-hazard chemicals during paper bleaching. (Sharma et al., 2020). Besides whitening the paper, it also, xylanase improves paper quality and reduces the processing costs (D. Kumar et al., 2021; Moteshafi et al., 2022).

1.3.2 Cellulases

Cellulases are biocatalysts responsible for the hydrolysis of one of the most abundant polymers in nature, cellulose. This biopolymer, through the action of cellulases, is converted into oligosaccharides and subsequently into simple sugars, such as glucose (Karnwal et al., 2019; D. Kumar et al., 2021; Sadeghi et al., 2023).

The cellulase enzyme complex consists of three enzymes, endo - glucanase, exo - cellulase, and β - glucosidase (Siva et al., 2022). The endo - glucanase is responsible for breaking the internal β - 1,4 - glycosidic bonds of cellulose in a randomly, exo - glucanase acts on the free and non - free ends of the cellulose chains so that there is a release of short - chain oligosaccharides and, finally, β - glucosidase hydrolyses the glucose - soluble oligosaccharides. The cooperative action of the three enzymes is necessary to achieve efficient enzymatic hydrolysis of lignocellulosic biomass (Hegedüs et al., 2012).

These enzymes are of great value to the industry due to their multiple applications (figure 1). They can be used to improve animal feed, in the production of biofuels, paper production, detergent formulations, and other industrial processes (Astuti et al., 2022; Siva et al., 2022).

Cellulases can be used to produce biofuels from lignocellulosic biomass, producing second generation biofuels (A. Singh et al., 2021; Wilson, 2009). The bioproduction process essentially consists of 3 phases: pre-treatment, hydrolysis, and fermentation (A. Singh et al., 2021). Lignocellulosic biomass is hydrolysed into simpler sugars which are then fermented to form bioethanol or biofuels (Ejaz et al., 2021; Srivastava et al., 2018). The cost of bioprocessing can be reduced by 40 % when using cellulase producing microorganisms (Ejaz et al., 2021).

In the food industry, cellulase can be applied to the extraction process of fruit juices (such as grape, apple, pear, and orange juices), allowing higher production volumes to be achieved (Pui and Saleena, 2023). The enzymatic treatment makes the final product more transparent, more flavourful, and with a better texture (Bhati et al., 2021).

In the production of beer, when the barley is of poor quality, precipitates of glucans are formed which increase the viscosity of the product (de Souza and Kawaguti, 2021). The application of cellulases in the malting process hydrolyses the glucans, increasing the quality of the malt and free the need for the

filtration process by around 30 %. In general, the use of cellulases, results in a higher fermentation rate, as well as a higher yield (Singh et al., 2021).

Cellulose enzymes can be used as a pre-treatment for animal feed to improve its nutritional value, by partially degrading the anti - nutritional components of the feed (Bhati et al., 2021; Ejaz et al., 2021).

The application of cellulases in the paper industry increases the bleaching capacity and the final gloss of the paper (Ejaz et al., 2021).

The pharmaceutical industry uses enzymes such as cellulase to manufacture supplements to facilitate the digestion of fibres in the intestine. These digestive products significantly improve the digestion of cellulose fibres (Singh et al., 2021).

Cellulase can also be used in the textile industry. Its action improves the quality of the end product and reduces the use of toxic chemicals (Korsa et al., 2023).

1.3.3 Amylase

There are 3 families of amylases, α - amylase, β - amylase and γ - amylase (Das, 2011). All of them degrade starch, however, they act on different bonds, with some degrading the α - 1,4 bond and others the α - 1,6 bond (Park et al., 2000). Starch is a polymeric carbohydrate that constituted by amylose and amylopectin. Amylose is a linear polymer made up of glucose units linked by α - 1, 4 - glycosidic bonds, while amylopectin, is made up of branched glucose chains. These branches are present every 15-45 glucose units and are joined by an α - 1, 6 - glycosidic bond (Farooq et al., 2021). Amylases not only act on starch molecules but also on glycogen and oligosaccharides (Deb et al., 2013).

α - amylase (EC 3.2.1.1) is a metalloenzyme whose activity depends on the presence of calcium. This enzyme breaks down long carbohydrate chains, producing maltotriose and maltose (Das, 2011). β - amylase cleaves the α - 1,4 glycosidic bond, working from the non - free end of the chain of the polysaccharide and releases two glucose units (maltose) at a time (Das, 2011; Reddy et al., 2003). This amylase is unable to break branched bonds (Reddy et al., 2003).

γ - Amylase (EC 3.2.1.3), in turn, breaks the α – 1, 6 - glycosidic bonds and the last α – 1,4 - glycosidic bonds at the non - free end (Das, 2011).

Microbial amylase is the most widely used due to its biochemical versatility, greater stability and high production rate (Simair et al., 2017). Amylases can be found in both eukaryotic and prokaryotic organisms. They are one of the most important biotechnological enzymes, accounting for 30 % of the world enzyme market (Das, 2011).

Amylase has numerous applications (figure 1), being used in detergents, in the textile industry, bakery, and animal feed (Das, 2011; Simair et al., 2017).

The food industry is one of the industries that uses amylase the most (Movahedpour et al., 2022). In the production of glucose and fructose syrups, amylase is used to saccharify starch by solubilising it, thus freeing the viscosity of the syrup (Farooq et al., 2021). Its use also increases the shelf life of the syrup (Karnwal and Nigam, 2013). In beer production, amylase is used in the clarification phase, where it is used to break down the starch and convert it into fermentable sugars (Far et al., 2020; Wefing et al., 2020). Moreover, in baking, amylase is used to produce dextrins from the hydrolysis of starch. The fermentation of dextrins by yeast reduces the viscosity of the dough and consequently improves the texture of the final product. Here, also, there is an increase in the shelf life of the products (Farooq et al., 2021).

During the formation of the paper, a protective layer of starch is applied to prevent the surface of the paper from being damaged during production (Movahedpour et al., 2022). At the end of the process, amylase is used to remove the starch layer. This way, the quality of the final product is not compromised (Mobini-Dehkordi and Afzal Javan, 2012).

In the pharmaceutical industry, amylase is used in products to aid digestion, helping to treat digestive disorders (Karnwal and Nigam, 2013; Mobini-Dehkordi and Javan, 2012).

Around 60 % of all amylase produced is used in detergent formulations. Detergents are composed of chemicals that are very difficult to break down, causing the eutrophication of rivers (Far et al., 2020). The use of amylases facilitates the process of removing starch and sugar stains from clothes. The use of amylase in detergents reduces the use of environmentally harmful chemicals. Furthermore, there is also a reduction in the energy spent on washing, since washing at high temperatures is no longer necessary (Movahedpour et al., 2022).

Amylase is also used in the production of biofuels. These are produced from lignocellulosic biomass, so amylase is needed to saccharify the starch into fermentable sugars, which are then used to produce ethanol by microorganisms such as *Saccharomyces cerevisiae* (Mobini-Dehkordi and Afzal Javan, 2012).

Amylase is also used in the textile industry. Like xylanase, it is also used to desizing process (Kalia et al., 2021).

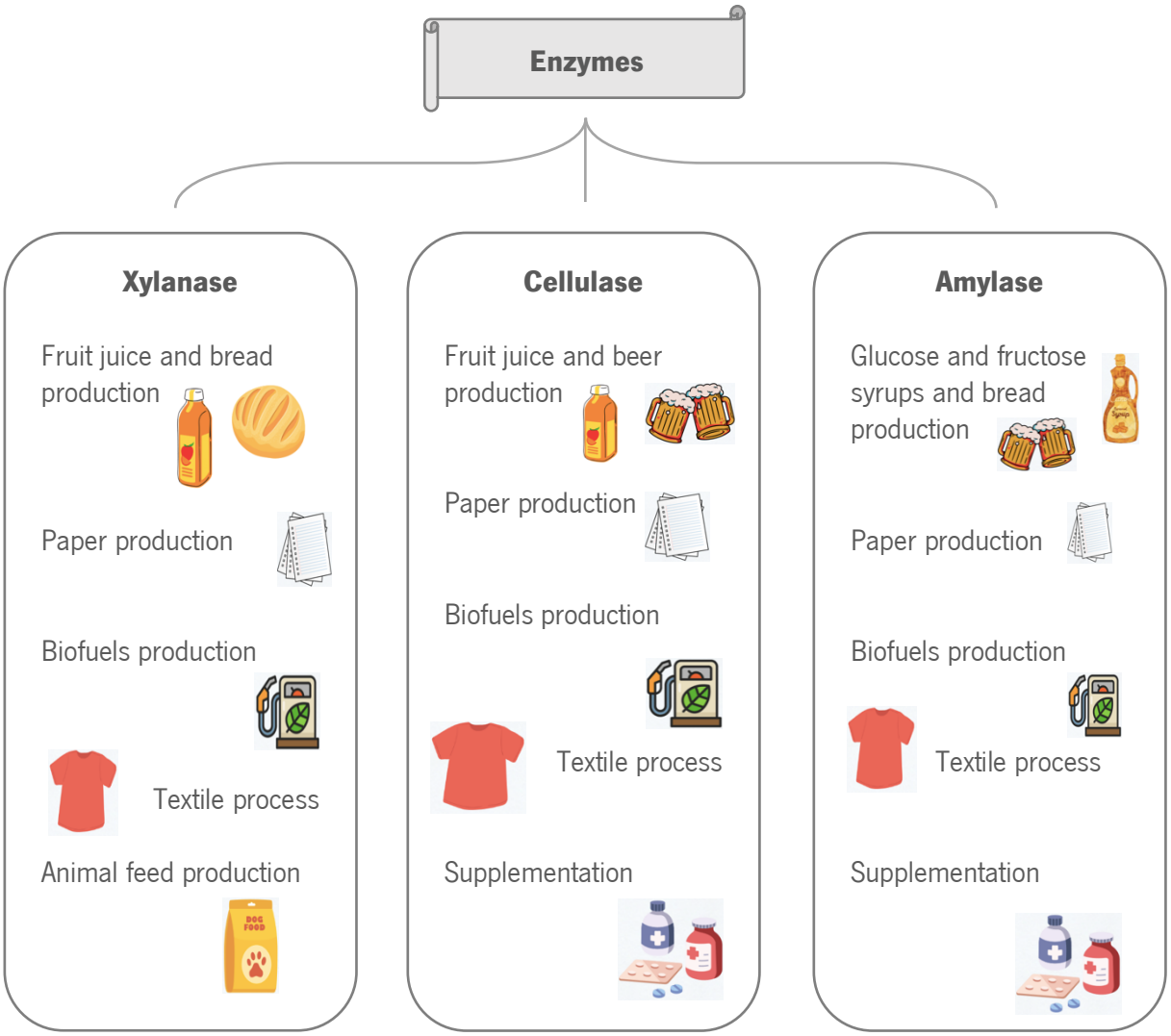

Figure 1. Enzymes application.

1.4 Enzymes use in the textile industry

In the textile industry, the use of enzymes is an example of white industrial biotechnology, which enhances the development of greener processing techniques and a higher quality of the final product (Araújo et al., 2008). Enzymes have applications across various stages of textile chemical processing (table 1). For instance, amylases can be used for textile desizing, cellulases can be employed for bio-polishing and denim washing (Madhu and Chakraborty, 2017).

In the desizing process, the action of amylase removes starch from the yarn. Thus, in the dyeing phase there is greater absorption of the dye (Kalia et al., 2021). As mentioned earlier, amylase is also used in the formulation of many detergents, both laundry detergents and dishwashing detergents. It is

also used in the fabric cleaning process. This step can therefore be carried out at a lower temperature (30 - 40 °C), thus free energy costs (Elmarzugi et al., 2014).

Additionally, enzymes can also be used in the treatment of the effluents resulting from the textile processing (Madhu and Chakraborty, 2017).

The use of cellulases in the textile industry began in the late 1980s, to give the effect of abrasion to jeans (Doshi and Shelke, 2001). Nowadays, besides being used in the denim finishing process, they are also used in the scouring process, and in the processing of cotton and other cellulose-based fibres (Araújo et al., 2008). To obtain an abrasive effect through the action of cellulases, denim garments are dyed with indigo, which adheres to the surface of the yarn, then the fibres on the surface of the fabric are hydrolysed by the cellulase. This partial hydrolysis of the surface creates lighter regions in the fabric (S. Singh and Khajuria, 2018). Cellulases can also reduce the tendency of a fabric to gain blubber. These biocatalysts can also hydrolyse microfibrils (fluff) on the surface of the fabric, which makes the surface of the fabric smoother (Singh and Khajuria, 2018).

On the other hand, xylanase removes the most difficult plant fibres so, they do not oxidise. Thus, the use of xylanase not only removes fibres that concentrated chemicals often do not remove, but also avoids the need for more aggressive fibre bleaching, since the lignin is not oxidised (Polizeli et al., 2005).

Table 1. Function of each of the enzymes being studied in the textile industry.

ENZYME	FUNCTION
Amylase	Desizing: removes the starch layer; Scouring: is used in the formulation of detergents.
Cellulase	Scouring: removes the fiber layer; Finish process: gives jeans a worn effect and improve the quality of final product removing the microfibrils
Xylanase	Desizing and Scouring: removes the fiber layer;

2 MATERIALS AND METHODS

2.1 Microorganisms

The fungi *Aspergillus niger* CECT 2088 obtained from the *Coleción Española de Cultivos Tipo* (CECT, Valencia, Spain) was stored at - 80 °C in an aqueous solution of 1 % (w/w) peptone and 30 % (v/v) glycerol and revived in PDA medium (potato extract 4 g/L, dextrose 20 g/L, agar 15 g/L,) for 7 days at 25 °C.

2.2 Agro-industrial by-products

The by-products used in this work were rice husk (RH), vine trimming shoots (VST), and brewer's spent grain (BSG). These were supplied, dried, and ground into different granulometries (10 mm, 4 mm, and 1 mm) and stored at room temperature. These by-products were supplied through CITEVE, on behalf of the Be@T project. RH was provided by the Nova Arroz company (Oliveira de Azemeis, Portugal), BSG by the LETRA craft brewery (Vila Verde, Portugal), and the VST is a mixture of VST from Loureiro and Touriga Nacional (Portugal).

2.3 Physical-chemical characterisation of the by-products

Solids were chemically characterised by the content of humidity, ash, free sugars, soluble proteins, total protein, lipids, and fibre (cellulose, lignin, and hemicellulose) and inorganic minerals (barium, calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, strontium, and zinc).

2.3.1 Humidity determination

For humidity determination, 1 g of the by-product was weighed into a crucible, previously dried and weighed. The crucible with the substrate was placed in a hot air convection oven at 105 °C for 24 h and placed in a desiccator to cool down. The humidity was calculated according to equation 1.

$$H\ (\%) = \frac{WCHS - WCDS}{WCHS - WC} \times 100 \quad (1)$$

Where *WCHS* is the weight (g) of the crucible with humid waste; *WCDS* is the weight (g) of crucible with dry waste; *WC* is the weight (g) of the crucible.

2.3.2 Ashes content

A known quantity of the by-product (1 g) was added to a crucible (previously dried and weighted) and placed in a muffle furnace at 550 °C for 2 h. After cooling in a desiccator, the crucible was weight. Ashes content (grams of ash per 100 g of dry solid) is calculated by equation 2.

$$Ash\ (\%) = \frac{WCA - WC}{(WCHS - WC) \times (1 - H)} \times 100 \quad (2)$$

In which: *WCA* is the weight (g) of the recipient with the ashes; *WC* is the weight (g) of the dry recipient; *WCHS* is the weight (g) of the recipient with the wet sample; *H* is the sample humidity.

2.3.3 Lignocellulosic characterization

To determine the content of cellulose, hemicellulose. and lignin in each by-product a quantitative hydrolysis was performed. The samples were digested with 72 % (v/v) sulphuric acid (1 m L of sulphuric acid to 0.1 g solid by-product) at 30 °C for 1 h and diluted to 4 % (w/w) with distilled water. The samples were autoclaved at 121 °C for 1 h and, after cooling, filtered into Gooch crucibles and dried at 105 °C for 24 h. The filtrate was used to quantify glucose, arabinose, xylose and acetic acid by HPLC using an Aminex HPX-87H column (300 mm × 7.8 mm, particle size 8 µm) at 60 °C and coupled to a UV detector (λ = 210 nm). The eluent used was 0.1 M sulphuric acid. The analysis was carried out at a flow rate of 0.5 mL /min. The concentration of sugars in the samples was obtained using calibration curves for glucose, xylose, arabinose and acetic acid.

Using the data of sugar concentrations, the content in polymers was calculated. The content in polymers of glucan (CGn), xylan (CXn), arabinan (CArn), and acetyl groups (CGA) were calculated according to equation 3 (expressed as grams of polymer per 100 grams of dry substrate).

$$Polymer\ content\ (\%) = F \times SCF \times \frac{[S]}{\rho} \times \frac{W + WHS \times H}{WHS\ (1 - H)} \times 100 \quad (3)$$

Where: *F* is a factor that corrects sugar degradation (1.04 for CGn, 1.088 for CXn/Carb and 1.00 for CGA); *SCF* is a stoichiometric correction factor to account for the increase in molecular weight during

hydrolysis (162/180 for CGn, 132/150 for CXn/CArn and 43/60 for CGA); *[S]* is the concentration of the monomer in g/L; ρ is the density of the analysed dissolution in g/L (as the samples are usually diluted in water for HPLC analysis, the ρ value is approximately 1000 g/L); *W* is the weight of added water in grams corrected to account for losses during the autoclave heating; WHS is the total weight of the residue in grams; H is the humidity content of the residue in g of water/g of wet by-product.

Cellulose and hemicellulose concentrations were obtained according to equation 4 and 5.

$$Cellulose\ (\%) = CG_n \quad (4)$$

$$Hemicellulose\ (\%) = CX_n + CAr_n + CG_a \quad (5)$$

The weight increase of the Gooch crucible corresponds to Klason lignin, and as a result, the lignin content (expressed in grams of lignin per 100 g of dry by-product) was determined using equation 6.

$$Lignin\ (\%) = \frac{WCDS - WC - Wash}{WCHS\ (1 - H)} \quad (6)$$

Where *WCDS* is the weight of Gooch crucible with dry solid filtrate in grams; *WC* is the weight of dry Gooch crucible in grams; *Wash* is the weight of ash, *WCHS* is the weight of Gooch crucible with humid initial sample in grams and *H* is the humidity of the solid.

2.3.4 Soluble protein determination

The soluble protein was extracted using distilled water (1 g dry solid to 10 mL distilled water), followed by incubation for 30 min at room temperature and 200 rpm of agitation. The mixture was then filtered through a nylon mesh and the filtered liquid was centrifuged at 8000 rpm for 10 min. The soluble protein in the filtrate was quantified using the Bradford method (Bradford, 1976), according to the following protocol: 150 µL of the previously extracted sample and 150 µL of "Coomassie Blue" reagent were placed in each well of a microplate. After shaking the plate for 30 seconds and incubating it for 10 min at room temperature, in the dark, the absorbance was measured at 595 nm. Protein concentration was obtained using a calibration curve constructed with standard solutions of bovine serum albumin.

2.3.5 Reducing free sugar determination

Extraction of the substrate for the determination of free sugars was performed as described in the previous section. Free sugars were determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Test tubes containing 100 µL of the extracted sample and 100 µL of DNS were placed in a hot water bath (100 °C for 5 min). The samples were cooled and 1 mL of distilled water was added. Absorbance was measured at 540 nm. The concentration of free sugars was obtained using a calibration curve using glucose as standard solutions.

2.3.6 Nitrogen determination

The total nitrogen content was determined by the Kjeldahl method (Kjeltec system; digestor model 1015 and distillation models 1026; Tecator Systems, Hoganas, Sweden) and total protein content was obtained using the factor 6.25.

2.3.7 Total lipid content determination

The total lipid content was quantified by the Soxhlet method using petroleum ether as solvent, at 70 °C (FOSS SoxTec 8000 Extraction Systems).

2.3.8 Mineral content determination

The content of minerals, such as barium, calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, strontium, and zinc of RH, BSG, and VST were determined by ICP-OES (Optima 8000 ICP-OES model, PerkinElmer, Inc.) after microwave assisted digestion (SpeedWave 4, Berghof). Briefly, 10 mL 69 % (v/v) HNO_3 and 2 mL 30 % (v/v) H_2O_2 were added to 500 mg of RH, 10 mL 69 % (v/v) HNO_3 was added to 600 mg of BSG, and 69 % (v/v) 10 mL HNO_3 and 3 mL 37 % (v/v) HCl were added to 250 mg of VST. Samples were digested at 170 °C for 10 min at 30 bar, followed by an digestion at 200 °C for 15 min, for RH and BSG. For VST, samples were digested at 200 °C for 35 min at 35 bar. The operation conditions of analysis were the following: radio frequency power of 1300 W, argon plasma flow of 10 L/min, auxiliary gas flow of 0.2 L/min and nebulizer gas flow of 0.7 L/min. Calibration solutions, with the exception of phosphorus, were prepared from a multi-element standard solution (Quality Controls STD-As 23 elements, 1000 µg/mL). Phosphorus calibration solution was prepared from phosphoric acid 85 % (v/v) H_3PO_4. Samples were diluted to an acid concentration of approximately 2.5 % (v/v) and filtered with 0.2 µm nylon filters. 2.5 % (v/v) HNO_3 was used as blank.

2.4 Solid-state fermentations

The SSF experiments were made in 250 mL Erlenmeyer flasks using 5 g of dry solid. Sterilisation was carried out at 121 °C for 15 min. Inoculation was made with 2 mL of spore solution of *A. niger* CECT 2088 with a concentration of 10^6 spores/mL. The initial humidity was adjusted to 75 % (w/w) with distilled water prior to sterilization. Flasks were incubated for 7 days at 25 °C.

2.4.1 Phosphate and nitrogen supplementation effect

The study of the effect of nitrogen and phosphorus supplementation on enzyme production was performed with RH and BSG. For that, SSF experiments were made with the addition of 2 % (w/w) $(NH_2)_4SO_4$ and 1 % (w/w) K_2HPO_4 to the wet solid. These were tested one at a time and in combination, and a control test without supplementation was also performed. All conditions were tested in duplicate.

2.4.2 Humidity effect

The effect of initial humidity of the SSF medium on enzyme production was studied at the conditions of 60 % (w/w), 70 % (w/w), 75 % (w/w), and 80 % (w/w). Each condition was tested in duplicate, using the substrate (10 mm RH) supplemented with 2 % (w/w) $(NH_2)_4SO_4$ and 1 % (w/w) K_2HPO_4.

2.4.3 Particle size effect

To test the influence on enzyme production of different particle sizes of each by-product, these were ground and divided, by passing them through sieves, in three different granulometries: 10 mm, 4 mm, and 1 mm. SSF was performed with the three solids sizes for each by-product, under the previously mentioned conditions of humidity, temperature, and fermentation time and using the substrate supplemented with 2 % (w/w) $(NH_2)_4SO_4$ and 1 % (w/w) K_2HPO_4.

2.4.4 Experimental design

To evaluate the best mixture of by-products to improve the production of amylase, β-glucosidase, cellulase and xylanase by *A. niger* CECT 2088, a *Simplex-Centroid* mixtures design was carried out. The design comprises a set of experiments that includes runs involving the individual factors, a combination of two factors at equal proportions, and a central point with equal proportions of all by-products. Hence,

this design allows the test of different agro-industrial by-products as substrates and the assessment of their interaction effects in SSF. The components and proportions of each experiment are shown in table 4. In this assay, all mixtures were supplemented with 2 % (w/w) $(NH_2)_4SO_4$ and 1 % (w/w) K_2HPO_4 and BSG and RH of 10 mm particle and VST of 4 mm particle size were used as substrate. In total, nine assays were made, with the central point (33.3 (w/w) % BSG, 33.3 % (w/w) RH and 33.3 % (w/w) VST) repeated three times.

The equations below (equations 7 and 8) represent the models that best fit the experimental results. The linear model (equation 7) was used for xylanase, cellulase and β-glucosidase, while a quadratic model was used for amylase (equation 8).

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 \quad (7)$$

$$Y = \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{1\,2} x_1 x_2 + \beta_{1\,3} x_1 x_3 + + \beta_{1\,2\,3} x_1 x_2 x_3 \quad (8)$$

Where Y represents the predicted response variable (enzyme activity per mass of dry solid mixture, U/g), β are the regression coefficients of the model and x the independent variables (mass fraction of each by-product).

In all the assays, inoculation, incubation parameters and extractions were performed as described previously.

2.4.5 Kinetics of enzyme production

The kinetics of enzyme production by SSF was analysed. Additionally, to the enzymes under study (amylase, β-glucosidase, cellulase and xylanase), protease activity was also determined. Fermentations were conducted over a 14-day period, using the best operation condition that resulted from the experimental design (100 %(w/w) BSG). SSF experiments were conducted using the same conditions mentioned above (75 % humidity, 25 °C). The final humidity of the fermented solid was determined as described before. Free sugars, soluble protein, and fibre content were also quantified as described before.

2.5 Analysis of enzyme activity

The enzymatic activity of xylanase, cellulase, β-glucosidase, amylase, and protease was determined from the crude extract resulting from SSF.

To extract the enzymes from the substrate solid, a liquid-solid extraction was performed with distilled water (10 mL water to 1 g dry solid) for 30 min under agitation of 200 rpm and 20 °C. The liquid extract was obtained by filtering the mixture through a fine mesh filter and centrifuging for 10 min at 4 °C and 7000 rpm. Possible residues were further removed by subsequent centrifugation (10000 rpm; 10 min; 20 °C). The resulting supernatant was stored at -20 °C until further analysis. For all assays, enzyme activity was determined in duplicated.

2.6 Enzyme quantification

2.6.1 Xylanase activity

Xylanase activity was determined using 1 % (w/v) xylan as a substrate in 50 mM sodium citrate buffer pH 4.8. The substrate (250 µL) was incubated with the sample (250 µL) at 50 °C for 15 min. The released xylose was quantified using the DNS method, as follows: 500 µL of DNS was added and the mixture was incubated at 100 °C for 5 min, after cooling the samples, 1 mL of distilled water was added and the absorbance was measured at 540 nm. Xylanase activity was calculated using a calibration curve of xylose standard solutions (g/L) and the equation 9:

$$Activity\ (U/g) = \left(\frac{\Delta\mathrm{Abs} - Y_{intercept}}{\mathrm{slope}}\right) \times \left(\frac{\mathrm{M} \times V_{total} \times \mathrm{df} \times 1000}{\mathrm{time}}\right) \times \frac{\mathrm{V_{extracted}}}{Weight_{\ solid}} \qquad (9)$$

Where $\Delta\mathrm{Abs}$ is the difference between the sample absorbance and the blank absorbance; M is the molecular mass of xylose (g/mol); V_{total} is the total reaction volume (mL); df is the dilution factor of the sample; time is the incubation time (min), $\mathrm{V_{extracted}}$ corresponds to the total volume of distilled water used in the extraction (mL) and $weight_{\ solid}$ to the dry weight of fermented solid that was extracted (g).

One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 µmol/min of xylose, in the conditions of the assay. Enzyme activity was expressed in U per gram of dry residue (U/g).

2.6.2 Cellulase activity

To quantify cellulase (endo-glucanase) activity, 250 μL of 2 % (w/v) carboxymethylcellulose (CMC) in 50 mM sodium citrate buffer pH 4.8 was incubated with the 250 μL of the crude extract at 50 °C for 30 min. The DNS method was used to quantify the released glucose using the same protocol as the one described for xylanase activity. Cellulase activity was calculated using a calibration curve of glucose standard solutions and equation 10, where $Y_{intercept}$ and slope are the origin ordinate and slope of the glucose calibration curve (g/L).

$$Activity\,(U/g) = \left(\frac{\Delta\mathrm{Abs} - Y_{intercept}}{\mathrm{slope}}\right) \times \left(\frac{\mathrm{M} \times V_{total} \times \mathrm{df} \times 1000}{\mathrm{time}}\right) \times \frac{\mathrm{V_{extracted}}}{Weight_{\,solid}} \qquad (10)$$

Where $\Delta\mathrm{Abs}$ is the difference between the sample absorbance and the blank absorbance; M is the molecular mass of glucose (g/mol); V_{total} is the total reaction volume (mL); df is the dilution factor of the sample; time is the incubation time (min), $\mathrm{V_{extracted}}$ corresponds to the total volume of distilled water used in the extraction (mL) and $weight_{\,solid}$ to the dry weight of fermented solid that was extracted (g).

One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 μmol/min of glucose, under the conditions of the assay. Enzyme activity was expressed in U per gram of dry residue (U/g).

2.6.3 β-Glucosidase activity

The activity of β-glucosidase was determined by incubating 100 μL of substrate 4 mM 4-nitrophenyl-β-D-glucopyranoside (pNPG) in 50 mM sodium citrate buffer, pH 4.8 with 100 μL of the crude extract at 50 °C for 15 min. To stop the reaction, 0.6 mL of sodium carbonate 1 M was added, following the addition of 1.7 mL distilled water to dilute the sample. The released p-nitrophenol was quantified at 400 nm. β-glucosidase activity was calculated using a calibration curve of p-nitrophenol standard solutions and equation 11.

$$Activity\,(U/g) = \left(\frac{\Delta\mathrm{Abs} - Y_{intercept}}{\mathrm{slope}}\right)\left(\frac{\mathrm{M}\,V_{total}\,\mathrm{df}}{\mathrm{time}\;1000}\right)\left(\frac{\mathrm{V_{extracted}}}{Weight_{\,solid}}\right) \qquad (11)$$

Where $\Delta\mathrm{Abs}$ is the difference between measured absorbance and the blank, $Y_{intercept}$ and slope are the origin ordinate and slope of the p-nitrophenol calibration curve (μg/mL); M is the molecular mass of p-nitrophenol, V_{total} is the total reaction volume (mL); df is the dilution factor of the sample; time is the incubation time (min), $\mathrm{V_{extracted}}$ corresponds to the total volume of distilled water used in the extraction (mL) and $weight_{\,solid}$ to the dry weight of fermented solid that was extracted (g).

One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 μmol/min of p-nitrophenol, under the conditions of the assay. Enzyme activity was expressed in U per gram of dry residue (U/g).

2.6.4 Amylase activity

Amylase activity was quantified using 2 % (w/v) starch in 50 mM sodium acetate pH 5.5 buffer, according to the protocol: 250 μL of the starch substrate was incubated with 250 μL of the sample at 40 °C for 30 min. The DNS method was used to quantify the released maltose using the same protocol as the one described for xylanase activity. Amylase activity was quantified using a calibration curve of maltose standard solutions and was and calculated by equation 12, where $Y_{intercept}$ and slope are the origin ordinate and slope of the maltose calibration curve (g/L).

$$Activity\ (U/g) = \left(\frac{\Delta\mathrm{Abs} - Y_{intercept}}{\mathrm{slope}}\right) \times \left(\frac{\mathrm{M} \times V_{total} \times \mathrm{df} \times 1000}{\mathrm{time}}\right) \times \frac{\mathrm{V_{extracted}}}{Weight_{\,solid}} \qquad (12)$$

Where $\Delta\mathrm{Abs}$ is the difference between the sample absorbance and the blank absorbance; M is the molecular mass of maltose (g/mol); V_{total} is the total reaction volume (mL); df is the dilution factor of the sample; time is the incubation time (min), $\mathrm{V_{extracted}}$ corresponds to the total volume of distilled water used in the extraction (mL) and $weight_{\,solid}$ to the dry weight of fermented solid that was extracted (g).

One unit of enzyme activity (U) was defined as the amount of enzyme needed to release 1 μmol/min of maltose, under the conditions of the assay. Enzyme activity was expressed in U per gram of dry residue (U/g).

2.6.5 Protease activity

Protease activity was quantified using 0.5 % (w/v) azocasein dissolved in 50 mM sodium acetate buffer pH 5 as a substrate. A volume of 500 μL of enzyme extract was incubated with 500 μL of substrate for 40 min at 37 °C. After adding 1 mL of 10 % (w/v) trichloroacetic acid (TCA), the solution was centrifuged (3000 rpm, 15 min). The supernatant was, then, collected and 1 mL of 50 mM potassium hydroxide (KOH) was added. The absorbance was read at 428 nm. Enzyme activity was calculated according to equation 12.

$$Activity\,(U/g) = \left(\frac{\Delta\mathrm{Abs}}{0.01 \times \mathrm{time} \times \mathrm{Vsample}}\right) \times\ df\ \times\ Vext\ \times\ \frac{100}{Ms} \qquad (12)$$

Where $\Delta\mathrm{Abs}$ is the difference between the absorbance measured and the absorbance of the blank; time is the incubation time (min), V_{sample} is the sample volume that was used in the quantification (mL); df is the dilution factor of the sample; $\mathrm{V_{extracted}}$ corresponds to the total volume of distilled water used in the extraction (mL) and $weight_{\,solid}$ is the dry weight of fermented solid that was extracted (g).

One unit of protease activity (U) is defined as the amount of enzyme that produces a change in absorbance of 0.01 per minute, under the reaction conditions. Enzyme activity was expressed in U per gram of dry residue (U/g).

In addition to xylanase, β-glucosidase, cellulase, amylase and protease activity quantification, in the early stages of this study lipase was also quantified, however, the enzyme activity detected was too low to justify the inclusion of this analysis in this work. There were also attempts to quantify laccase, but once again there was no activity detected.

2.7 Statistical analysis

All results are presented as the mean ± standard deviation (SD) of two replicates. Microsoft Office Excel software was used to perform these analyses. Statistically significant differences were analysed by one-way ANOVA, applying the Tukey multiple-comparisons test. A significant difference was considered for $p \leq 0.05$. The experimental design was analysed using Statgraphics Centurion XVI software (Manusgisties, Inc, Rockville MD).

3 RESULTS AND DISCUSSION

3.1 Physical - chemical characterization of by-products

Substrate composition is of major importance to assess the feasibility of its use in SSF as it impacts the production of the compounds of interest (Costa et al., 2022). The solid substrate acts as a physical support and source of nutrients for microorganisms during the fermentation process (Leite et al., 2021). Table 2 shows the characterization of RH, VST and BSG obtained in this study and the by-products characterization according to the literature.

Table 2. Characterization of RH, VST and BSG. Experimental values are the mean ± standard deviation of replicates. Values with the same letter in each row do not present statistically significant differences ($p \leq 0.05$). Values from literature taken from the following references: Amorim et al., 2004; Jin et al., 2022; Meneses et al., 2013; Mohidem et al., 2022; Nabais et al., 2010; Pedro Silva et al., 2004; Sun et al., 2020; Wei et al., 2022; Zaid and Ganiyat, 2008.

PARAMETERS (% w/w, dry basis)	**EXPERIMENTAL VALUES**			**LITERATURE VALUES**		
	RH	**VST**	**BSG**	**RH**	**VST**	**BSG**
Total protein	2.5 ± 0.1^{a}	3.4 ± 0.1^{a}	15.4 ± 1.2^{b}	3.1	3.4 – 4.2	15.2 – 23.9
Soluble protein	0.02 ± 0.01^{a}	0.03 ± 0.02^{a}	0.03 ± 0.04^{a}	-	2.5	23.1
Total lipids	0.4 ± 0.3^{a}	0.3 ± 0.1^{a}	5.3 ± 0.6^{b}	1.5 – 1.7	0.4 – 0.7	4.2 – 6.5
Ashes	12.6 ± 0.3^{a}	2.7 ± 0.1^{b}	2.6 ± 0.2^{b}	20.4	1.9	3.1 – 3.4
Cellulose	37.1 ± 0.1^{a}	39.7 ± 0.1^{a}	40.9 ± 1.1^{a}	35.9	33.5	21.7
Hemicellulose	19.8 ± 1.3^{a}	22.7 ± 0.1^{a}	14.9 ± 0.4^{a}	20.3	21.7	19.2
Lignin	25.1 ± 6.8^{a}	21.0 ± 0.2^{a}	6.6 ± 3.6^{b}	13.3	14.8	19.4
Free sugars	0.13 ± 0.01^{a}	2.4 ± 1.7^{b}	1.1 ± 0.3^{c}	-	42	15.2

BSG is the by-product with the highest total protein content, with 6-fold more total protein than RH and 4.5-fold than VST. However, there were no statistically significant differences between the soluble protein content among the solids. VST is the by-product with the more free sugars content followed by BSG. RH free sugars content is very low when compared to the other two solids (VST has 17-fold and BSG has 7-fold more sugar content than RH). RH have also a low content of soluble protein. BSG also presents the highest content of total lipids, with RH and VST presenting a significantly lower lipid content. Considering the lignocellulosic characterization, the content of cellulose and hemicellulose did not vary significantly ($p \leq 0.05$) between the three solids. This content is of particular importance, as cellulose and hemicellulose induce the production of enzymes, specifically cellulases and xylanases (Leite et al., 2016). On the other hand, although RH and VST have similar lignin content, lignin content in BSG is significantly lower. RH ash content is 4-fold higher than in the other by-products.

Comparing the results obtained in this study with the ones presented in the literature, it is possible to conclude that the total protein content of the three solids is similar to the ones reported in literature. The same occurs for lipid content. Ash content, which represents the inorganic compounds of the solids, of RH is 38 % lower than the value reported by the literature. BSG ash content is also lower that the values reported in the literature. The mineralogical composition of these two solids is also lower than that reported in the literature (table 3). This can be explained by the lack of minerals in the soil where the rice, from which the husk used in the study was removed, was planted. It may also have been due to the use of chemicals, such as pesticides, in the soil where the rice plant reported in the literature grew (Hasan et al., 2022). However, VST and BSG ash content is similar to the values reported by the literature. Regarding the fiber content of the by-products, the cellulose and hemicellulose content of RH and VST are like the ones reported by the literature. However, the BSG cellulose and hemicellulose contents obtained in this experiment are different than the ones in the literature. Cellulose content is about 2-fold, higher and hemicellulose content is 25 % lower than the ones reported. Free sugar and soluble protein contents obtained on this experiment are also lower than what is reported by the literature. Baiano et al (2023) report that more than one cereal can be used in the production of beer, and Harris and Smith et al (2006) reported the composition of the cell wall varies from species to species. Therefore, the use of different cereals implies a different composition of lignin, cellulose, hemicellulose and soluble protein.

The differences between free sugars reported in the literature and in this study may be related to inadequate storage of BSG when it was removed from the tank where the must was stored. At this time, the BSG is quite wet and when stored at room temperature it could accidentally grow fungus that

degraded some of the free sugars. However, drying it allowed the BSG to have a very low humidity content, which made fungal growth impossible.

Minerals are essential for the growth of microorganisms (Spann and Schumann, 2010). Thus, the mineralogical composition of the substrates will also influence the growth of the fungus and, consequently, the production of enzymes. Table 3 represents the mineral composition of RH, VST, and BSG.

Table 3 . Mineral elements in RH, BSG, and VST composition. Values from literature taken from the following references: Carmona et al., 2013; Jin et al., 2022; Li et al., 2014; Mansouri et al., 2012; Mendívil et al., 2013; Meneses et al., 2013; Prakongkep et al., 2013; Ziarati and Azizi, 2013.

MINERALS (mg/Kg)	**EXPERIMENTAL VALUES**			**LITERATURE VALUES**		
	RH	**BSG**	**VST**	**RH**	**BSG**	**VST**
Barium	6.4	12.8	36.6	15	8.62	-
Calcium	2686.4	615.2	7770.2	2245	3600	2400
Copper	13.3	13.1	31.6	10	11.40	-
Iron	77.0	99.6	61.5	43	111	43
Potassium	962.4	1799.1	3024.1	1150	1618	800
Magnesium	2179.6	572.0	835.0	900	1900	1000
Manganese	42.3	80.1	40.4	35	40.0	-
Sodium	1020.5	1521.2	177.0	29628	369	20-100
Phosphorus	6626.0	680.1	965.2	1033	4603	4270
Strontium	6.0	3.1	23.4	-	10.36	-
Zinc	79.4	112.7	33.6	35	82.10	100

According table 3, RH is the by-product with the lowest concentration of barium, potassium and manganese. However, it is the by-product with the highest magnesium and phosphorus content. The silica content, quantified by the supplier, was 5.7 % (w/w). Todkar et al (2016) reported that silica content could vary between 18-20 %. RH used in this experiment have less 97 % of sodium than what is reported by the literature.

The iron, manganese, sodium, and zinc content of BSG are higher while the calcium and strontium content are lower than that of the other solids. In general, the results obtained are according to the literature. Although the calcium and magnesium content are lower, and the sodium and phosphorus content are higher than that reported in the literature, there is not much discrepancy between the ash values obtained in this work and those in the literature. As mentioned earlier, these differences may be due to the use of BSG made up of different cereals, which have different compositions.

VST is the by-product with the highest content of barium, calcium and copper in its composition. On the other hand, it has the lowest iron, manganese, sodium and zinc. Comparing the experimental results obtained with the values in the literature, it is possible to conclude that the calcium and magnesium content of the VST analyzed in this work are lower than those presented in the literature, while the sodium and phosphorus content are higher than the values in the literature. As in the case of BSG, the difference between the literature values and the values obtained in VST is not expressed in the ash content, since this is in line with what is reported in the literature.

Leite et al (2021), reported that higher lignin concentration could inhibit fungi growth, which consequently could reduce enzyme production. Therefore, the lowest lignin content of BSG could the favorable for enzyme production. Additionally, Ferani de Souza et al (2001) conclude that substrate colonization by the fungus is faster when there are free sugars at the beginning of SSF, so the high concentration of free sugars of VST and BSG could also be favorable for enzyme production.

As RH is the solid with the highest ash content, it was expected that its mineral concentration would be higher than that of the other by-products. However, its mineral composition is mostly made up of silica. Ahmaruzzaman and Gupta et al (2011) reported that about 95 % of ash weight in RH corresponded to the silica. Therefore, it can be concluded that the higher ash content of RH is due to the high concentration of silica. However, this value is also lower than expected and reported in the literature. Macronutrients (nitrogen, potassium, magnesium and phosphorus) are essential to fungal growth and required in higher concentrations than micronutrients (such as iron, copper, zinc and manganese) (Steinberg, 1950). Therefore, it is expected that by-products with a higher concentration of macronutrients will obtain better results in fungal growth and enzyme production.

BSG is the by-product with the most total protein, potassium, and cellulose content, and with the lowest lignin value, and it also has a high free sugar concentration. Therefore, it is expected that BSG would be a suitable substrate for the production of enzymes. VST has a high potassium content and high soluble sugar concentration, which makes this substrate a potential option for the production of enzymes by SSF. As RH, is, nutritionally, the poorest substrate with low protein and sugar values, it is expected a lower enzymatic activity, than the other two by-products.

3.2 Effect of potassium and nitrogen supplementation on enzyme production

According to RH characterization (Table 2 and Table 3), this by-product is very poor in nitrogen and potassium, so the effect of supplementation with a source of nitrogen and a source of potassium was tested.

Figure 2 shows the effect of supplementation, in RH, with 1 % (w/w) K_2HPO_4, 2 % (w/w) $(NH_4)_2SO_4$ and the combination of both compounds, in xylanase, cellulase, and β-glucosidase activity obtained by SSF.

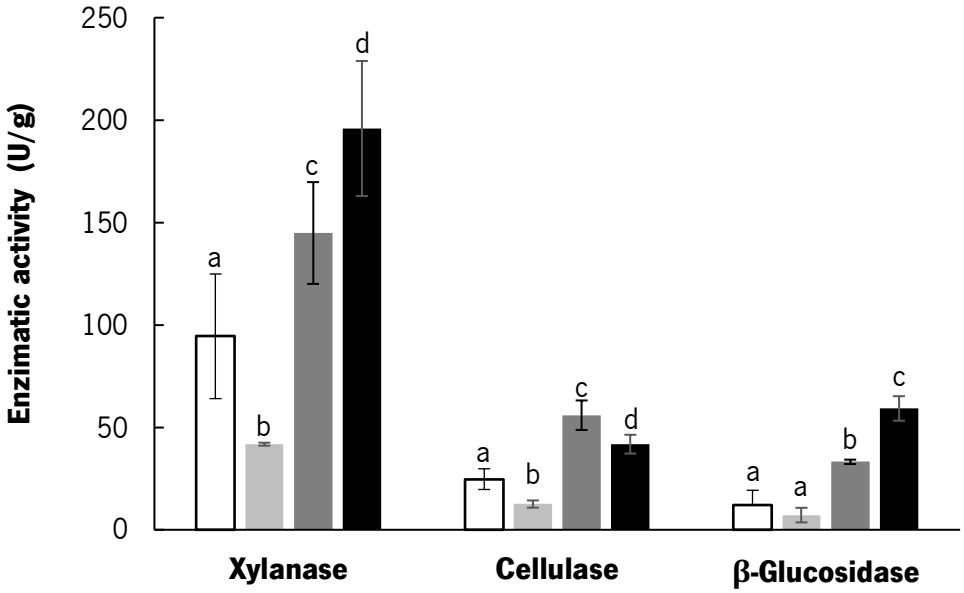

Figure 2. Production of cellulase, xylanase and β-glucosidase by *A. niger* CECT 2088, in RH, without supplementation (□) and with 3 different supplementations: 1% (w/w) K_2HPO_4 (▒), 2% (w/w) $(NH_4)_2SO_4$ (▓) and combination of 1% (w/w) K_2HPO_4 and 2% (w/w) $(NH_4)_2SO_4$ (■), after 7 days of incubation at 25 °C. The error bars represent the standard deviation of two independent replicates. Values with the same letter for the same enzyme do not present statistically significant differences ($p \leq 0.05$).

It is possible to observe that supplementation with both 1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$ was the condition that most increased the production of xylanase and β-glucosidase. Xylanase activity, increased about 2-fold, and β-glucosidase activity of 5-fold, when compared with the control fermentation (without supplementation). The condition that most increased cellulase activity was the supplementation with 2 % (w/w) $(NH_4)_2SO_4$, however, with both supplementations combined there was an increase of cellulase production of approximately 2.5-fold regarding the control test. Overall, considering the result for the three studied enzymes, the combination of 1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$, was considered the best supplementation condition.

After concluding that phosphate and nitrogen supplementation improved the production of enzymes under study using RH as a substrate, the effect of this supplementation (1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$) was study using BSG as substrate of SSF. This solid is poor in phosphorus, so K_2HPO_4 was used as a source of this mineral and although BSG has a higher nitrogen content than RH and VST, it is not known whether or not a higher concentration of nitrogen would improve the production of enzymes, so this substrate was also supplemented with a source of nitrogen ($(NH_4)_2SO_4$). The results are shown in Figure 3.

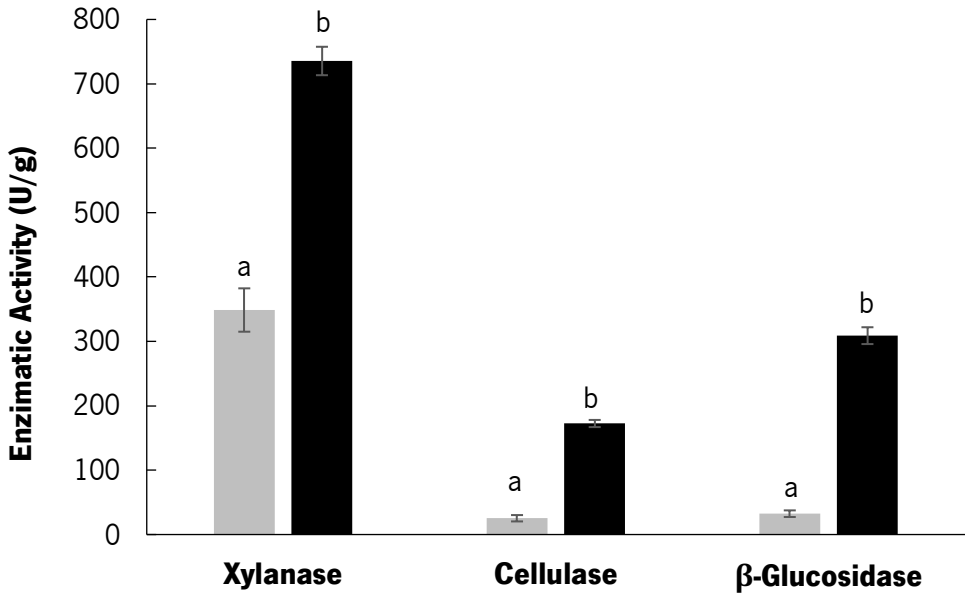

Figure 3. Production of cellulase, xylanase and β-glucosidase by *A. niger* CECT 2088 in BSG without supplementation (▒) and with supplementation: 1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$ (■), after 7 days of incubation at 25 °C. The error bars represent the standard deviation of two independent replicates. Values followed by the same letter in enzyme do not present statistically significant differences ($p \leq 0.05$).

The beneficial effect of supplementation with 1 % (w/w) K_2HPO_4 and 2 % of (w/w) $(NH_4)_2SO_4$ on enzyme production using BSG as substrate is clear, with the results showing a significant increase in the activity of all tested enzymes. Xylanase activity increased by 2-fold when compared to the control test, cellulase activity increased by 6-fold, and β-glucosidase increased by 8-fold. This result was already expected once BSG has a low content of phosphorus.

Analyzing the enzyme production of each supplemented by-products it can be concluded that there is a greater increase in enzyme activity when supplementing BSG than when RH is supplemented, although the opposite was expected. As BSG has a higher nitrogen content than RH, it was expected that the enzymatic activity of RH would increase more than BSG with $(NH_4)_2SO_4$ supplementation. However, RH has more 6-fold phosphorus than BSG. And BSG only has the double of potassium content than RH.

The increase in enzymatic activity when the medium was supplemented with a source of nitrogen and potassium was already expected, since nitrogen and potassium are essential macronutrients for fungi metabolism (Walker and White, 2017), and since the availability of the nitrogen source influences the formation of extracellular enzymes (Selim et al., 2017). As RH have low nitrogen and potassium content, it was expected that supplementation with ammonium sulphate, would increase fungal growth and consequently enzyme production, as it was verified in this study.

The positive effect of $(NH_4)_2SO_4$ supplementation has been reported by some authors. Bernal-Ruiz et al (2021) concluded that the production of xylanases by *Penicillium sp.*HC1 increased significantly when BSG was supplemented with 5g/L of $(NH_4)_2SO_4$. Xylanase activity increased 1-fold. Liu et al (2008) concluded that xylanase production by *A. niger* SL-05 increased 10 % when 2% (w/w) $(NH_4)_2SO_4$ was used. Selim et al (2017) also obtained a significant increase of xylanase, by *A. niger* production when 1 % (w/w) $(NH_4)_2SO_4$ was used. Oberoi et al (2014), when comparing inorganic and organic sources of nitrogen conclude that inorganic sources increased more the cellulase activity, by *A. niger* HN-2. In that study, it was also reported that β-glucosidase activity increased by 50 % when the medium was supplemented with 0.2 % (w/w) $(NH_4)_2SO_4$. Cahyaty et al. (2021), reported the positive effect of 2.5 % (w/v) $(NH_4)_2SO_4$ supplementation in recombinant xylanase production by *Pichia pastoris* KM71. It was also reported that cellulase production increased with increasing concentration of ammonium sulphate up to a concentration of 1 % (w/w). Furthermore, it was reported that the growth of *Bacillus subtilis* CBTK 106 and enzyme production was lower in a medium not supplemented with an inorganic nitrogen source (Krishna, 1999).

Although potassium is a very important macronutrient, as it regulates the volume and pH of cells (Shantappa et al., 2013), it was found that supplementation of by-products with 1 % (w/w) K_2HPO_4 affects negatively the production of the three studied enzymes. It was observed a decrease of 55 %, 48 %, and 41 % for xylanase, cellulase, and β-glucosidase, respectively, when compared to the non-supplemented control.

Other authors developed studies about the effect of different medium components on the enzymatic activity of xylanase. In a study using *A. niger* AS-1 in soybean hulls it was concluded that K_2HPO_4 had a positive effect on the production of this enzyme, but this effect was only observed up to a concentration of 0.04 %. For higher concentrations, the authors observed a decrease in the enzymatic activity of xylanase (Salihu, 2015). Thus, the decrease in the enzymatic activity observed in this assay, with only K_2HPO_4 supplementation, can be explained by the high concentration used (1 %).

According to the by-product's characterization, the contents of total protein, and thus nitrogen, in RH and VST are similar. Mineral analysis shows that, even though VST is richer in potassium than RH, it would not be necessary to supplement with a source of potassium. However, VST has a lower content of phosphorus. Thus, the results obtained with RH supplementation were extrapolated to the VST and K_2HPO_4 was assumed as a source of phosphorus. Therefore, the best supplementation condition (1% (w/w) K_2HPO_4 + 2% (w/w) $(NH_4)_2SO_4$) for RH and BSG was extrapolated to VST.

3.3 Humidity effect on enzymes production

The initial humidity content of SSF influences the fungi growth and enzymes production (Chutmanop et al., 2008). It is important to study the optimum initial humidity for each fungus, since a high level of humidity reduces the porosity of the substrate and limits the transfer of oxygen (Darabzadeh et al., 2019; Kheng and Omar, 2005). On the other hand, a low initial humidity level reduces the solubility of nutrients (Kheng and Omar, 2005) and inhibits fungal growth (dos Santos et al., 2012).

To understand which initial humidity was the most favourable for enzyme production, humidity contents of 60 % (w/w), 70 % (w/w), 75 % (w/w) and 80 % (w/w) were studied in SSF using RH supplemented with 1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$.

It was concluded that enzymatic activity was not affected by humidity values between 70 % (w/w) and 80 % (w/w). However, a significant decrease in enzyme activity was observed when the humidity was reduced to 60 % ($p \leq 0.05$), resulting in a decrease in enzyme activity of 24 %, 52 %, and 35 % for xylanase, cellulase, and β-glucosidase, respectively, when compared with enzyme activity obtained with 70 % (w/w) humidity.

Studies by other authors, concluded that the best initial humidity condition was 70 % (w/w) for the production of xylanase, cellulase and β-glucosidase by *A. niger* DFR-5 (Pal and Khanum, 2010). Selim et al (2017), reported that maximal xylanase activity, by *A. niger* was quantified at 80 % humidity.

Knowing that at the end of the 7 days of SSF, the loss of humidity was lower than 4 %, it was considered that the best initial humidity condition would be 75 % humidity. Although the influence of varying initial humidity on enzyme production was only analysed using RH, 75 % initial humidity was considered the best condition for SSF with VST and BSG as well.

3.4 Effect of substrate's particle size on enzyme production

One of the most critical parameters of the fermentation process is the granulometry of the substrate. The particle size influences its efficiency, so it is important to study the effect of this parameter to optimize the results. The size of substrate particles plays an important role in microbial colonization, air penetration and CO_2 removal (Manpreet, S. et al., 2005). Obtaining an ideal particle size, generally involves a compromise between nutrient accessibility and oxygen availability. Fungal colonization and growth, and thus enzymatic activity, depends on the accessible surface area and, thus of the particle size of the substrate (Poorna and Prema, 2007). Free particle size implies an increase in surface area, increasing the fungi's accessibility to the substrate. However, very small particles can agglomerate and limit the availability of oxygen between particles, interfering with fungal growth (Baysal et al., 2003; Pérez-Rodríguez et al., 2017).

Figure 4 shows the activity of cellulase, xylanase, and β-glucosidase produced by *A. niger* CECT 2088, in function of the particle size (1, 4, and 10 mm) of each by-product studied, during 7 days of incubation at 25 °C.

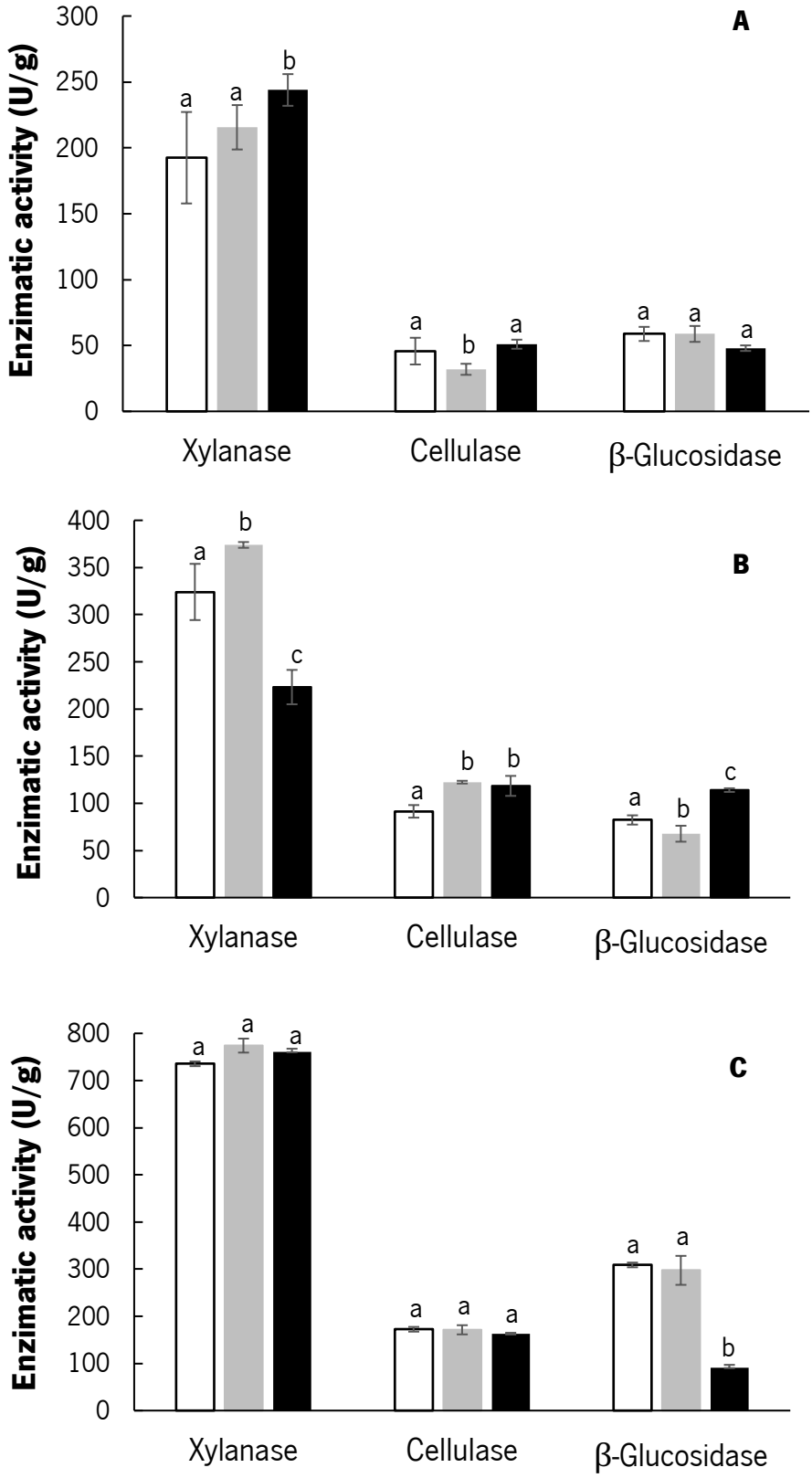

Figure 4. Production of xylanase, cellulase and *β*-glucosidase by *A. niger* CECT 2088 in (A) RH; (B) VST and (C) BSG of different particles' sizes, 10 mm (□), 4 mm (▒), 1 mm (■), during 7 days of incubation at 25 °C. All tests were supplemented with 1 % (w/w) K_2HPO_4 and 2 % (w/w)

$(NH_4)_2SO_4$. The error bars represent the standard deviation of two independent replicates. Values followed by the same letter in enzyme do not present statistically significant differences ($p \leq 0.05$).

In the assay using RH as substrate (Figure 4A), it was found that xylanase activity increased 27 % as the particle size decreased from 10 mm to 1 mm. Cellulase and β-glucosidase activity were not affected significantly ($p \leq 0.05$) when RH particle size decreased from 10 mm to 1 mm. There was a decrease of 30 % in cellulase activity when particle size decreased from 10 mm to 4 mm. Although there was an increase in xylanase activity with the reduction of RH particle size, it was considered that this increase in enzymatic activity does not justify the energy costs of free particle size. Therefore, for RH, 10 mm was determined as the best particle size, considering the particle sizes selected, to produce the enzymes under study.

VST was the by-product in which there was the more variation in enzymatic activity with the reduction in granulometry. The 4 mm particle size was the condition where the highest xylanase activity was quantified, resulting in an increase of 15 %, when compared with 10 mm particle VST. However, when the particle size was reduced to 1 mm, the enzyme activity fell by 40 %. Cellulase activity increased 34 % when particle size was reduced from 10 mm to 4 mm. No significant differences ($p \leq 0.05$) were found in cellulase activity with the reduction in particle size from 4 mm to 1 mm. The particle size of 1 mm was the condition in which the highest β-glucosidase activity was quantified. This value represents an increase of 39 % when compared to 10 mm and 69 % when compared to 4 mm. Even though 4 mm granulometry is the condition that produces less β-glucosidase, it is the best particle size to produce xylanase and cellulase, thus it was chosen for further studies.

BSG particle size reduction did not show any significant differences ($p \leq 0.05$) in the activity of xylanase and cellulase as the particle size decreased. However, it was observed a decrease of 70 % in the activity of β-glucosidase when the granulometry was reduced from 10 mm to 1 mm. Therefore, the largest particle size (10 mm) was defined as the best particle size for enzyme production.

Other authors reported the influence of particle size in enzyme activity. Srivastava et al (2022) concluded that larger particle sizes were better to maximize cellulase activity, achieving a the maximum cellulase activity with a particle size of 2.2 mm. Pérez-Rodríguez et al (2017) studied two particle sizes of VST, one ≤ 5 mm and the other ≤ 0.5 mm, and concluded that the activity of xylanase and cellulase was highest when the larger particle size was used.

The results obtained in this study are in accordance with those reported in the literature, since it was concluded that larger particle sizes are better for maximizing enzymatic activity.

Comparing the enzymatic activity obtained by the best condition of each by-product, it can be concluded that BSG was the substrate with the highest enzymatic activity obtained for all the three enzymes monitored, which was already expected, considering BSG's low content of lignin, high content of nitrogen and free sugars. On the other hand, RH was the substrate that led to the lowest enzyme production. This is in accordance with the recalcitrant nature of RH, with high content of ashes and low content of free sugars and proteins.

Other authors reported that BSG was a good substrate for enzyme production for the production of xylanase, cellulase, and β-glucosidase. Leite et al (2019), after comparing the production of these enzymes, by *A. niger* CECT 2088*,* from several substrates, including VST and BSG, concluded the BSG was the most suitable substrate for enzyme production (Leite, Belo, et al., 2021).

High lignin content of the substrate inhibits the activity of the enzymes under study. Berlin et al (2006) concluded that lignin reduced the activity of cellulase, β-glucosidase, and xylanase by 8 % to 84 %. Analysis of the ligning content of the substrates (Table 2) shows that the lignin content of RH and VST is similar, with no significant differences ($p \leq 0.05$), and that the lignin content of BSG is much lower when compared to the other substrates. Thus, the difference in enzymatic production yield between the three substrates under study can also be linked to with the different lignin contents.

Previous studies mentioned that enzyme-lignin interactions occur due to hydrophobic and electrostatic interactions. These enzymes are made up of two domains, one of which is a carbohydrate-binding molecule and the other a catalytic domain. The carbohydrate-binding module contains the charged and aromatic amino acids. It is also these charged amino acids that are involved in binding the enzyme-lignin (Berlin et al., 2006; Kellock et al., 2017). As the enzyme-carbohydrate binding site is the same as the enzyme-substrate bond, when the enzyme binds to lignin, it cannot bind to the substrate, therefore its activity is not quantified.

Leite et al (2019) reported that a high hemicellulose and cellulose content, favoured the production of xylanase and cellulase, respectively. However, the hemicellulose and cellulose did not vary significantly between the three solids.

3.5 Optimization of substrate mixtures in enzyme production

Although BSG is the substrate in which the highest enzymatic activity is obtained, it is also the substrate with most applications. There is a great interest in BSG because of its high protein content,

which makes this by-product suitable to be used in the feed and even for the food industry (Jackowski et al., 2020). In order to study the impact on enzymes production of a mixture of the by-products, a simplex-centroid mixtures design was used. The goal was to determine the optimum mixture to maximize enzyme production and valorise of the other two solids (RH and VST). As amylase has many applications in the food and textile industry, it was decided to also quantify its activity in the following set of experiments.

The simplex-centroid mixtures design of experiments was performed using BSG 10 mm, VST 4 mm, and RH 10 mm, all the solids were supplemented with 1 % (w/w) K_2HPO_4 and 2 % (w/w) $(NH_4)_2SO_4$, at initial humidity of 75 % (w/w) and 5 g of dry solid. SSF was conducted for 7 days at 25 °C. The results are shown in table 4.

Table 4. Substrate composition for each SSF experiment of the simplex-centroid design and results of the dependent variables (enzymatic activity) obtained.

RUNS	**INDEPENDENT VARIABLES**			**DEPENDENT VARIABLE**			
	By-products (%, w/w)			Enzymatic activity (U/g)			
	BSG	VST	RH	Xylanase	Cellulase	β-Glucosidase	Amylase
1	100	0	0	616	174	368	243
2	0	100	0	206	167	65	48
3	0	0	100	116	28	39	107
4	50	50	0	333	204	148	134
5	50	0	50	533	145	221	231
6	0	50	50	247	123	77	48
7	33.3	33.3	33.3	405	159	193	171
8	33.3	33.3	33.3	380	96	191	171
9	33.3	33.3	33.3	396	117	189	189

According to table 4, the use of BSG as a substrate produced the highest enzyme activity for xylanase, β-glucosidase and amylase, as it was concluded before. Moreover, in the case of binary

mixtures, the use of BSG in combination with the other 2 by-products significantly improved the enzymatic activity when compared to the enzymatic activity obtained with the other solids alone. The 50 % (w/w) BSG and 50 % (w/w) RH mixture produced a slightly lower amount of xylanases than BSG alone, but represents a 2.5-fold increase compared to 100 % (w/w) RH. The 50 % (w/w) BSG and 50 % (w/w) VST mixture was the condition with the highest cellulase activity. Although this value is similar to the ones obtained using 100 % (w/w) BSG and 100 % (w/w) VST. As already mentioned, higher β-glucosidase activity was obtained using 100 % (w/w) BSG. However, when RH was used in a binary mixture with BSG, the activity of β-glucosidase increased about 5-fold when compared with RH alone. The same was verified for amylase activity.

The mathematical models to maximize enzyme activity obtained for each enzyme by the simplex centroid mixture design are shown in table 5. For xylanase, cellulase and β-glucosidase a linear model fitted well to the data, and for amylase a quadratic model was applied.

Table 5. Mathematical models that maximize enzyme activity, p-value and R^2 of each fitting, and predicted optimal enzyme activity by the model.

ENZYME	MODEL	PREDICTED OPTIMAL ENZYME ACTIVITY (U/g)
Xylanase	Xylanase = 651.1*BSG + 209.1*VST + 216.8*RH $p \leq 0.05$ $R^2 = 0.82$	651
Cellulase	Cellulase = 189.1*BSG + 175.0*VST + 40.0*RH $p \leq 0.05$ $R^2 = 0.79$	189
β-Glucosidase	β-Glucosidase = 362.8*BSG + 62.7*VST + 71.16*RH $p \leq 0.05$ $R^2 = 0.91$	363
Amylase	Amylase = 237.7*BSG + 43.7*VST + 102.7*RH + 41.9*BSG*VST + 311.9*BSG*RH – 32.1*VST*RH $p \leq 0.05$ $R^2 = 0.96$	263

The statistical parameters of the model were also obtained. The determination coefficient, R^2, was between 0.79 and 0.96, which demonstrates that there was a good adjustment of the model, indicating that 79 to 96 % of the variability of the response is explained by the model. P- value was lower than 0.05, for the four enzymes analyzed, which explains a statistically significant relationship between enzyme activity of each enzyme and the components of the mixture, with a confidence level of 95 %.

Each independent variable impact on the activity of each enzyme is directly proportional to the values of the linear or quadratic regression coefficients. Positive coefficients mean that the by-products do not have an antagonistic effect on enzyme production while, negative coefficients indicate that the by-product or mixture of by-products has an antagonistic effect on the production of a particular enzyme. In linear models used for xylanase, cellulase, and β-glucosidase it is possible to see the influence of each substrate alone in enzyme activity, while the quadratic model, used for amylase activity, also shows the influence of binary mixtures on enzyme production. Therefore, it can be concluded that no substrate or mixture of substrates has a negative effect on the production of any enzyme, except for the 50 % (w/w) VST and 50 % (w/w) RH mixture that has an antagonistic effect on amylase production.

It should be noted that BSG is the by-product with the highest coefficient for four enzymes: xylanase, cellulase, β-glucosidase, and amylase, demonstrating its capacity to be used in enzyme production. The same can be seen in the contour plots shown in figure 5. The red region in the BSG apex of figure 5A and figure 5C indicates that the best substrate, predicted by the experimental design, for maximising β-glucosidase and xylanase activity is 100 % (w/w) BSG. Figure 5B shows that although the optimum substrate composition to maximise cellulase production is 100 % (w/w) BSG, the red region extends almost up to VST, indicating that a mixture of the two by-products is favourable for enzyme production. Figure 5D, on the other hand, shows that the optimum mixture for amylase production, predicted by the experimental design, is a mixture of BSG and RH. According to the adjusted model the optimal mixture to maximize amylase activity is 72 % (w/w) BSG and 28 % (w/w) RH.

The positive effect of BSG mixture with other substrates for SSF has been reported by several authors. Leite et al (2021) reported that the highest enzymatic activity of xylanases, cellulase and β-glucosidase was obtained with a mixture of crude olive pomace (COP) and BSG.

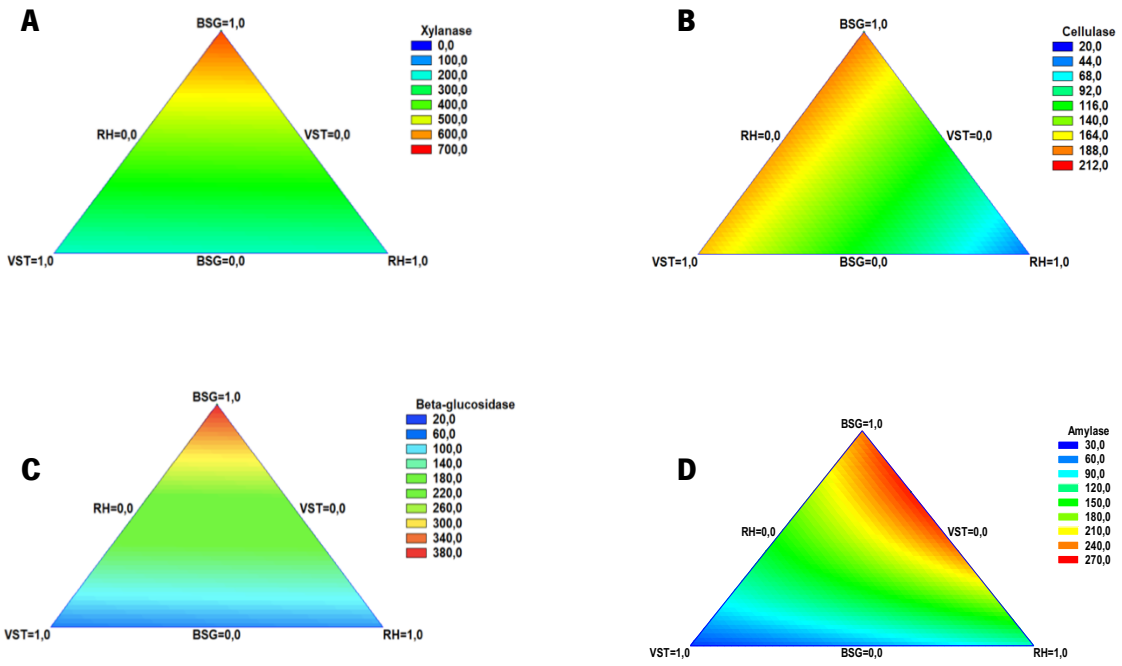

Figure 5. Contour plots for the dependent variables obtained in the simplex centroid mixture design. (A) xylanase, (B) cellulase, (C) β-glucosidase, and (D) amylase.

3.6 Enzyme production kinetics

After determining that BSG was, overall, the substrate that resulted in the best enzyme production, the enzyme production by SSF in BSG using *A. niger* CECT 2088 was evaluated after 0, 3, 5, 7, 10 and 14 days. Fermentations were conducted with BSG supplemented with 2 % (w/w) $(NH_4)_2SO_4$ and 1 % (w/w) K_2HPO_4, 75 % (w/w) humidity content and incubated at 25 °C. Figure 6 represents the enzymes production over time during SSF.

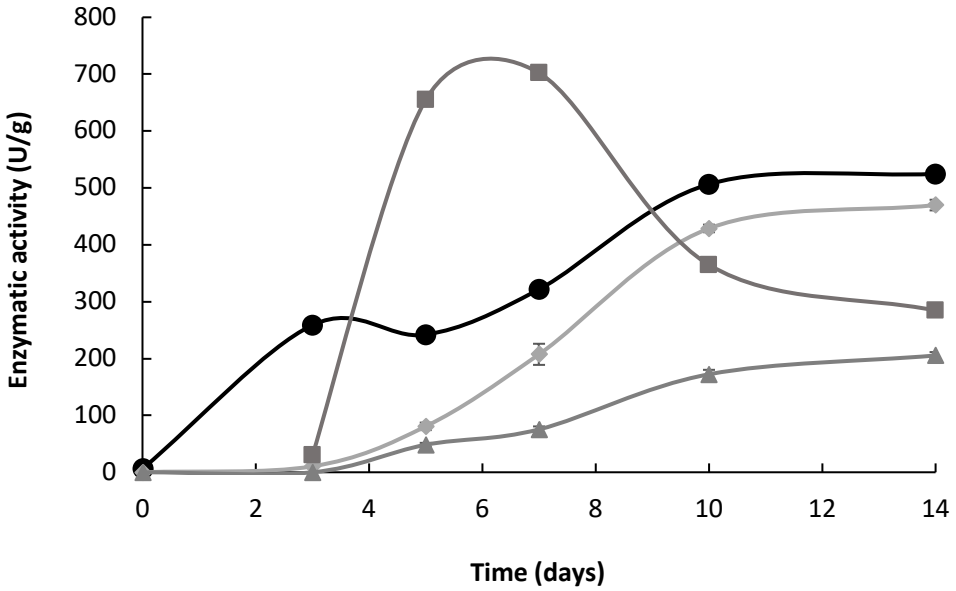

Figure 6. Enzymatic activity variation with time of xylanase (-■-), cellulase (-▲-), β-glucosidase (-◆-) and amylase (-●-), produced by SSF of BSG supplemented with 1 % (w/w) K_2HPO_4 + 2 % (w/w) $(NH_4)_2SO_4$, using *A. niger* CECT 2088. The error bars represent the standard deviation of two independent replicates.

Through the results obtain, it is possible to verify that on the fifth day, xylanase activity was at its maximum and remained at this maximum until the seventh day. However, it decreased significantly after 10 days and further decreasing after 14 days. This reduction in activity could be due to the action of proteases, but in this case, this was not verified since the measured protease activity was low in all the assays (less than 25 U/g). Thus, the loss of xylanase activity can be probably explained by its low stability. Fernandes et al (2022) studied the thermostability of cellulase and xylanase enzymes and concluded that xylanase is much more unstable with increasing temperature than cellulase, reporting a 36 % loss of activity when xylanases were kept at 45 °C for 2 hours.

Some authors have already reported that xylanase maximum activity is obtained after 6 or 7 days, and after which their activity starts to decline. Irfan et al (2014) reported that the maximum xylanases activity was obtained at 7 days, using *Trichoderma viride* - IR05 and that from that day onwards their activity decreased. Shata et al (2014) obtained the maximum activity of the xylanase produced by *A. niger* NRC 9A at day 6 and Pal and Khanum et al (2010) also found that maximum enzyme activity (2596 U/gds), using *A. niger* DFR-5, occurred in day 6, and after that, xylanase activity began to decline.

The activity of cellulase, amylase, and β-glucosidase were similar over time, with the 3 enzymes reaching their maximum activity at 10 days and maintaining their activity until 14 days. The maximum enzymatic activity of cellulase reported by Leite et al (2016) was 35 U/g was obtained after 11 days of SSF. On the other hand, Irfan et al (2012) obtained maximum amylase activity after only 4 days. Ramasamy et al (2011) reported that maximum amylase production using groundnut oil cake and *A. niger* BAN3E was reached after 6 days. Liguori et al (2021), reported a maximum xylanase activity (1315 U/g), using BSG after 4 days. However, cellulase maximal activity (118 U/g) was quantified on the 10th day. The maximal cellulase activity obtained in this study is about 4 - fold higher than the cellulase activity reported by Leite et al (2016). As for amylase, Irfan et al (2012) obtained the maximal amylase activity 6 days earlier than obtained in this study. The layer of lignin and hemicellulose in the by-product used for Irfan et al (2012) was smaller than the lignin and hemicellulose of BSG used on this study, so the fungus was able to easily penetrate the fibre to access the starch.

According to Heredia et al (1995), in the cell wall, the cellulose microfibrils are lined with other fibres such as lignin and hemicellulose. Xylanase is the first enzyme to be produced because in order for the fungus to penetrate the cellulose fibrils, thus it has to produce enzymes that hydrolyse the hemicellulose. Between the third and fourth day of fermentation, the fungus succeeds in penetrating the fibre and accessing the cellulose and starch. In this way, the production of cellulase and amylase begins to increase in order to hydrolyse the cellulose and starch into simpler sugars, which will be consumed by the fungus as a carbon source.

If the aim was to produce xylanase, fermentation should be carried out until the fifth day, which is the day when xylanase is most productive and its purification is easiest, since production of the other enzymes is still low. On the fifth day, the specific activity of xylanase was 1.87 U/mg $_{\text{soluble protein}}$. However, the specific activity of xylanase only peaked on day 7 (1.98 U/mg $_{\text{soluble protein}}$). The specific activity of xylanase increased up to day 7 and, from day 10 onwards, decreased to 0.64 U/mg $_{\text{soluble protein}}$.

In the case of the other enzymes, fermentation should take place until the tenth day, when the maximum values are reached. The variation of soluble proteins and free sugars throughout SSF is shown in Table 6. The free sugar values quantified over the 14 days of SSF correspond to the balance between the sugars consumed by the fungus as a carbon source and the sugars liberated by the enzymatic hydrolysis of the fiber (Varzakas et al., 2008). The decrease of 85 % in free sugars indicates that the rate of consumption of sugars by the fungus is higher than the rate of sugars production resulting from fiber degradation by enzyme activity.

The concentration of soluble proteins quantified also represents a balance between the proteins consumed by the fungus and the enzymes being produced. In the first 5 days of SSF, the soluble protein, decreased by around 17 %, despite the fact that xylanase activity was at its maximum. This indicates that during the first 5 days of SSF, the rate of protein consumption by the fungus was higher than the rate of enzyme production. However, from the fifth day on forward, the soluble protein content began to increase, increasing by 25 % until the end of the SSF. During this period, although the activity of xylanase decreased abruptly, the enzymatic activity of cellulase, amylase, and β-glucosidase increased. As a result, the balance between protein consumption and production was altered, with more protein being produced than consumed. Similary, Viniegra-González et al (2003) reported a decrease in soluble protein during SSF, due to the fungus' need for nitrogen.

Table 6. Variation in free sugars and soluble protein during a SSF with BSG, for 14 days at 25 °C, using *A.niger* CECT 2088. Experimental values are the mean of two replicates ± standard deviation.

TIME (DAYS)	FREE SUGARS (mg/g)	SOLUBLE PROTEIN (mg/g)
0	39.9 ± 1.8	422.9 ± 5.8
3	32.7 ± 4.5	407.8 ± 12.2
5	10.8 ± 0.8	350.0 ± 2.7
7	8.2 ± 0.6	354.9 ± 8.5
10	5.6 ± 3.5	439.3 ± 6.8
14	6.1 ± 0.4	442.2 ± 8.2

The variation in fibre content was also analysed over the 14 days of SSF (figure 7). The lignin content, as well as the hemicellulose content, remained constant throughout the 14 days of SSF. Cellulose content was the only value fibre that decreased, by around 35 %, after 14 days of SSF.

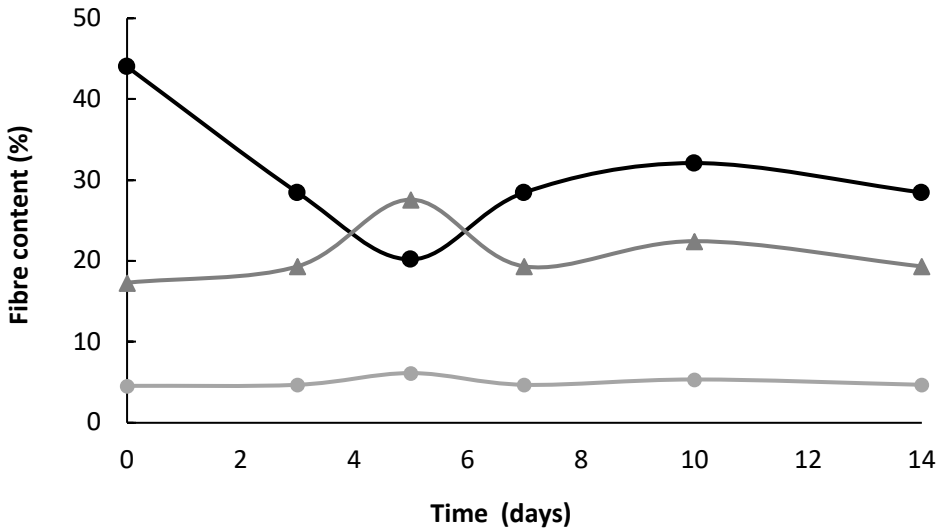

Figure 7. Variation of fibre content: lignin (—●—), hemicellulose (—▲—) and cellulose (—●—) for 14 days fermentation using BSG supplemented with 1 % (w/w) K_2HPO_4 + 2 % (w/w) $(NH_4)_2SO_4$ as substrate and *A. niger* CECT 2088, at 25 °C. The error bars represent the standard deviation of two independent replicates.

Some authors have studied the influence of the presence of lignin and hemicellulose on cellulose hydrolysis. It was reported by Valchev et al (2016), Loelovich et al (2011) and Turon et al (2008), that the degree of polymerisation of hemicellulose and lignin and their quantity is related to the hydrolysis of cellulose. As the amount of hemicellulose and lignin present in BSG under study are small comparing to the cellulose content (table 2), it is easy for the fungus to penetrate the fibre and hydrolyse the cellulose.

According to Houfani et al (2020) and Díaz et al (2011) the solubility of hemicellulose increases when it is exposed to heat treatment, such as the sterilization process. Thus, heat treatment increases the accessibility of the fungus to cellulose. However, comparing the hemicellulose content on day 0 of SSF (that has undergone sterilization) and the initial hemicellulose composition of the by-product (non-sterilized) (Table 2), it is possible to conclude that they are quite similar. Therefore, it is possible that sterilization had no effect on the solubilization of hemicellulose.

4 CONCLUSIONS:

The objective of this work was to produce lignocellulosic enzymes, speficically xylanase, cellulase, and β-glucosidase and amylase, by SSF from by-products obtained from the agro-industrial industry. The use of by-products in this biotechnological processes adds value to them and contributes to a circular economy.

This study proved that SSF with *A. niger* CECT 2088 is a suitable biotechnological process for the production of lignocellulosic enzymes from rice husk, vine pruning shoots, and brewer's spent grain.

When the by-products were supplemented with a source of nitrogen (ammonium sulfate) and a source of phosphorus and potassium (dipotassium hydrogen phosphate) the enzymatic activity obtained increased significantly. The study of granulometry, determined that the best particle size for enzyme production in RH and BSG was 10 mm, and that 4 mm was the best using VST. It was also confirmed that enzyme activity is affected by the initial humidity content of the SSF. However, the enzymatic activity remained constant when the huminity content of the SSF varied between 70% (w/w) and 80% (w/w).

The simplex centroid design allowed the determination of the best substract or mixture of substrates to maximize enzyme production. It was concluded that the best substrate composition, predicted by the experiemntal design, for the production of xylanase, cellulase, and β-glucosidase is 100% BSG, however, using a mixture of 50% (w/w) BSG/VST as substrate also achieved high cellulase production. The ideal mixture to maximize amylase activity was a mixture of 72 % (w/w) BSG and 28 % (w/w) RH. The optimal activities of each enzyme were obtained 651 U/g for xylanase, 189 U/g for cellulase, 363 U/g for β-glucosidase and 263 U/g for amylase.

When studing the kinetic's of enzyme production it was verified that maximum xylanase production was obtained on the 5th day of SSF and that cellulase, β-glucosidase, and amylase require 10 days of fermentation to reach maximum activity.

This work demonstrates that production of xylanase, cellulase, β-glucosidase, and amylase by SSF using BSG, RH and VST supplemented with 1 % (w/w) K_2HPO_4 + 2 % (w/w) $(NH_4)_2SO_4$, 75 % humidity and using *A. niger* CECT 2088, is a biotechnological process with potential for the production of lignocellulosic enzymes.

5 FUTURE PERSPECTIVES:

This work demonstrated the potential of using SSF for enzymes production, that have many industrial applications including in textile industry. Thus, it would be interesting to study the stability of the enzymes produced over time according to storage methods, as well as their purification.

The scale-up of SSF process should also be studied in order to subsequently apply this approach to industrial level. Different bioreactors could be tested, such as tray-type and rotary-drum, and otimization of operational conditions should be performed

The final composition of fermented and extrated solid by-products could also be studied in order to investigate their potential application to promote a circular economy and a zero waste integrated process.

Other byproducts can also be studied in order to produce other enzymes of industrial interest, such as for instance lipase and laccase.

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