Universidade do Minho
Escola de Engenharia

Helena Isabel Santos Vilela

Production of single-cell protein: species selection and optimization of operational conditions

outubro de 2023

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biológica
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Doutora Marlene Alexandra da Silva Lopes
e da
Professora Rita Isabel Couto Pinheiro

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STATEMENT OF INTEGRITY

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

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

PRODUÇÃO DE PROTEÍNA MICROBIANA: SELEÇÃO DE ESPÉCIES E OTIMIZAÇÃO DE CONDIÇÕES OPERACIONAIS

RESUMO

O crescimento atual da população mundial está a levar a uma procura insustentável por proteína animal e vegetal. De modo a responder ao desafio de alimentar o mundo, este trabalho focou-se na obtenção de um produto rico em proteína e com valor nutricional, obtido a partir da fermentação em estado sólido (SSF) de cascas de fruta (laranja e banana) por fungos filamentosos.

Inicialmente, foram estudados os efeitos de vários fatores (humidade - 60 % e 75 %; tempo de fermentação - 7 dias e 14 dias; tamanho do inóculo - 1×10^7 esporos/g e 5×10^7 esporos/g; concentração de sulfato de amónio - 0 g/g e 0.01 g/g; concentração de licor de maceração de milho (CSL) - 0 g/g e 0.01 g/g; e fungo filamentoso - *Aspergillus ibericus* e *Rhizopus oryzae*) na composição das cascas de fruta (nomeadamente proteína total, açúcares redutores, fenóis totais e atividade antioxidante) usando um desenho experimental de Plackett-Burman. Observou-se crescimento significativo das 2 espécies nos meios compostos por cascas de laranja e de banana. Para além disso, o conteúdo de proteína total, fenóis totais e atividade antioxidante das cascas de fruta aumentaram após a fermentação com os fungos filamentosos usados. A humidade (50 %, 60 %, 70 %), a concentração de sulfato de amónio (0 g/g, 0.005 g/g, 0.01 g/g) e a concentração de CSL (0 g/g, 0.005 g/g, 0.01 g/g) foram identificados como os fatores mais importantes para aumentar o valor nutricional das cascas de fruta (em particular o teor em proteína total) e foram posteriormente estudados através de um desenho experimental de Box-Behnken. Estes ensaios foram realizados com *Aspergillus ibericus* (concentração inicial de 5×10^7 esporos/g) durante 7 dias. O valor máximo de proteína total nas cascas de laranja fermentadas (16.29 %) foi obtido nas condições de 70 % de humidade, 0.005 g/g de sulfato de amónio e sem CSL. O valor máximo de proteína nas cascas de banana (17.67 %) foi obtido nas condições de 70 % de humidade, 0.005 g/g de CSL e sem sulfato de amónio. Por fim, as cascas fermentadas foram caracterizadas quanto ao seu teor em hidratos de carbono, açúcares redutores, fibra bruta, pectina, lípidos totais, minerais e atividade antioxidante. A SSF reduziu o conteúdo de hidratos de carbono, açúcares redutores e pectina das cascas, enquanto aumentou o conteúdo de proteína, fibra, lípidos, minerais, e atividade antioxidante, aumentando assim o seu valor nutricional.

Palavras-chave: Cascas de banana; Cascas de laranja; Fermentação em estado sólido; Fungos filamentosos; Proteína microbiana.

PRODUCTION OF SINGLE-CELL PROTEIN: SPECIES SELECTION AND OPTIMIZATION OF OPERATIONAL CONDITIONS

ABSTRACT

The actual growth of global population is leading to an unsustainable rising demand for animal and plant-based protein. To address this challenge, this work focused on the production of a nutritious and protein-rich product, obtained by solid-state fermentation (SSF) of orange and banana peels with filamentous fungi.

Firstly, the main effect of several factors (moisture content - 60 % and 75 %; fermentation time – 7 days and 14 days; inoculum size - 1×10^7 spores/g and 5×10^7 spores/g; concentration of ammonium sulfate - 0 g/g and 0.01 g/g; concentration of corn steep liquor (CSL) - 0 g/g and 0.01 g/g; and fungal species - *Aspergillus ibericus and Rhizopus oryzae*) on fruit peels composition (specifically, total protein, reducing sugars, total phenols, and antioxidant activity), were studied in SSF by a variable screening Plackett-Burman experimental design. A significant growth of both fungal species on orange and banana peels was observed. Additionally, the content of total protein, total phenols, and antioxidant activity increased in fruit peels after SSF with filamentous fungi. Moisture (50 %, 60 %, 70 %), ammonium sufate concentration (0 g/g, 0.005 g/g, 0.01 g/g), and CSL concentration (0 g/g, 0.005 g/g, 0.01 g/g) were considered important factors for upgrading the nutritional value of fruit peels (particularly protein content) and were studied with a Box-Behnken experimental design. These experiments were carried out with *Aspergillus ibericus* (initial concentration of 5×10^7 spores/g), for 7 incubation days. The highest protein content of fermented orange peels (16.23 %) was obtained with 70 % moisture content, 0.005 g/g ammonium sulfate, without CSL. For banana peels, the highest content of protein (17.49 %) was obtained with 70 % moisture content, 0.005 g/g CSL, without ammonium sulfate. Finally, the fermented fruit peels were characterized for carbohydrates, reducing sugars, crude fiber, pectin, total lipids, minerals, and antioxidant activity. Carbohydrates, reducing sugars, and pectin content of fruit peels decreased after SSF, while total protein, fiber, lipids, minerals, and antioxidant activities increased, enhancing the nutritional value of orange and banana peels.

Key words: Banana peels; Filamentous fungi; Microbial protein; Orange peels; Solid-state fermentation.

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A – Constant for experimental design models

a_i – Main effect for a factor x_i

a_{ij} – Interaction effect for factors x_i and x_j

ANOVA – Analysis of variance

AS – Ammonium sulfate

BP – Banana peels

CO_2eq – Carbon dioxide equivalent

CSL – Corn steep liquor

DNS – Dinitro-salicylic acid

DPPH – Diphenyl picrylhydrazyl

e.g. – *Exempli gratia* (for example)

EAAs – Essential amino acids

et al. – *Et alia* (and others)

FAO – Food and Agriculture Organization of the United Nations

GHG – Greenhouse gas

GRAS – Generally recognized as safe

i.e. – *Id est* (that is)

MUM – Micoteca da Universidade do Minho

OP – Orange peels

PDA – Potato dextrose agar

RNA – Ribonucleic acid

rpm – Rotations per minute

SCP – Single-cell protein

SDG – Sustainable Development Goal

SSF – Solid-state fermentation

v/v – Volume per volume

w/v – Weight per volume

w/w - Weight per weight

x_i – Factor or independent variable

Y – Response variable

CHAPTER 1. STATE OF THE ART

1.1. Current challenges and efforts for food production

The exponential demographic growth is putting too much pressure on Earth's resources, at risk of compromising the equilibrium of land and water systems. In 2019, the population reached the 7.7 billion mark, and it is estimated that, by 2030, this number will be close to 8.5 billion, 9.7 billion by 2050, and 10.9 billion by 2100 (United Nations, 2019). Currently, there are approximately 2.37 billion people facing food insecurity, roughly 30 % of the entire world population. This number includes the 720 - 811 million people that went through hunger in 2020, most of which live in Asian and African countries (FAO, 2022). Several factors contributed to the setback in the efforts made to reduce world hunger and poverty, such as climate change, the global COVID-19 pandemic, and, more recently, the Ukraine-Russia and Israel-Hamas wars. Due to COVID-19, 119 to 124 million people went through extreme poverty in 2020, and the same happened to an additional 175 to 228 million in 2021 (Lakner *et al.*, 2021). As a consequence of Ukraine-Russia war, Ukrainian companies reported an increase of 93 % of production costs for agriculture and of 76 % for livestock production, as well as damages, losses, land contamination, and difficulty to export commodities. Ukraine and Russia together have a 53 % share of the global sunflower oil and seeds market, 27 % for wheat, 23 % for barley, 16 % for colza seeds and 14 % for corn. From 2021 to 2022, food prices increased 34 %. Additionally, Russia is the largest and second-largest exporter of natural gas and oil, respectively, and, along with Belarus, they export around 20 % of the world's total fertilizers. In result, crude oil, gas, and fertilizers prices also increased drastically. These events had negative impact, particularly in developing countries, whose economies usually rely mainly on food and energy trades (FAO, 2022; FAO, 2023a; United Nations, 2022; UNCTAD, 2022).

The Food and Agriculture Organization predicts that it is unlikely that the global Sustainable Development Goals (SDG) for 2023 for eradicating hunger, malnutrition and food insecurities will be met since it would be necessary to increase the production of food, fiber, and biofuel to meet the needs of the estimated 2030's population. Furthermore, agriculture's productivity has declined in recent years due to the deterioration of land (from which 95 % of food is obtained) and water resources, mainly owing to agricultural activity itself. From 1961 to 2019, the land used for crop cultivation increased by 15 % (208 million ha), and this increase consisted of a substantial rise in the use of land destined to irrigation (110 %) and only an increase of 2.6 % of use of rainfed land. Irrigation for agricultural purposes accounts

for 70 % of total groundwater withdrawal, and groundwater accounts for 30 % of total water used for irrigation, increasing 2.2 % per year. This leads to an exhaustive exploitation of aquifers. Estimates indicate that 250 km^3 of aquifer capacity is lost every year (FAO, 2022). In addition to water scarcity problems, agriculture contributes to climate change, as soils emit carbon dioxide, nitrous oxide and methane due to deforestation, use of fertilizers, and flooding of rice fields (Bailey *et al.*, 2014; UNICEF, 2020; FAO, 2022). In 2019, the agricultural sector alone emitted 31 % of global carbon dioxide equivalent (CO_2-eq) emissions, corresponding to a 16 % increase in CO_2-eq emissions since 1990 (FAO, 2022).

The sustainable production of food to feed the growing population is limited due to the constrains of agriculture. The expansion of agricultural activities potential is reduced since everyday agriculture-grade land is lost to urbanization. In 2018, 55 % of people lived in urbanized areas, in which 80 % of total world food is consumed, and, by 2050, the population percentage might increase to 67 % (United Nations, 2018; FAO, 2022). Additionally, urbanization is associated with intensive agriculture and livestock exploitation due to the rising demand for fast food, particularly meat products. Meat consumption has increased almost 60 % over the last two decades and continues to increase every year at a 2 % rate. Near 80 % of agricultural land is used for livestock production, of which a third is destined for feed crop cultivation (FAO, 2022). Livestock productivity increased between 2000 and 2019 due to less use of land for this industry (a decrease of 0.2 billion ha), while techniques like zero-grazing allowed higher meat production yields. This productivity intensification, however, negatively impacts the environment due to overgrazing and poor livestock management, resulting in soil erosion and compaction and overuse of local water resources and their contamination with nutrients from animal waste and antibiotics. The production of meat requires large quantities of feed crops and water, having a higher impact on the environment than agriculture. For example, 15 415 L, 8 763 L, 5 988 L, and 4 325 L of water are needed to produce, respectively, 1 kg of bovine, sheep or goat, pig, and chicken meat. By contrast, 9 063 L, 1 644 L, 962 L, 387 L, and 322 L of water are needed to produce 1 kg of nuts, cereals, fruits, starchy roots and vegetables, respectively (Mekonnen and Hoekstra, 2012, 2010). A reduction in the consumption of meat, not only saves water, but also reduces land use and greenhouse gas emissions (GHG) (UNICEF, 2020).

Due to their lower environmental footprint, high protein content and other health benefits (Thrane *et al.*, 2017), soy products are among the plant-based foods that are gaining popularity as an alternative protein source. Almost 14 % of the USA citizens avoid meat in their diet (Statista, 2022). The USA led soybean production between 2012 and 2017, but Brazil surpassed it in 2018. In 2022-2023, Brazil harvested

153 mega metric tons of soybeans, and the USA harvested 116.4 mega metric tons (Statista, 2023a). While these two American countries are the biggest producers of soy, Asian countries are the biggest consumers of soy products, with a total consumer sales market share of 72.2 % in 2020. China and Japan are the largest consumers of soy products, excluding natto, miso, and soy sauce (USSEC, 2021). The global market for plant-based foods had estimated worth of 44.2 billion U.S. dollars in 2022, and in 2025 it is estimated to be 77.8 billion U.S. dollars, and more than double of that in 2030 (Statista, 2023b). However, the soy and other plants production sectors are not currently capable of answering to the exponential rising demand of alternative protein-rich products to feed the future generations (Ahmad *et al.*, 2022). Besides, the high phytoestrogen content, allergenicity in some populations, low essential amino acids (EAAs) content, low bioavailability, and negative environmental and socioeconomic impacts are discouraging soy product consumption (Jargin, 2014; Taylor *et al.*, 2021; Mulalapele and Xi, 2021; Vallikkadan *et al.*, 2023). Moreover, the environmental and socioeconomic negative impacts also contribute to the reduction of soy market (da Silva *et al.*, 2021; Dreoni *et al.*, 2022; Gollnow *et al.*, 2018; Lopes *et al.*, 2021).

Another common alternative to meat products is fish. Fish accounts for 10 % of protein consumption and less than 5 % of global food production. The most common means for fish production are inland fisheries and inland aquaculture, which, together, account for 75 % of total fish production. Inland fisheries and inland aquaculture rely on local freshwater resources, such as rivers or lakes, and currently more than 40 % of inland fish comes from poor food-scarce countries. However, these systems are vulnerable to human and climate change-induced changes on local freshwater bodies, such as reduced water availability due to competition with agriculture for irrigation water and damns for water storage, pollution, and eutrophication accompanied by fish mortality (FAO, 2022; Funge-Smith, 2018). From 1984 to 2015, 90 000 km^2 of water bodies were lost and another 70 000 km^2 transitioned from permanent water bodies to seasonal water bodies (Pekel *et al.*, 2016), thus reducing growth potential of inland fish capture/production.

New protein sources are being explored, such as insect protein, cultured meat (created *in vitro* with animal cell cultures or from genetically modified animals), and microbial protein produced by microorganisms (Hashempour-Baltork *et al.*, 2020; Ahmad *et al.*, 2022; Vallikkadan *et al.*, 2023; Mateti *et al.*, 2022). Insect protein is a viable protein source due to its easily scalable production, high bioavailability, and high content of protein, essential amino acids, vitamins, minerals, and unsaturated fatty acids. There have been identified around 2000 edible insect species so far (Vallikkadan *et al.*, 2023).

Generally, protein content in insects ranges from 50 % to 82 %, containing high amounts of threonine and lysine (often not present in plants), and low amounts of methionine and cysteine, which can be supplemented with plant protein (Vallikkadan *et al.*, 2022; Agbidye *et al.*, 2009). Additionally, Fe, Na, Zn, P, Ca, Mg, Cu, Mn, pantothenic acid, riboflavin, and biotin can also be found at high concentrations in insect protein (Vallikkadan *et al.*, 2023). In contrast to meat and plant-based protein, insect protein has low requirements for water, land, and feed, however, its main downside is the low acceptability of the market in developed countries, due to a preconception that insect protein is unclean (Lee *et al.*, 2023). Insect protein is already consumed in African and Asian countries, but allergic reactions to insect protein, such as urticaria, angioedema, dyspnea, nausea, vomiting, and diarrhea have been reported (Vallikkadan *et al.*, 2023).

Cultured meat refers to meat or fish analogs produced using cell engineering technology, where tissue cells from animals are collected and proliferated *in vitro* in order to culture animal tissues (Lee at al., 2023; Zhang *et al.*, 2020; Stephens *et al.*, 2018). This emerged as an alternative to obtain animal flesh products without requiring farming or slaughter, while also responding to the food and economic challenges that the population faces due to agriculture, livestock, and fishing's constraints (Zhang *et al.*, 2020; Stephens *et al.*, 2018). In the cultured meat production process, animal stem cells are often used, specifically muscle satellite cells, a common type of adult stem cells, due to their capacity to differentiate into muscle and fat cells, commonly found in animal flesh, but already existing tissue can also be used. Already existing techniques are able to produce and structure cultured meat in a way to potentially confer it pleasant organoleptic properties, similar to those of natural meat, such as tissue engineering, the use of scaffolds (*e.g.* chitosan, gelatin), microcarriers and hydrocolloids, freeze structuring, 3D printing, electrospinning, shear cell, biophotonics, nanotechnology, thermo-extrusion and cloning/genetically modifying animals (Vallikkadan *et al.*, 2023; Zhang *et al.*, 2020: Mateti *et al.*, 2022). However, the main disadvantages of this technology are that it is still expensive and stem cell maintenance is hard, which consequently limits its scale-up (Lee at al., 2023).

1.2. Microbial protein as a potential protein source

Microbial protein is defined as a protein produced by microbial species such as bacteria, yeast, fungi, or microalgae (Upadhyaya *et al.*, 2016; Ahmad *et al.*, 2022). Microbial protein can be used as food additives or in industrial processes (*e.g.*, as foam stabilizers and in leather and paper processing) (Junaid *et al.*,

2020) but its most common application is for food and feed consumption. Microbial protein has the potential to substitute, partially or entirely, animal protein (Vallikkadan *et al.*, 2023). Microbial protein is commonly denominated single-cell protein (SCP) when it is obtained in the isolated microorganism form. Although it gained popularity after mycoprotein Quorn™ products entry into the market in 1985 (Ahmad *et al.*, 2022; Hashempour-Baltork *et al.*, 2020), microbial protein production goes way back in time, with the cultivation of microalgae *Spirulina* for food by African people in Lake Chad and the Aztecs in Mexico. Microbial protein produced by *Saccharomyces cerevisiae*, *Candida utilis*, *Aspergillus oryzae,* and *Rhizopus arrhizus* was used as a protein-rich food during the World Wars. Since 1960, some energy companies have been studying the production of microbial protein by yeasts from paraffin wax (Chama, 2019).

Microbial protein has several advantages compared to other protein sources, including fast growth of microbial species (doubling time: microalgae: 2 h – 6 h; yeast: 1 h – 3 h; bacteria: 0.3 h – 2 h), high protein content, the possibility of using several by-products from agriculture and food processing activities, which are usually cheap and abundant, thus contributing to a circular economy, its production independency from climate conditions (*i.e.*, production is viable in all seasons) and the fact that it requires less land and water comparatively to agriculture and livestock production (Chama, 2019; Hashempour-Baltork *et al.*, 2020; Bratosin *et al.*, 2021).

Bacterial protein has a high protein concentration in the cellular wall and is more commonly used for feed rather than for human food. Its protein content ranges from 50 % to 80 %, however it is necessary to treat and refine it before its consumption (Junaid *et al.*, 2020; Raziq *et al.*, 2020; Pereira *et al.*, 2022). The main disadvantages of microbial protein produced from bacteria for human consumption are the low amount of sulfur-containing amino acids, the high nucleic acid content, and unattractive organoleptic properties without previous processing (Chama, 2019; Raziq *et al.*, 2020). The presence of nucleic acids in food for human consumption can have a negative impact on health, such as high levels of uric acid in the blood, which in turn can lead to kidney stones (Raziq *et al.*, 2020; Ritala *et al.*, 2017). Common bacterial species used for microbial protein production are *Methylobacterium extorquens, Methylococcus capsulatus*, *Rhodobacter sphaeroides*, *Afifella marina,* and *Corynebacterium ammoniagenes* (Pereira *et al.*, 2022). Imperial Chemical Industries used to produce a feed for pigs named Pruteen, composed of 70 % of bacterial protein but was discontinued due to the lack of competitivity in the market (Ahmad *et al.*, 2022).

Another protein-producing group is microalgae, which are very popular in the vegetarian/vegan communities. Microalgae have been used for animal feed and human consumption for decades as protein

and vitamin supplements. Some popular microalgae belong to the genera *Chlorella* and *Spirulina*, but other commercially important microalgae are *Dunaliella salina*, *Aphanizomenon flos-aquae*, *Euglena*, *Tetraselmis suecica*, and *Isochrysis galbana* (Junaid *et al.*, 2020; Pereira *et al.*, 2022). An important aspect of microalgae is that they contain high concentrations of protein (40 % to 70 %) (Harun *et al.*, 2010; Draaisma *et al.*, 2013, Junaid *et al.*, 2020; Pereira *et al.*, 2022), and low content of nucleic acids (Ritala *et al.*, 2017). Other advantages of microalgae include the consumption of carbon dioxide for their metabolism, the production of metabolites under both fermentation and photosynthesis, and the production of omega-3 fatty acids (Ritala *et al.*, 2017; Pereira *et al.*, 2022). However, the main disadvantages of microalgae protein are the economically limited scale-up of production plants and the need to break the microalgae cell wall to increase digestibility (Pereira *et al.*, 2022).

Filamentous fungi and yeasts are considered the most important microorganisms for protein production. Yeasts are the most studied microorganism group and the most accepted among consumers. In addition to *S. cerevisiae* and *C. utilis*, *Yarrowia lipolytica* is a non-pathogenic and food-grade yeast commonly studied to produce microbial protein (Sotiris *et al.*, 2020; Lopes *et al.*, 2022; Carranza-Méndez *et al.*, 2022). The main advantages of using yeasts include the high protein content (38 % - 65 %), the nutritional quality, the easy recovery, the excretion activity, the lower nucleic acid content compared with bacterial species, the high lysine and malic acid content, and the low risk for spoilage due to their capability to grow in acidic media (Sotiris *et al.*, 2020; Raziq *et al.*, 2020; Pereira *et al.*, 2022). Filamentous fungi have several advantages compared to yeasts and other microbial species regarding protein production. Firstly, fungal protein (also known as mycoprotein) has a lower content of nucleic acids and higher digestibility than bacteria or yeasts. Filamentous fungi can also penetrate solid substrates, which allows the fermentation of solid food-grade by-products. Protein from filamentous fungi can easily be transformed into foods with diverse pleasant textures, and its aroma and taste are similar to those of mushrooms, thereafter, having high acceptability by consumers (Chama, 2019). It is estimated that the market of mycoprotein will grow 20.3 % between 2022 and 2031 (PR Newswire, 2023), reaching more than 460 million EUR in 2027 (Derbyshire & Delange, 2021). Examples of filamentous fungi species used for mycoprotein production are *Fusarium venenatum*, *Aspergillus niger*, *Aspergillus oryzae*, *Rhizopus oligosporus*, *Trichoderma reesei*, and *Neurospora intermedia*. The protein content of filamentous fungi could reach up to 50 % (Pereira *et al.*, 2022; Chama, 2019).

Table 1.1 summarizes the protein content and characteristics of microbial protein produced by microalgae, fungi, yeasts and bacteria.

Table 1.1. Average protein content and characteristics of protein produced by microbial species. Adapted from Matassa *et al.* (2016)

Microorganism	Protein content	Characteristics	References
Microalgae	40 % - 70 %	Similar to eggs, soy, and wheat proteins. Cell wall digestibility is an issue. Contains omega-3 fatty acids.	Harun *et al.* (2010), Draaisma *et al.* (2013), Pereira *et al.* (2022)
Filamentous fungi and yeasts	0.23 % to 50 % (filamentous fungi) 38 % - 65 % (yeasts)	Amino acids profile and digestibility of mycoprotein are similar to those of eggs and milk. Low fat. High unsaturated/saturated fatty acids ratio.	Raziq *et al.* (2020), Chama (2019), Pereira *et al.* (2022)
Bacteria	50 % - 80 %	Amino acid's profile and digestibility are similar to those of fishmeal.	Junaid *et al.* (2020), Raziq *et al.* (2020), Pereira *et al.* (2022)

Microbial protein is a potential nutritious alternative to animal-based protein. The target markets of microbial protein include vegetarian/vegan populations and Muslims, Jews, and Buddhists, which have religious restrictions regarding the meat consumption of some animal species. Microbial protein not only mitigates the excessive use of antibiotics, pesticides, fertilizers, water, and land used for animal and vegetable protein production but is also a solution to food shortages (Hashempour-Baltork *et al.*, 2020; Ahmad *et al.*, 2022).

1.3. Microbial protein produced by fungi (mycoprotein) – high quality protein

The protein produced by food-grade fungi (mycoprotein) is classified as Halal by the Halal Food Authority and as GRAS (Generally Recognized as Safe) by the U.S. Food and Drug Administration and is approved

for human consumption by the Ministry of Agriculture, Fisheries, and Food of the UK since 1984 (Hashempour-Baltork *et al.*, 2020; FDA, 2002; Thavamani *et al.*, 2020). The production of microbial protein by filamentous fungi requires 20 times less water and 23 times less land than animal protein, thus having a carbon footprint 4 to 10 times lower than meat products (Hashempour-Baltork *et al.*, 2020; Thavamani *et al.*, 2020). The commercial brands of mycoprotein products use a set of processes of fermentation, denaturation, separation, freezing, and mixing with food additives in order to produce mycoprotein products, such as chunks, sausages, burgers, nuggets, and minced "meat", with pleasant textures and flavors similar to animal meat (Vallikkadan *et al.*, 2023; Thavamani *et al.*, 2020; Wiebe, 2002).

Although there were some reports of sensitivity and intolerance to mycoprotein in humans, the data relative to these reactions is still scarce, and intolerance seems to be statistically lower than soy and egg intolerance (Finnigan *et al.*, 2017; Vallikkadan *et al.*, 2023). Mycoprotein contains all essential amino acids (EAAs) and in higher content than vegetable protein, is rich in fiber, specially β-glucans and chitin poly-N-acetyl glucosamines, and poor in fats (predominantly constituted by polyunsaturated fatty acids such as linoleic and linolenic acids). Mycoprotein is also a source of minerals, such as copper, zinc, selenium, manganese, phosphorous, iron, and sodium, and vitamins, such as vitamins B2 and D, which makes mycoprotein a perfect option for consumers who are concerned with their health (Ahmad *et al.*, 2022; Hashempour-Baltork *et al.*, 2020; Thavamani *et al.*, 2023; Vallikkadan *et al.*, 2023). However, the content of iron, vitamin B12, and sodium is lower in mycoprotein than in beef and it does not contain vitamins A, C, and E (Ahmad *et al.*, 2022). It was demonstrated that mycoprotein reduces blood cholesterol levels and energy intake, promotes healthy muscle growth, and increases satiety and blood sugar control (Cherta-Murillo *et al.*, 2020; Coelho *et al.*, 2021; Monteyne *et al.*, 2020). Mycoprotein is safe for children and babies, however, due to its high fiber content and low energetic value, they are not recommended for children under 3 years (Denny *et al.*, 2008). Table 1.2 summarizes the nutritional value of mycoprotein compared with other protein sources.

The content of essential amino acids and nucleic acids are important quality factors in microbial protein. Generally, mycoprotein has high amount of lysine and threonine but low content of cysteine and methionine (though methionine levels still meet international recommendations) (Upadhyaya *et al.*, 2016; Ritala *et al.*, 2017). The amino acids profile of mycoprotein depends not only on the microorganism but also on the substrate used for the fermentation and on the processing techniques (Upadhyaya *et al.*, 2016). The protein quality of mycoprotein can also be verified by its high PDCAAS (Protein Digestibility

Corrected Amino Acid Score). Table 1.3 summarizes the PDCAAS values of mycoprotein and of other protein sources.

Table 1.2. Nutritional value of mycoprotein, animal protein, and soy protein. Adapted from Ahmad *et al.* (2022)

Food (100 g)	Mycoprotein	Milk	Eggs	Beef	Chicken	Soybean
Energy (kcal)	85	46	151	172	106	141
Protein (g)	11.25	3.40	12.56	20.20	24.00	14.00
EAAs (g)	4.59	1.53	5.55	7.92	9.11	8.50
Carbohydrates (g)	3.0	4.7	Trace	0.06	Trace	5.10
Total fat (g)	2.90	1.70	9.51	4.30	1.10	7.30
Saturated fat (g)	0.60	1.10	3.13	1.70	0.30	0.90
Fiber (g)	6.0	Trace	Trace	Trace	Trace	6.10
Sodium (mg)	5.00	43.00	142.00	43.00	60.00	1.00
Iron (mg)	0.50	0.02	1.75	3.50	0.40	3.00
Zinc (mg)	9.00	0.40	1.29	0.40	0.70	0.90
Vitamin B12 (mg)	Trace	0.40	0.89	0.40	Trace	Trace
Selenium (mg)	20.00	1.00	11.00	1.00	12.00	5.00

Table 1.3. Protein Digestibility-Corrected Amino Acid Scores (PDCAAS) of some traditional sources of protein and microbial protein sources

Protein source	PDCAAS (%)	Reference
Egg	100	Hoffman and Falvo (2004)
Milk/Casein	94 - 100	Yamada and Sgarbieri (2005) Hoffman and Falvo (2004)
Whey protein	100	Hoffman and Falvo (2004)
Beef	92	Hoffman and Falvo (2004)
Soy	100	Hoffman and Falvo (2004)
Mycoprotein	99.6	Edwards and Cummings (2010)
Baker's yeast	62 - 84	Yamada and Sgarbieri (2005)

Other important factor for mycoprotein quality is its concentration in nucleic acids, specifically RNA. Microbial biomass generally contains high concentrations of RNA, depending on the microorganism species, which can be detrimental to health for humans and animals. While the safe limit for human

consumption of RNA in food is a concentration of 2 %, this nucleic acid is usually found at concentrations between 7 % to 10 % in dry basis in mycoprotein, thereafter it must be removed or inactivated prior to consumption (Nasseri *et al.*, 2011). The methods for RNA removal or inactivation include chemical, enzymatic, and physical treatments. Chemical treatments, such as nucleic acid removal by extraction with alcohol, salt, acids, and alkalis, are not the most suitable for animal and human consumption applications, since most solvents are not food-grade and toxic compounds may be formed, especially if high temperatures are also applied (*e.g.*, lysinoalanine) (Nasseri *et al.*, 2011). Enzymatic treatment is another possible method for nucleic acids inactivation, for example with ribonucleases. Optimal conditions for the degradation of ribonucleic acids by ribonucleases are also optimal for protein degradation by endogenous proteases. Therefore, using exogenous ribonucleases or immobilized enzymes is preferable to not reduce protein yield in mycoprotein. Disadvantages of enzymatic treatments are: 1) the process is slow, and 2) is costly (Nasseri *et al.*, 2011). Physical treatments, such as high temperature, are the most used methods for the reduction of nucleic acid content. In mycoprotein production, the fungal biomass is generally heated to 72 °C – 74 °C for 30 min – 45 min (Ahmad *et al.*, 2022). In this case, the disadvantages are: (1) loss of protein and (2) loss of nutritional value (Nasseri *et al.*, 2011; Wiebe, 2002; Souza Filho *et al.*, 2018).

1.4. Factors affecting microbial protein production

Generally, microbial species require carbon and nitrogen sources for cellular growth and metabolite production. Substrates for fermentation can either be pure components, for example as in Quorn™'s method of production (where glucose is used directly as a substrate, along with ammonium salts and biotin), or they can be by-products of the food processing industry, agricultural and forest cleaning activities, or the oil refining industry (*e.g.,* simple sugars, starches, cellulose, hydrocarbons). In mycoprotein production for human consumption, it is a requirement that the substrate used is food-grade. Thus, by-products like fruit peels, rice bran and straw, corn cobs, wheat straw, vegetable pulps, and fruit pomace are examples of appropriate and cheap substrates for mycoprotein production for food application (Junaid *et al.*, 2020; Barzee *et al.*, 2021; Bratosin *et al.*, 2021).

Depending on the substrate and microorganism, three types of fermentation can be carried out to produce mycoprotein: submerged (SmF), semi-solid and solid-state (SSF). Filamentous fungi are known for their ability to use solid by-products as substrates for SSF. This has several advantages in substrate acquisition

(abundant and cheap) and preparation (fewer pre-treatments are needed) and energy costs (Barzee *et al.*, 2021). With solid-state fermentation, it is possible to achieve high yields, and the substrate can simultaneously be a physical support and a nutrient source for fungi. Using low-cost food by-products reduces the global costs of the process and is more environmentally friendly. The seasonality and distance to production centers should be considered in the industrial production of mycoprotein from food by-products (Hashempour-Baltork *et al.*, 2020; Koukoumaki *et al.*, 2023).

The operational conditions that may affect mycoprotein production by SSF are nutrient availability, aeration, the particle size of the substrate, temperature, pH, moisture content, water activity, and inoculum's size, age, and physiological state (Junaid *et al.*, 2020; López-Gómez *et al.*, 2020). SSF ideally must have an aeration system, as oxygen is an important factor for aerobic species. Forced humid air and the mixture of the solid matrix are commonly applied to overcome the diffusion limitations between the solid particles and the air and to prevent moisture gradients and heterogenicity (López-Gómez *et al.*, 2020). The particle size of the substrate also plays a role in oxygen diffusion. Fungi easily penetrate substrates with small particle size, however, the oxygen diffusion coefficient is also smaller due to smaller interparticle space. A compromise between substrate penetration and oxygen diffusion has to be evaluated (López-Gómez *et al.*, 2020).

Solid-state fermentation with filamentous fungi enables to produce high quality mycoprotein or a protein-enriched food from GRAS by-products. For SSF, substrate is collected, homogenized and pre-treatments are applied, and nutritional supplements are added, if needed. After sterilization of the medium and equipment, the medium is inoculated with the fungal species. During SSF, aeration of the medium or other growth factors are regulated. For food application purposes, a reduction of nucleic acid concentration step is necessary. Table 1.4 summarizes some operational conditions used for mycoprotein production by filamentous fungi.

Schmidt and Furlong (2012) studied the effect of particle size of rice bran and supplementation with ammonium sulfate on protein enrichment of rice bran and phenolic compounds production by *Rhizopus oryzae* under semi-solid-state fermentation. The authors found that an increase in particle size decreased protein production, whereas biomass production was improved. This factor was significative for protein, biomass, and phenolic compounds production. Additionally, the authors concluded that ammonium sulfate supplementation was significative for biomass and phenolic compounds production but not for protein production. They reported an increase of 53 % in total protein content and 56 % of total phenolic

content in rice bran, relatively to unfermented rice bran, with semi-solid-state fermentation by *Rhizopus oryzae* with an 0.18 mm average particle size and supplementation with 8 g/L ammonium sulfate.

Kamal *et al.* (2019) studied the effect of temperature (20 °C – 40 °C), pH (4 – 8), substrate concentration (5 % – 25 %), and fermentation time (1 day – 5 days) on biomass and protein yields by *Aspergillus niger* under submerged fermentation of banana peel hydrolysate. The authors concluded that all factors had a positive and significant effect on biomass and protein production. Protein production was optimized (60.15 %) when the operational conditions were fixed at 31.02 °C, pH of 6.19, substrate concentration of 19.92 %, and fermentation period of 4 days.

Olorunnisola *et al.* (2016) also studied the effects of temperature (28 °C and 36 °C), pH (4 – 7), fermentation time (1 day – 10 days), inoculum size (4.0 g/L – 8.0 g/L), and moisture content (60 % – 80 %) on protein enrichment of fruit peels (a mixture of banana, pineapple and papaya peels), by co-cultivating *Schizophyllum commune* and *Phanerochaete chrysosporium* in solid-state fermentation. The authors reported an optimal crude protein synthesis of 198.77 mg/g, at 32 °C, pH 5.4, 6.1 % inoculum size, and 70 % moisture content, at the 6th incubation day. pH, inoculum size and interaction between pH and moisture were significative factors for protein production, in the range of values studied by the authors.

Table 1.4. Operational conditions for mycoprotein protein production by filamentous fungi

Filamentous fungi	Substrate	Operational conditions	Protein content (%)	Reference
Trichoderma reesei	rice straw and coffee husks	SSF; 30 °C; pH 5.0; 20:1 C/N ratio with urea as nitrogen source; 75 % initial moisture; 1:1 (w/v) inoculum size to substrate	22	Said *et al.* (2019)
Neurospora intermedia and *Rhizopus oryzae*	stale bread and brewer's spent grain	SSF; 35 °C; 40 % initial moisture; 6.7 % (w/w) inoculum size for both microorganisms; 6 days	21.1	Gmoser *et al.* (2020)

Table 1.4. Operational conditions for mycoprotein protein production by filamentous fungi (*continued*)

Filamentous fungi	Substrate	Operational conditions	Protein content (%)	Reference
Rhizopus oryzae	rice bran	Semi-SSF; 30 °C; substrate moistened with 2 g/L KH_2PO_4, 1 g/L $MgSO_4$, and 2-8 g/L $(NH_4)_2SO_4$ at 45 % w/v; 4×10^6 spores/g inoculum size; 50 % initial moisture; 96 h	20.4	Schmidt and Furlong (2012)
Geotrichum candidum	Orange peels	SmF; OP were pretreated (100 °C, 2 h) with sulfuric acid 98 % and water and washed. The solid fraction was used for hesperidin extraction, and the hydrolysate was used for continuous (D = 0.078) microbial protein production. 3 % N and 0.5 % P supplementation; 28°C; 450 rpm; 0.6 vvm – 1.0 vvm air flow; pH 5	35 - 40	Lo Curto *et al.* (1992)
Aspergillus niger	Banana peels	SmF with banana peel hydrolysate (10:1 HCl 10 %, 100 °C, 1.5 h), 2.9×10^7 cfu/mL inoculum size, 31.02 °C, pH 6.19, 19.92 % substrate concentration, 4 days	60.15	Kamal *et al.* (2019)
Neurospora Intermedia, Aspergillus oryzae, Monascus purpureus, Fusarium venenatum and *Rhizopus oryzae*	Pea-processing by-products	SmF; pre-treated 2 % - 3 % substrate with α-amylase, amyloglucosidase, and cellulase; 35 °C; 48 h; 0.42 vvm; 3.9×10^5 –3.8×10^6 spores/mL inoculum size	54.53 - 54.11, 43.13 - 46.36, 53.61 - 58.66, 55.28 - 59.75, 50.03 - 54.79, respectively for each species	Souza Filho *et al.* (2018)
Schizophyllum commune and *Phanerochaete chrysosporium* (co-culture)	Mixture of banana, pineapple and papaya peels	SSF; 32 °C, pH 5.4, 6.1 % inoculum size, 70 % moisture content, 6 days	Production of 198.77 mg/g	Olorunnisola *et al.* (2016)

1.5. Microbial species: filamentous fungi

Filamentous fungi are microorganisms capable of producing a wide range of added-value products such as enzymes, organic acids, pigments, antioxidants, and antibiotics for application in the food, textile, paper, leather, detergent, biodiesel, and biotechnological industries (Abdel-Azeem *et al.*, 2021; Barzee *et al.*, 2021; Londoño-Hernández *et al.*, 2017).

A new *Aspergillus* species was isolated from wine grapes and raisins from Portugal and Spain by Serra *et al.* (2006). *Aspergillus ibericus* is a promising species that does not produce ochratoxin A at detectable concentrations, a mycotoxin commonly found in contaminated wine, contrary to its morphological closest relative, *A. carbonarius* (the main mycotoxin producer in grapes). *Aspergillus ibericus* grows well between 25 °C and 37 °C, but not at 5 °C (Serra *et al.*, 2006). This species has already been studied for enzyme and fructo-oligosaccharides production (Abrunhosa *et al.*, 2013; Oliveira *et al.*, 2017; Makesh *et al.*, 2016; Salgado *et al.*, 2016, 2014; Nobre *et al.*, 2018a, 2018b), however, no data concerning microbial protein production is available in the literature.

Rhizopus oryzae is a GRAS-status filamentous fungus, traditionally used to ferment protein-rich foods such as tempeh and ragi, and alcoholic beverages, and industrially to produce enzymes (*e.g.*, glucoamylases, polygalacturonases) and organic acids (*e.g.*, lactic acid, fumaric acid) (Londoño-Hernández *et al.*, 2017). This species is able to grow in a wide range of temperatures (7 °C - 45 °C, optimum at 37 °C) and pH values (4 - 9) (Londoño-Hernández *et al.*, 2017). *Rhizopus oryzae* uses several substrates as carbon sources, including xylan, tannic acid, cellulose, xylose, glucose, sucrose, mannose, galactose, mannitol, ethanol, citrus pectin, fructose, inulin, and starch for enzyme and organic acids production (Liao *et al.*, 2007; Bakir *et al.*, 2001; Banerjee *et al.*, 2005; Kupski *et al.*, 2015; Bulut *et al.*, 2004; Maas *et al.*, 2006; Park *et al.*, 2004; Battaglia *et al.*, 2011; Ghosh and Ray, 2011; Mertens *et al.*, 2008; Ramachandran *et al.*, 2005; Roa Engel *et al.*, 2011; Lv *et al.*, 2012, 2015). Fermentation of solid substrates with *Rhizopus oryzae* results in an overall improvement of nutritional properties (increase in protein, vitamin B, mineral and phenolic contents and antioxidant activity, while reducing energy and fat contents) and organoleptic properties (mainly texture) (Cai *et al.*, 2012; Yadav *et al.*, 2013; Bhanja Dey and Kuhad, 2014).

1.6. Fruit peels – an agro-industrial by-product with a valorization potential

Roughly one third of fruits is made of peels and seeds. After consumption or industrial processing (*e.g.*, fruit juices and pulps industries), these by-products are often used for animal feed, incinerated or discharged in landfill, at a cost for the producer (O'Shea *et al.*, 2012).

Orange peels (OP) are a particularly abundant food by-product, since around 85 % of harvested oranges are processed for juice production (O'Shea *et al.*, 2012), generating large quantities of peels every year since peels represents, roughly half of the total fruit mass (Suri *et al.*, 2022; López *et al.*, 2010). The Food and Agriculture Organization estimated that a total of 76.3 million metric tons of oranges were produced worldwide in 2019 (FAO, 2021). Traditionally, orange peels are either discarded in landfills, negatively impacting the environment due to often uncontrolled emissions of methane and carbon dioxide, used for incorporation in feed for cattle (Siyal *et al.*, 2016; Bampidis and Robinson, 2006), and human food, such as crystalized fruit in traditional desserts (*e.g.*, the Portuguese "Bolo Rei") or for beverage or medicinal purposes (*e.g.*, as a botanic for gin manufacturing or for orange tea), or to produce energy via combustion. Other alternatives for valorization of orange peels have been studied such as the production of fertilizers, natural herbicides, biofuels (*e.g.*, biogas and ethanol), enzymes, organic acids (succinic, citric, malic, malonic, and oxalic acids), and microbial protein. The extraction of pectin, essential oils, vitamin C, citric acid, and the use of OP as pollutant absorbents for methylene blue and heavy metals, or as a supplement to pulp in paper manufacturing has also been explored (López *et al.*, 2010; Erukainure *et al.*, 2016; Adoki, 2008; Qureshi *et al.*, 2017; Rivas *et al.*, 2008). Orange peels are rich in sugars (*e.g.*, glucose, fructose and sucrose), dietary fiber, folic acid, minerals (*e.g.*, calcium and potassium), phytochemicals (*e.g.*, β-carotene and ferulic acid), flavonoids (*e.g.*, hesperidin, naringin, and neodiosmin), and terpenoids (*e.g.*, limonene and linalool) with anti-oxidative, anti-inflammatory and anti-carcinogenic properties (Suri *et al.*, 2022; O'Shea *et al.*, 2012; Erukainure *et al.*, 2016; Carranza-Mendez *et al.*, 2022).

Banana peels (BP) are another abundant food-grade by-product with valorization potential, since it is the world's 4th most consumed crop, with an average annual consumption per capita of 12 kg, and the peel constitutes 35 % – 50 % of the fruit's weight (Zaini *et al.*, 2022). Banana production reached 114 million metric tons in 2017 and it is estimated to grow 1.5 % every year to 135 million metric tons by 2028 (FAO, 2019). Approximately 36 million metric tons of banana peels are produced every year, due to industrial pulp, flour, and chips production and domestic consumption, among other applications (Zaini *et al.*, 2022). The traditional valorization of banana peels includes production of organic fertilizers, incorporation

in animal feed, transformation into charcoal for energy production, manufacturing of banana peel flour, medicinal purposes (*e.g.*, remedy for burns, anemia, diarrhea, snakebite, excessive menstruation) and, ultimately, disposal at landfills (Zaini *et al.*, 2022; Siyal *et al.*, 2016; Anhwange *et al.*, 2009; Rebello *et al.*, 2014). The main applications of banana peels take advantage of their antioxidant, antibacterial, and anti-inflammatory properties due to the high content of phenolic compounds (Zaini *et al.*, 2022; Rebello *et al.*, 2014; Anhwange *et al.*, 2009; Aboul-Enein *et al.*, 2016; Agourram *et al.*, 2013). Flavonoids, tannins, phlobatannins, alkaloids, glycosides, anthocyanins, and terpenoids are common compounds found in banana peels (Zaini *et al.*, 2022). Banana peels are also rich in carbohydrates, both dietary fiber and available carbohydrates, ashes (minerals), and small amounts of lipids (mainly polyunsaturated fatty acids such as linoleic acid and α-linolenic acid) and starch (Zaini *et al.*, 2022; Tsado *et al.*, 2021; Anhwange *et al.*, 2009). Alternative applications of banana peels include incorporation in human food such as sausages, patties, bread, and cookies, to enhance dietary fiber, ash, and lipid contents and antioxidant and organoleptic properties; production of xylitol, edible food wrapper and bioplastic films; production of microbial biomass and protein, and enzymes (Zaini *et al.*, 2022; Essien *et al.*, 2005; Kamal *et al.*, 2019; Adoki, 2008; Abera *et al.*, 2023; Qureshi *et al.*, 2017; Bishnoi *et al.*, 2023).

Banana peels contain antinutrients (*e.g.*, tannins, oxalates, phytates, glycosides/hydrogen cyanide) that may interfere with nutrient absorption, and cause inflammation and damage to the organs. However, some treatments can be used to reduce the antinutrient components such as fermentation by microorganisms, microwave drying, boiling, and air and oven-drying (Zaini *et al.*, 2022; Anhwange *et al.*, 2009).

The upgrade of fruit peels through solid-state fermentation (SSF) to improve their nutritional value for human consumption is still underexplored. This would increase the economic and nutritional value of fruit peels, as well as contribute to mitigate world hunger and environmental issues associated with their uncontrolled disposal. Orange and banana peels are presented as attractive substrates for SSF, due to their abundance, high nutrient content, and ability to acquire at low cost.

1.7. Objectives

The main objective of this work was to upgrade the nutritional value of fruit peels (orange and banana peels), namely protein content and antioxidant activity, by solid-state fermentation (SSF) with filamentous fungi (*Aspergillus ibericus* and *Rhizopus oryzae*). The specific aims include:

- Evaluate the nutritional potential of FP for filamentous fungi species, after an intensive characterization;
- Exploit the potential of using FP directly in SSF;
- Optimize the operational conditions to improve the nutritional value of fruit peels under SSF using untreated fruit peels;
- Characterize the fermented solid (fruit peels + fungal biomass) – carbohydrates, reducing sugars, protein, lipids, fiber, *etc*.
- Evaluate the final fermented solid as a potential food for human consumption considering its nutritional composition.

CHAPTER 2. MATERIALS AND METHODS

2.1. Raw material: fruit peels

Orange (*Citrus sinensis* Linnaeus) and banana (*Musa acuminata* Colla AAA Cavendish cultivar) were bought from a local supermarket in Braga (Portugal), in March 2023. 2 batches of orange (OP) and banana (BP) peels were washed with water, grinded, dried at 60 °C, and stored at - 20 °C (Figure 1.1).

Figure 1.1. Preparation of (A) orange and (B) banana peels before storage at – 20 °C.

2.2. Microorganisms

Aspergillus ibericus MUM 01.294 and *Rhizopus oryzae* MUM 10.260, stored in cryostocks at – 80 °C, were obtained from the culture collection of Micoteca da Universidade do Minho (MUM - Braga, Portugal). The fungal strains were grown in potato dextrose agar (PDA) petri dishes (4 g/L potato extract, 20 g/L dextrose, 15 g/L agar) at 25 °C for 5 days and stored at 4 °C for a maximum of 2 weeks.

2.3. Preparation of spore suspension

Spore suspensions of *A. ibericus* MUM 01.294 or *Rhizopus oryzae* MUM 10.260 were prepared by adding a sterile peptone solution (1 g/L peptone, 0.1 g/L Tween 80) to 5-day-grown agar plates. After appropriate dilution, 1×10^{7} spores/g solid or 5×10^{7} spores/g solid solutions were used to inoculate the fruit peels medium.

2.4. Effect of medium composition and operational conditions on nutritional value of fruit peels

The dried orange (OP) and banana (BP) peels were weighted (5 g of dry basis) to 250-mL Erlenmeyer flasks and the nitrogen supplements (ammonium sulfate and corn steep liquor, CSL) were added, according to the experimental assay matrixes (Chapters 2.4.1 and 2.4.2). The flasks containing the medium for SSF were sterilized at 121 °C for 15 min. A spore suspension prepared according to Chapter 2.3 was added to each flask, according to the experimental assay matrixes. The initial moisture of the OP and BP was adjusted with sterilized distilled water. The fermentations were carried at 25 °C in a ventilated oven without agitation for 7 days or 14 days (Figure 1.2).

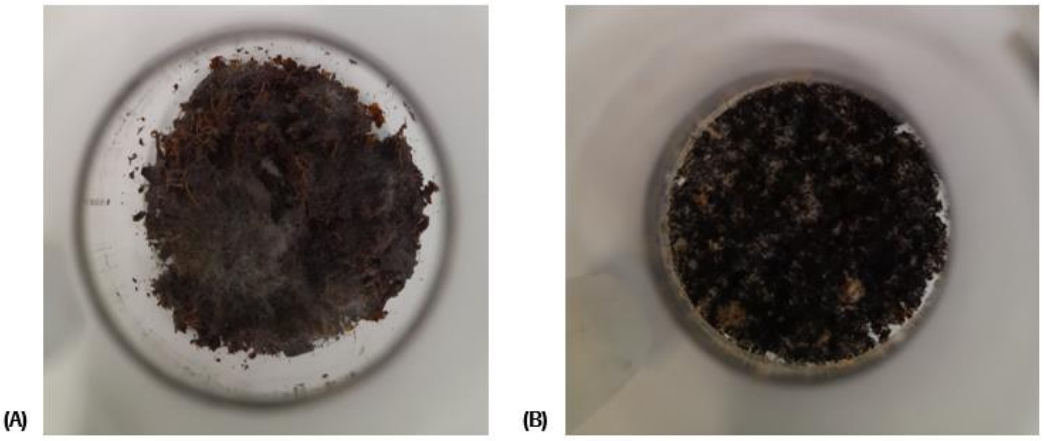

Figure 1.2. Visible growth of filamentous fungi in orange and banana peels. (A) *Rhizopus oryzae* growing in banana peels. (B) *Aspergillus ibericus* growing in orange peels.

2.4.1. Plackett-Burman design

The Plackett-Burman design (Plackett and Burman, 1946) is a statistical experimental design derived from the full factorial design that was created in order to study the main effects of several independent variables (n), called factors, in a dependent variable, called response, generally at 2 levels (L=2), with the least possible number of experiments, when the complete factorial number of experiments (L^n) is realistically unfeasible. For this design, a Hadamard matrix is created, where "1" and "-1" represent the 2 quantitative levels studied for each factor. This experimental design is commonly used for variable screening, since it assumes that factor interaction is negligible, thus only considering main effects. The

Plackett-Burman design adjusts the experimental data to a linear model, that establishes a relationship between response variables (Y) and factors (x_i) and their main effect coefficients (a_i):

$$Y = A + \sum_i a_i \bullet x_i$$

The main effect of 6 SSF variables (time, inoculum size, moisture, ammonium sulfate concentration, CSL concentration and fungus species) on protein production, reducing sugars, total phenols, and antioxidant activity responses in fermented OP and BP (Batch 1) were studied at 2 levels, in order to evaluate the potential to upgrade fruit peels' nutritional value. Table 2.1 depicts the matrix of factors and levels studied with Plackett-Burman design in SSF of OP and BP, by filamentous fungi.

Table 2.1. Plackett-Burman design matrix used to study the main effects of fermentation time, inoculum size, moisture content, ammonium sulfate (AS) concentration, corn steep liquor (CSL) concentration and fungal species, at 2 levels, on protein production, reducing sugars, total phenols, and antioxidant activity responses in fermented orange and banana peels

Run/Level	Time	Inoculum size	Moisture	AS concentration	CSL concentration	Fungus species
1	-1	1	1	1	-1	1
2	-1	1	1	-1	1	-1
3	1	-1	1	-1	-1	-1
4	1	-1	1	1	-1	1
5	1	1	1	-1	1	1
6	-1	-1	-1	-1	-1	-1
7	-1	-1	1	1	1	-1
8	1	-1	-1	-1	1	1
9	1	1	-1	1	-1	-1
10	-1	-1	-1	1	1	1
11	1	1	-1	1	1	-1
12	-1	1	-1	-1	-1	1
-1	7 days	1×10^7 spores/g	60 %	0 g/g	0 g/g	*Aspergillus ibericus*
1	14 days	5×10^7 spores/g	75 %	0.01 g/g	0.01 g/g	*Rhizopus oryzae*

2.4.2. Box-Behnken design

The Box-Behnken design (Box and Behnken, 1960) is a statistical experimental design which generates response surfaces that requires 3 equally spaced levels and 3 central points. This experimental design is used for optimization of quantitative response variables. The Box-Behnken design has the advantage of needing fewer experimental assays (runs) with 3 to 4 factors, relatively to other optimization experimental designs (*e.g.*, Central Composite Circumscribed design and Central Composite Inscribed design). The Box-Behnken design adjusts experimental data to a quadratic model, that establishes a relationship between response variables (Y), factors (x_i), main effect coefficients (a_i), and interaction effects coefficients (a_{ii}):

$$Y = A + \sum_i a_i \bullet x_i + \sum_i a_{ii} \bullet x_i^2 + \sum_{ij} a_{ij} \bullet x_i \bullet x_j$$

After variable screening with the Plackett-Burman design, a Box-Behnken design was employed to maximize the production of microbial protein from OP and BP (Batch 2). Table 2.2 depicts the matrix of factors and levels studied with Box-Behnken design for the optimization of total protein from OP and BP.

Table 2.2. Box-Behnken design matrix used to study the effect of different levels of moisture content, ammonium sulfate (AS) concentration, and corn steep liquor (CSL) concentration on total protein production from orange and banana peels

Run/Level	Moisture	AS concentration	CSL concentration
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1

Table 2.2. Box-Behnken design matrix used to study the effect of different levels of moisture content, ammonium sulfate (AS) concentration, and corn steep liquor (CSL) concentration on total protein production from orange and banana peels (*continued*)

Run/Level	Moisture	AS concentration	CSL concentration
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0
-1	50 %	0 g/g	0 g/g
0	60 %	0.005 g/g	0.005 g/g
1	70 %	0.01 g/g	0.01 g/g

The fermentations were carried at 25 °C in a ventilated oven without agitation for 7 days. Each Erlenmeyer flask was inoculated with 1×10^{7} spores/mL of *A. ibericus*. A control experiment without inoculation was also performed.

2.5. Sample processing for analysis

After solid-state fermentation, the final solid (fruit peels and grown fungi) was mixed to reduce its heterogeneity and it was separated into 2 fractions: 2 g - 3 g of solid were saved for final moisture and total protein analysis and its remainder was utilized for a room temperature distilled water extraction (1:10 w/v, 200 rpm, 30 min) for analysis of soluble compounds (reducing sugars and total phenols) and antioxidant activity. In the final experiments (verification of conditions predicted by Box-Behnken design), fermented solid was also characterized for carbohydrates, crude fiber, pectin, and lipids, in addition to moisture and total protein.

2.6. Analytical methods

2.6.1. Moisture

Moisture content of non-fermented and fermented fruit peels was measured according to a modified standard method from AOAC (2023). Briefly, aluminum cups were identified and placed in the drying oven at 105 °C for a minimum of 6 hours or until constant weight. Once cooled in a desiccator, the aluminum cups were weighed empty and 0.5 g to 1.0 g of sample were weighed into the aluminum cups, with an analytical balance. The samples were dried at 105 °C until constant weight. After reaching room temperature in the desiccator, the cups' final weigh was registered.

Moisture content was determined by:

$$\text{Moisture}\ (\%) = \frac{(\text{Mass of cup and fresh sample}) + (\text{Mass of cup and dried sample})}{(\text{Mass of cup and fresh sample}) + (\text{Mass of empty cup})} \times 100$$

2.6.2. Total protein

The total protein content was quantified by the Kjeldahl method (Kjeldahl, 1883). The nitrogen content was converted into crude protein using a factor of 6.25. Briefly, 0.5 g of sample, 10 mL of concentrated sulfuric acid and a selenium tablet were added into each digestion tube. After digestion and cooling (1 h + 15 min, 420 °C) in the digestor (FOSS Kjeldahl Digestion Systems, Thermo Fisher Scientific, USA), the tubes were transferred into a distiller (FOSS Kjeltec 8400 system, Thermo Fisher Scientific, USA), where the samples were neutralized.

2.6.3. Reducing sugars

Reducing sugars content was measured by the dinitro-salicylic (DNS) acid reagent (Miller, 1959), using glucose as standard. Briefly, 100 µL of extracts (or distilled water for the blank) and 100 µL of DNS reagent were added to the test tubes. The tubes were boiled in a water bath at 100 °C, for 5 min, placed

immediately in a cold-water bath, and 1 mL of distilled water was added. After vortex-mixing, the absorbance was read at 540 nm (Multiskan Sky, Thermo Fisher Scientific, USA), in 96-well plates.

2.6.4. Carbohydrates

Carbohydrates were determined after acidic hydrolysis and the quantification of the resulting total sugar monomers by the DNS method. 0.1 g to 0.2 g of sample were weighed to hydrolysis tubes, and 10 mL of H_2SO_4 1.5 M were added. The tubes were boiled in a water bath for 20 min and 12 mL of NaOH 10 % were added to the tubes, once cooled. The mixture was vortex-mixed and filtered into a 100-mL volumetric flask and the remaining volume was made up with distilled water. For the quantification of sugar monomers, the DNS method protocol mentioned in Chapter 2.6.3. was employed.

The carbohydrates content was determined by:

$$\text{Carbohydrates (\%)} = \frac{\text{Concentration of reducing sugars}}{\text{Mass of sample used for hydrolysis}} \times 0.1\ \text{L} \times 100$$

2.6.5. Crude fiber

The crude fiber was quantified according to the AOAC Official Method 962.09, with some modifications. Briefly, 2 glass microfiber filters were calcined at 500 °C for 4 h in a muffle furnace and allowed to cool in a glass desiccator. 2 g of sample and 150 mL of H_2SO_4 0.13 M were added into a 250 mL reflux flask. The mixture was boiled for 30 min, vacuum filtered and washed, along with the reflux flask, with 90 mL of boiling water. The filter was then placed inside the reflux flask, and 150 mL of KNO 0.23 M were added. The mixture was boiled again for 30 min and repeated the washing step with boiling water. The mixture and the reflux flask were washed with 75 mL of acetone. Afterwards, the 2 filters were dried at 105 °C for 4 h in a drying oven and weighed after cooling in the desiccator. The filters were calcined for 30 min, at 500 °C, in the muffle furnace. Finally, the 2 filters were weighed after cooling in the desiccator.

The crude fiber content corresponds to the fraction of residue left in the sample:

$$\text{Crude Fiber (\%)} = \frac{\text{Mass of 2 filters after drying} - \text{Mass of 2 filters after calcination}}{\text{Mass of sample}} \times 100$$

2.6.6. Pectin

Pectin was quantified according to the method described by Rodsamran & Sothornvit (2018) with some modifications. Briefly, 5 g of sample powder was weighed into a bottle Schott, and 100 mL of HCL 0.05 M were added. The bottle Schott was placed in an agitated water bath at 95 °C for 1 h. The content was filtered through a nylon net and vacuum filtered, and the solid residues went through a second extraction, following the same previous steps. The solution volume was measured and absolute ethanol (1:2, v/v) was added. The bottle Schotts were agitated for 10 min in an incubator (100 rpm, room temperature) and left to rest for 2 h to precipitate the pectin. The pectin was vacuum filtered and washed with absolute ethanol (3 times) and acetone (1 time). The pectin was dried overnight at 40 °C and it was finally weighed.

The pectin content was determined by:

$$\text{Pectin (\%)} = \frac{\text{Mass of pectin}}{\text{Mass of sample}} \times 100$$

2.6.7. Total lipids

The total lipids content was determined by the Soxhlet method, with petroleum ether as extraction solvent, in a Soxtec 8000 system. Firstly, the Soxhlet aluminum cups were dried at 105 °C for 2 h with 5 glass spheres inside. Once cooled in the desiccator, the Soxhlet cups were weighed. 1 g of sample was weighed into the cellulose thimbles, which were covered with cotton. 50 mL of petroleum ether were added to the Soxhlet cups and these, along with the cellulose thimbles were inserted and adapted into the Soxtec system, and the extraction occurred overnight (70 °C). In the morning, the cups were removed and placed in the drying oven at 105 °C for 15 min to evaporate the residual petroleum ether. The weight of the Soxhlet cups was registered, after cooling in the desiccator.

The total lipids content of the sample was determined as followed:

$$\text{Total lipids (\%)} = \frac{\text{Mass of Soxhlet cups after extraction} - \text{Mass of Soxhlet cups before extraction}}{\text{Mass of sample}} \times 100$$

2.6.8. Ashes

Ashes content was determined according to the standard methods from AOAC (2023). Firstly, porcelain crucibles were calcined at 550 °C, for 4 h, cooled in the desiccator and weighed. 0.5 g to 1 g of sample were weighed into the crucibles, which were heated at 550 °C for 4 h, cooled in the desiccator, and weighed.

The ashes content of the fruit peels was determined as:

$$\text{Ashes (\%)} = \frac{\text{Mass of crucible and ash} - \text{Mass of crucible}}{\text{Mass of sample}} \times 100$$

2.6.9. Free carbon and nitrogen in aqueous extracts

Free carbon and nitrogen concentrations in fruit peels were determined in aqueous fruit peel extracts (Chapter 2.5). For free carbon, a Total Organic Carbon LCK387 (300 mg/L – 3000 mg/L, Hach-Lange GmbH, Berlin, Germany) kit was used and, for free nitrogen, a Total Nitrogen LCK 138 (1 mg/L – 16 mg/L, Hach-Lange GmbH, Berlin, Germany) kit was used.

2.6.10. Total phenols

Total phenols in fruit peels extracts were quantified by the Folin-Ciocalteau method (Commission Regulation (EEC) No. 2676/90), using gallic acid as standard. Briefly, 20 µL of sample (or distilled water for blank), 400 µL of Na_2CO_3 15 %, 100 µL of Folin-Ciocalteau reagent, and 1480 µL of distilled water were added to a tube, in triplicate for each sample. The assay tubes were placed in a 50 °C water bath for 5 min and then cooled with water and vortex-mixed for 30 s. The absorbance was read at 740 nm (Multiskan Sky, Thermo Fisher Scientific, USA) and converted to total phenols concentration using the calibration curve.

2.6.11. Antioxidant activity

Antioxidant activity was measured in fruit peels aqueous extracts by the modified DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant assay method (Blois, 1958). Briefly, 200 μL of diluted sample extracts (trolox solution for calibration curve) and 100 μL of a methanolic solution of DPPH 0.5 mM were added into microplate wells. The mixture was incubated for 30 min in the dark at room temperature. The absorbance was measured at 517 nm (Multiskan Sky, Thermo Fisher Scientific, USA). Two controls were also carried out: (1) 200 μL of diluted sample extracts and 100 μL of methanol (individual control); and (2) 200 μL of methanol and 100 μL of DPPH solution (microplate control).

Antioxidant activity was expressed as micromoles of trolox per g of sample and obtained through the trolox concentration *vs* free radical scavenging activity calibration curve. The absorbance was converted to scavenging activity with the following equation:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{Corrected\ absorbance\ of\ sample\ with\ individual\ blank}{Absorbance\ of\ control\ blank}\right) \times 100$$

2.6.12. Minerals

Mineral composition of fruit peels was analyzed by Inductively coupled plasma optical emission spectrometry (ICP-OES). Fruit peels were previously digested using a Berghof microwave digestion system (Speedwave four DAP-60+, Berghof Products + Instruments GmbH, Germany). Briefly, 0.25 g of dried peels or 0.5 g of humid sample were weighed into the digestion vessels, and 6 mL of HNO_3 69 % and 1 mL of H_2O_2 30 % were added. The vessels were slowly shaken and, after 10 min, closed. The digestion program was chosen, based on the equipment user manual chapter "Microwave Digestion of Fruits". Firstly, samples were subjected to temperature and pressure values of 170 °C and 30 bar, respectively, for 10 min, and then of 200 °C and 30 bar, for 15 min. The final step of digestion consisted of applying temperature and pressure of 50 °C and 25 bar, for 10 min.

The digested liquid samples were filtrated with 0.22 μm pore syringe filters and analyzed in the ICP-OES equipment (Optima 8000, PerkinElmer, Waltham, MA, USA). The operating conditions were as follows: radio frequency power 1400 W; 12 L/min argon plasma flow; 0.2 L/min auxiliary gas flow; 0.75 L/min nebulizer gas flow. The plasma view was axial for all analyzed elements. The wavelengths (nm) used for

each element were: Cu, 324.752; K, 766.490; Mg, 280.271; Na, 588.995; P, 213.617; Ca, 317.933; Fe, 238.204; Mn, 259.372; Ni, 221.648; Zn, 213.857; Sr, 407.771; and Ba, 493.408.

2.7. Statistical analysis

The data for Plackett-Burman experimental design were analyzed in the Statgraphics Centurion 19.05.01 version program, and the data for Box-Behnken experimental design were analyzed in the Design Expert 13 program, by a one-way ANOVA (analysis of variance). Statistical significance was considered with a confidence interval of 95 %.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Characterization of fruit peels

Orange and banana peels are by-products of the food processing industry (*e.g.*, fruit juice, fruit pulp, baby food industry, *etc.*), which are rich in sugars and fiber, making them potential substrates for biotechnological processes. Fruit peels from 2 different batches were characterized for moisture, carbohydrates, reducing sugars, crude fiber, pectin, total protein, lipids, ashes, total phenols, antioxidant activity, and total carbon and nitrogen in aqueous extracts (Tables 3.1 and 3.2).

The orange peels from the 2 batches had in general similar compositions, except in their carbohydrate content. Their initial moisture content was slightly higher than other values found in the literature (Chau and Huang, 2003; Carranza-Méndez *et al.*, 2022; Mahmoud *et al.*, 2015). Orange peels were composed of 36.59 % - 63.12 % carbohydrates, 25.17 % - 28.20 % of reducing sugars, 11.06 % - 12.55 % of crude fiber, and 18.69 % - 19.82 % of pectin. Similar compositions were reported by other authors (Chau and Huang, 2003; Rivas *et al.*, 2008; Carranza-Méndez *et al.*, 2022; Mahmoud *et al.*, 2015), except for the pectin content which was higher than the content reported by Chau and Huang (2003) but lower than the content reported by Rivas *et al.* (2008). The compounds found in smaller percentages were protein, lipids, and ashes. The protein content was similar to the that reported by Mahmoud *et al.* (2015) and Rivas *et al.* (2008), but different from the results obtained by other authors (Chau and Huang, 2003;

Carranza-Méndez *et al.*, 2022). The analysis of orange peels extracts showed that these peels had low amount of total free nitrogen. Phenolic compounds and antioxidant activity were also detected in orange peels extracts.

Table 3.1. Main composition of orange peels. Data (w/w, in dry basis) are the average ± standard deviation of 3 independent analyses

Component	Batch 1*	Batch 2*	Chau and Huang (2003)**	Rivas *et al.* (2008)*	Carranza--Méndez *et al.* (2022)*	Mahmoud *et al.* (2015)*
Moisture (%)	81.65 ± 0.69	82.42 ± 0.76	75.30 ± 1.02	-	73.32 ± 0.94	61.58 ± 0.43
Carbohydrates (%)	63.12 ± 2.57	36.59 ± 4.07	57.00 ± 1.00 (dietary fiber) 27.30 (carbohydrates)	63.05 (fiber)	-	-
Reducing sugars (%)	25.17 ± 0.95	28.20 ± 0.16	-	16.9	37.49 ± 1.78	-
Crude fiber (%)	12.55 ± 0.38	11.06 ± 0.50	-	-	8.75 ± 1.06	10.24 ± 0.38
Pectin (%)	18.69 ± 0.84	19.82 ± 1.27	9.41 ± 0.09 (soluble dietary fiber)	42.5	-	-
Total protein (%)	4.78 ± 0.18	6.10 ± 0.06	10.20 ± 0.37	6.50	0.97 ± 0.13	5.59 ± 0.34
Total lipids (%)	1.67 ± 0.07	1.73 ± 0.12	2.22 ± 0.61	1.95	0.71 ± 0.74	1.16 ± 0.03
Ashes (%)	3.65 ± 0.02	2.52 ± 0.02	3.30 ± 0.05	3.50	3.42 ± 0.05	4.43 ± 0.04
Total phenols (mg/g)	10.57 ± 0.25	8.09 ± 0.37	-	-	-	-
Antioxidant activity (μmol/g)	8.74 ± 0.30	5.91 ± 0.28	-	-	-	-
Total carbon (mg/g)	5.96 ± 0.01	-	-	-	-	-
Total nitrogen (mg/g)	0.071 ± 0.003	-	-	-	-	-

* *Citrus sinensis* Linnaeus "Valencia". ** *Citrus sinensis* Linnaeus "Liucheng".

Table 3.2. Characterization of banana peels. Data (% w/w, in dry basis) are the average ± standard deviation of 3 independent analyses

Component	Batch 1*	Batch 2*	Aboul-Enein *et al.* (2016)**	Anhwange *et al.* (2009)***	Abera *et al.* (2023)*
Moisture (%)	91.04 ± 0.29	92.19 ± 0.83	88.10 ± 0.18	-	-
Carbohydrates (%)	37.50 ± 2.06	20.76 ± 0.53	68.31 ± 0.83	59.00 ± 1.36	37.8 ± 0.11
Reducing sugars (%)	11.20 ± 1.71	19.71 ± 0.65	-	-	-
Crude fiber (%)	18.82 ± 0.48	23.84 ± 1.07	-	31.70 ± 0.25	34.37 ± 0.23
Pectin (%)	3.67 ± 0.34	4.55 ± 0.65	-	-	-
Total protein (%)	7.54 ± 0.10	8.29 ± 0.02	7.57 ± 0.30	0.90 ± 0.25	2.17 ± 0.81
Total lipids (%)	4.04 ± 0.31	7.53 ± 0.45	10.44 ± 0.89	1.70 ± 0.10	6.66 ± 0.10
Ashes (%)	17.44 ± 0.19	9.59 ± 0.05	13.42 ± 0.35	8.50 ± 1.52	8.50 ± 0.50
Total phenols (mg/g)	3.31 ± 0.10	1.61 ± 0.24	9.89 ± 0.16	-	-
Antioxidant activity (μmol/g)	5.43 ± 1.15	2.91 ± 0.22	-	-	-
Total carbon (mg/g)	0.97 ± 0.06	-	-	-	-
Total nitrogen (mg/g)	0.0064 ± 0.003	-	-	-	-

* *Musa acuminata* Colla AAA Cavendish cultivar, ** *Musa paradaisica* L., *** *Musa sapientum*.

Banana peels used in this work had a high moisture content, similar to the moisture content obtained by Aboul-Enein *et al.* (2016). Banana peels were composed of 20.76 % - 37.50 % of carbohydrates, similarly to banana peels characterized by Abera *et al.* (2023), but these values were slightly different from each other and lower than the ones reported by Aboul-Enein *et al.* (2016) and Anhwange *et al.* (2009). Banana peels had lower contents of carbohydrates, reducing sugars and pectin relatively to orange peels, but higher contents in crude fiber, protein, lipids, and ashes. These compositions are in the range of values found in the literature (Aboul-Enein *et al.*, 2016; Anhwange *et al.*, 2009; Abera *et al.*, 2023), except for

crude fiber content which was lower than the values reported by Anhwange *et al.* (2009) and Abera *et al.* (2023). Additionally, banana peels had lower total phenols content, antioxidant activity and amounts of total free carbon and nitrogen than orange peels, but higher ashes content, due to a richer minerals composition overall (Tables 3.3 and 3.4).

Table 3.3. Characterization of mineral elements of orange peels. Data (in dry basis) are the average ± standard deviation of 2 independent analyses

Component	Batch 1*	Batch 2*	Czech *et al.* (2020)**	Mahmoud *et al.* (2015)*
Potassium (mg/g)	1.119 ± 0.047	1.174 ± 0.026	1.54	2.033 ± 0.021
Calcium (mg/g)	1.413 ± 0.088	0.963 ± 0.015	0.419	1.572 ± 0.020
Sodium (mg/g)	0.032 ± 0.002	0.052 ± 0.003	0.0054	1.352 ± 0.020
Phosphorous (mg/g)	0.171 ± 0.004	0.111 ± 0.004	0.253	0.292 ± 0.013
Magnesium (mg/g)	0.182 ± 0.007	0.134 ± 0.007	0.132	1.947 ± 0.021
Iron (μg/g)	1.119 ± 0.590	Below detection limit	5.1	3.323 ± 0.002
Copper (μg/g)	3.094 ± 0.050	0.653 ± 0.050	1.5	0.093 ± 0.002
Zinc (μg/g)	2.574 ± 0.740	1.715 ± 0.080	2.5	0.913 ± 0.002
Manganese (μg/g)	0.804 ± 0.020	0.843 ± 0.020	1.3	-
Strontium (μg/g)	8.193 ± 1.040	7.654 ± 0.160	-	-
Nickel (μg/g)	0.108 ± 0.010	0.116 ± 0.000	-	-
Barium (μg/g)	1.099 ± 0.040	1.053 ± 0.060	-	-

* *Citrus sinensis* L. "Valencia". ** *Citrus sinensis* L. "Navelina".

Orange peels used in this work contained a mineral composition similar to those found by other authors (Czech *et al.*, 2020; Mahmoud *et al.*, 2015). Orange peels contained macrominerals, such as potassium, calcium, sodium, phosphorous, and magnesium, and microminerals, such as iron, copper, zinc, and manganese. Trace elements like strontium, nickel and barium were also detected. Banana peels, on the other hand, were particularly rich in potassium, but also contained other minerals even though at lower amounts than reported by Aboul-Enein *et al.* (2016) and Anhwange *et al.* (2009). Nevertheless, both orange and banana peels were reported to be a source of minerals, a potential source for microorganism growth factors.

Table 3.4. Characterization of mineral elements of banana peels. Data (in dry basis) are the average ± standard deviation of 2 independent analyses

Component	Batch 1*	Batch 2*	Aboul-Enein *et al.* (2016)**	Anhwange *et al.* (2009)***
Potassium (mg/g)	4.835 ± 0.354	4.167 ± 0.056	93.9 ± 17.4	78.10 ± 6.58
Calcium (mg/g)	0.492 ± 0.014	0.555 ± 0.002	4.4 ± 0.8	19.20 ± 0.00
Sodium (mg/g)	0.040 ± 0.000	0.042 ± 0.000	1.8 ± 0.2	24.30 ± 0.12
Phosphorous (mg/g)	0.250 ± 0.000	0.253 ± 0.002	0.9 ± 0.1	-
Magnesium (mg/g)	0.212 ± 0.008	0.188 ± 0.002	7.1 ± 0.5	-
Iron (μg/g)	1.972 ± 0.027	2.812 ± 0.152	96.50 ± 19.73	0.61 ± 0.22
Copper (μg/g)	0.876 ± 0.033	1.036 ± 0.002	3.73 ± 1.14	-
Zinc (μg/g)	2.972 ± 0.007	2.638 ± 0.220	27.95 ± 3.31	-
Manganese (μg/g)	7.799 ± 0.091	5.332 ± 0.141	35.01 ± 4.25	76200 ± 0
Strontium (μg/g)	4.203 ± 0.340	4.822 ± 0.246	-	30 ± 10
Nickel (μg/g)	0.130 ± 0.012	0.093 ± 0.005	-	-
Barium (μg/g)	1.736 ± 0.316	1.620 ± 0.050	-	-

* *Musa acuminata* Colla AAA Cavendish cultivar, ** *Musa paradaisica* L., *** *Musa sapientum* .

3.2. Effect of medium composition and operational conditions on nutritional value of fruit peels

Firstly, the effect of several factors (fermentation time, inoculum size, moisture content, ammonium sulfate (AS) concentration, corn steep liquor (CSL) concentration, and fungal species) on nutritional value of orange (OP) and banana (BP) peels fermented by filamentous fungi in solid-state fermentation (SSF) was evaluated using a Plackett-Burman experimental design. The three independent variables with more effect on dependent responses (total protein content, reducing sugars concentration, total phenols and antioxidant activity) were further studied using a Box-Behnken experimental design.

3.2.1 Variable screening: Plackett-Burman design

Tables 3.5 and 3.6 summarize the defined responses analyzed for the Plackett-Burman experimental designs obtained after SSF of orange and banana peels. Values obtained for protein content ranged from 5.44 % (run 12) to 13.33 % (run 7) after fermentation of orange peels (Table 3.5) and from 7.74 %

(run 12) to 11.01 % (run 4) after fermentation of banana peels (Table 3.6). In general, protein content increased in all experiments comparatively to unfermented peels, especially in orange peels experiments. Particularly in run 7 (orange peels) and run 4 (banana peels), protein content increased 178.9 % and 31.5 %, respectively. The highest protein content in fermented orange peels was obtained in experiments 7 and 11, which had in common the inoculation with *A. ibericus*, suggesting that this fungal species have high ability to grow in these peels since the high protein content is also related with high biomass concentration (Schmidt and Furlong, 2012).

The reducing sugars content ranged from 2.71 % (run 2) to 37.34 % (run 9) after fermentation of orange peels and from 2.22 % (run 4) to 14.99 % (run 6) after fermentation of banana peels. In all experiments with banana peels, reducing sugars content decreased after peels fermentation and the lowest amount of reducing sugars was obtained in the run with the highest protein content (run 4). This suggests that filamentous fungi assimilated the soluble sugars for biomass proliferation and metabolite production. By contrast, in orange peels experiments, the amount of reducing sugars increased in some runs, decreased in others and no direct relation between reducing sugars and protein content was observed. The increase in soluble sugars may suggest that fungal species excreted hydrolytic enzymes that hydrolyzed complex carbohydrates into sugar monomers (Abdel-Azeem *et al.*, 2021; Barzee *et al.*, 2021).

In general, the content of total phenols did not suffer a significant change after the fermentation of fruit peels with filamentous fungi, ranging from 5.85 mg/g (run 9) to 10.80 mg/g (run 12) in orange peels experiments and from 1.59 mg/g (run 9) to 2.28 mg/g (run 1) in banana peels experiments.

The antioxidant activity varied between 13.43 μmol/g (run 5) and 16.12 μmol/g (run 9) in orange peels experiments and between 5.22 μmol/g (run 9) to 14.66 μmol/g (run 12) in banana peels experiments. A considerable improvement in antioxidant activity was obtained after the fermentation of fruit peels with filamentous fungi. Particularly in run 9 (orange peels) and run 12 (banana peels), antioxidant activity increased 84.4 % and 170.0 %, respectively. Although antioxidant activity is usually directly related to phenols content, in this work no proportionality was observed between both parameters. This is often observed due to different phenolic compounds profiles in samples (Jacobo-Velázquez and Cisneros-Zevallos, 2009). A similar behavior is discussed in the literature for the relation between biomass production and protein content (Schmidt and Furlong, 2012). Despite the direct association between biomass concentration (not quantified in this work) and protein production, the biomass and protein content increase are not always proportional to each other.

Table 3.5. Experimental results of total protein, soluble sugars, total phenols, and antioxidant activity responses obtained in Plackett-Burman variable screening analysis after fermentation of orange peels. Data are represented in w/w (dry basis)

Run	Total protein (%)	Reducing sugars (%)	Total phenols (mg/g)	Antioxidant activity (μmol/g)
1	6.30	15.54	10.08	15.56
2	10.50	2.71	8.21	15.08
3	10.07	22.34	7.19	14.02
4	6.60	32.92	10.46	13.60
5	6.02	10.34	9.71	13.43
6	7.92	15.68	8.71	13.52
7	13.33	36.44	7.06	14.00
8	5.64	20.07	8.27	14.55
9	10.73	37.34	5.85	16.12
10	7.07	9.02	10.17	15.92
11	12.28	29.97	6.12	13.81
12	5.44	21.92	10.80	14.53

Table 3.6. Experimental results of total protein, soluble sugars, total phenols, and antioxidant activity responses obtained in Plackett-Burman variable screening analysis after fermentation of banana peels. Data are represented in w/w (dry basis)

Run	Total protein (%)	Reducing sugars (%)	Total phenols (mg/g)	Antioxidant activity (μmol/g)
1	9.21	8.44	2.28	9.36
2	9.01	5.31	2.13	9.37
3	9.44	3.60	1.92	10.14
4	11.01	2.22	1.63	7.36
5	8.39	2.66	1.78	7.33
6	7.95	14.99	2.11	6.30
7	9.39	2.44	2.18	8.23
8	8.24	11.00	1.76	8.07
9	9.05	12.81	1.59	5.22
10	9.04	13.39	2.10	12.89
11	8.45	11.67	1.99	12.93
12	7.74	12.26	1.84	14.66

Tables 3.7 and 3.8 show coefficients of regression and correlation coefficients (R^2) of Plackett-Burman design for orange and banana peels, respectively. Significant coefficients were identified by asterisk. Regression coefficients allow the determination of the effect of each constituent. A high value of regression coefficient indicates that the factor has a large impact on dependent variable. A R^2 close to 100 indicates a good correlation between the predicted response value and the actual response value. However, in the range of values tested in this work, it was not possible to predict the entire variability of the results, particularly for protein content (orange and banana peels) and total phenols (banana peels). Furthermore, due to the inability of the Plackett-Burman test to account for interactions between factors, the results may miss some factors where significant interactions occurred. In the experiments carried out with orange peels, only time was found as a significant factor for the antioxidant activity (Table 3.7). In the SSF with banana peels, inoculum size had a significant impact on protein and reducing sugars content. Moisture and CSL had a significant effect on antioxidant activity (Table 3.8).

Table 3.7. Regression coefficients and parameters for the linear model obtained by Plackett-Burman analysis for each response (total protein, reducing sugars, total phenols, and antioxidant activity) in orange peels solid-state fermentation

Linear coefficients	Total Protein	Reducing sugars	Total phenols	Antioxidant activity
Average	8.49167	21.1917	855.25	14.5
Time	-0.546667	8.17	-142.833	-1.06667*
Inoculum size	0.88	-4.45667	27.1667	-0.333333
Moisture	-1.11333	-12.5467	128.5	0.433333
AS	-0.493333	8.67667	13.1667	0.9
CSL	-1.72	-2.74	-13.8333	0.433333
Fungus species	-1.99333	-2.04	161.5	0.466667
R^2 (%)	34.056	71.9304	59.1577	78.7789
Adjusted R^2 (%)	0	38.247	10.147	53.3135

Table 3.8. Regression coefficients and parameters for the linear model obtained by Plackett-Burman analysis for each response (total protein, reducing sugars, total phenols, and antioxidant activity) in banana peels solid-state fermentation

Linear coefficients	Total Protein	Reducing sugars	Total phenols	Antioxidant activity
Average	8.91	8.39917	194.25	9.325
Time	-0.08	-2.715	-15.8333	0.883333
Inoculum size	0.94*	-6.925*	2.83333	-0.216667
Moisture	-0.54	1.11167	15.5	3.55*
AS	0.66	0.388333	-1.16667	0.616667
CSL	0.483333	-2.65167	-16.1667	-3.05*
Fungus species	-0.52	1.775	-22.1667	0.783333
R^2 (%)	76.8629	77.3715	67.9501	77.5342
Adjusted R^2 (%)	49.0984	50.2173	29.4901	50.5752

The main effects of each studied factor on total protein, reducing sugars, total phenols, and antioxidant activity after SSF of orange peels are depicted in Figure 3.1. A negative effect of time was observed for protein, total phenols, and antioxidant activity, while a positive effect was observed for reducing sugars. This indicates that, as time increases from 7 days to 14 days, reducing sugars concentration increases. By contrast, total protein, total phenols, and antioxidant activity were favored by a 7-day fermentation. In fact, the highest amount of protein and total phenols were obtained in runs 7 and 12, respectively, which were carried out for 7 days (Tables 2.1 and 3.5). The highest concentration of reducing sugars was obtained in a 14-day SSF (run 9).

A positive effect of inoculum size was observed for protein and total phenols, while a negative effect was observed for reducing sugars and antioxidant activity. This indicates that, as the inoculum size increases from 1×10^7 spores/g to 5×10^7 spores/g, reducing sugars and antioxidant activity reduce. In fact, the highest concentration of total phenols was obtained in run 12, which was inoculated with 5×10^7 spores/g (Tables 2.1 and 3.5). By contrast, reducing sugars and antioxidant activity were favored by a SSF inoculated with 1×10^7 spores/g (Figure 3.1).

A negative effect of moisture was observed for total protein and reducing sugars, while a positive effect was observed for total phenols and antioxidant activity (Figure 3.1). This indicates that, as moisture increases from 60 % to 75 %, total phenols and antioxidant activity increase.

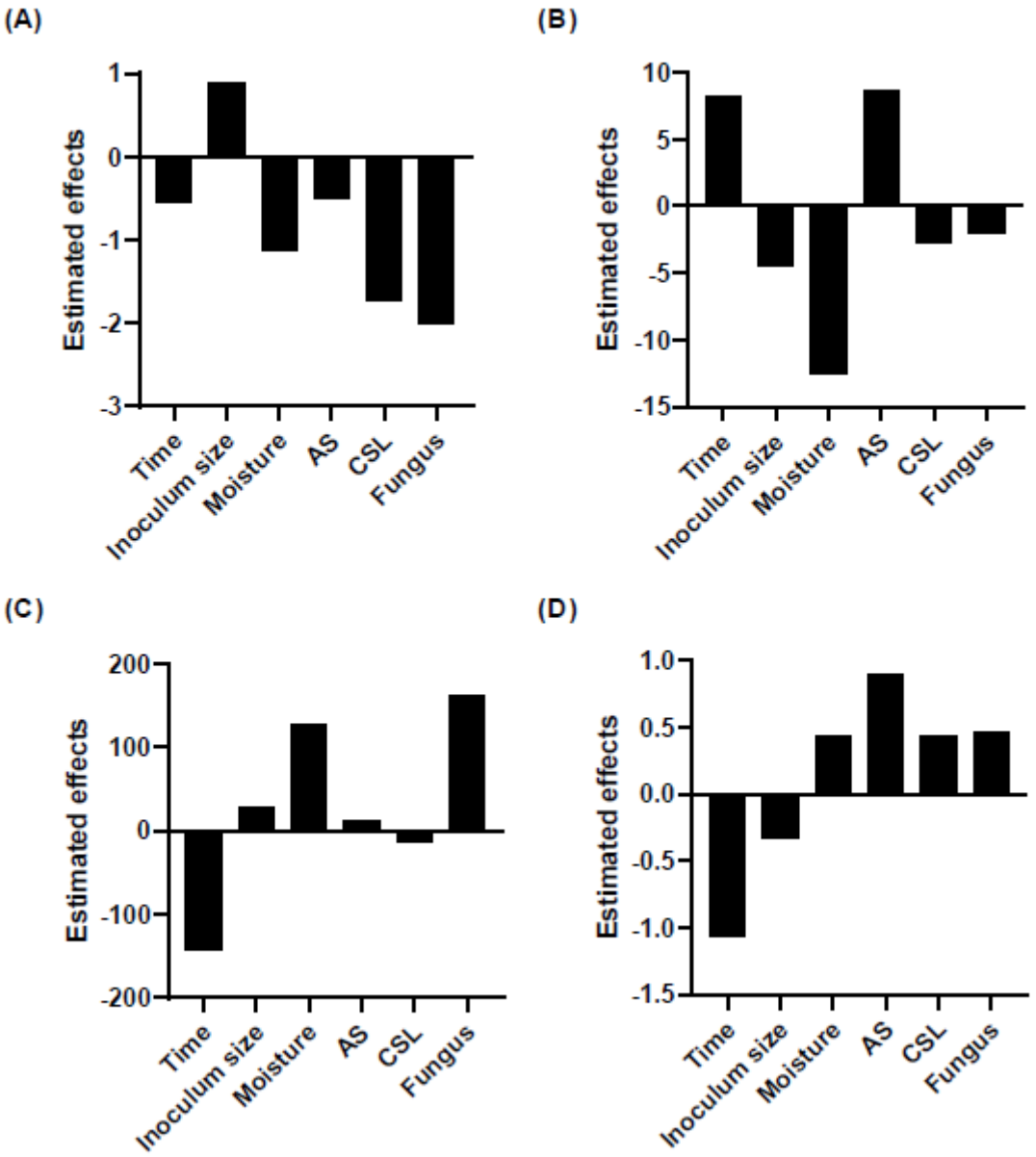

Figure 3.1. Estimated effects of several parameters (fermentation time, inoculum size, humidity of peels, ammonium sulfate (AS) and corn steep liquor (CSL) supplementation, and fungal species) on (A) total protein, (B) reducing sugars, (C) total phenols, and (D) antioxidant activity, obtained for solid-state fermentation of orange peels.

A positive effect of ammonium sulfate was observed for reducing sugars, total phenols, and antioxidant activity, while a negative effect was observed for total protein (Figure 3.1). This indicates that the supplementation of orange peels with ammonium sulfate did not increase protein content. By contrast, the supplementation of orange peels with 0.01 g/g ammonium sulfate during SSF, favored the content of reducing sugars, total phenols, and antioxidant activity. The highest antioxidant activity and reducing

sugars concentration were obtained in run 9, which was supplemented with 0.01 g/g ammonium sulfate (Tables 2.1 and 3.5).

A negative effect of corn steep liquor (CSL) was observed for protein, reducing sugars, and total phenols, while a positive effect was observed for antioxidant activity (Figure 3.1). This indicates that the supplementation of orange peels with 0.01 g/g CSL favored the antioxidant activity of orange peels after SSF.

The level (+1) of fungal species had a positive effect on total phenols and antioxidant activity, while the level (-1) favored total protein and reducing sugars (Figure 3.1). This indicates that SSF of orange peels with *A. ibericus* favored protein and reducing sugars. In fact, the highest protein content and the second highest reducing sugars concentration were obtained in the experiments inoculated with *A. ibericus* (run 7). By contrast, SSF of orange peels with *R. oryzae* favored total phenols and antioxidant activity. The highest content of total phenols was obtained in the experiments inoculated with *R. oryzae* (run 12) (Tables 2.1 and 3.5).

The main effects of each studied factor on total protein, reducing sugars, total phenols, and antioxidant activity after SSF of banana peels are depicted in Figure 3.2. A negative effect of time was observed for protein, reducing sugars, and total phenols, while a positive effect was observed for antioxidant activity. This indicates that, as time increases from 7 days to 14 days, antioxidant activity increases. By contrast, total protein, reducing sugars, and total phenols were favored by a 7-day fermentation. In fact, the highest amount of reducing sugars and total phenols of fermented banana peels were obtained in runs 6 and 1, respectively, which were carried out for 7 days (Tables 2.1 and 3.6).

A positive effect of inoculum size was observed for protein and total phenols, while a negative effect was observed for reducing sugars and antioxidant activity (Figure 3.2). This indicates that, as the inoculum size increases from 1×10^7 spores/g to 5×10^7 spores/g, reducing sugars and antioxidant activity reduce. In fact, the highest concentration of total phenols was obtained in run 1, which was inoculated with 5×10^7 spores/g. The highest concentration of reducing sugars was obtained in run 6, which was inoculated with 1×10^7 spores/g (Tables 2.1 and 3.6).

A negative effect of moisture was observed for protein content, and a positive effect for all the other responses (Figure 3.2). This indicates that, as moisture increase from 60 % to 75 %, reducing sugars, total phenols, and antioxidant activity increase. In contrast, an increase moisture content did not increase

protein production. The highest total phenols content in fermented banana peels was obtained in run 1, which was carried out with 75 % of moisture (Tables 2.1 and 3.6).

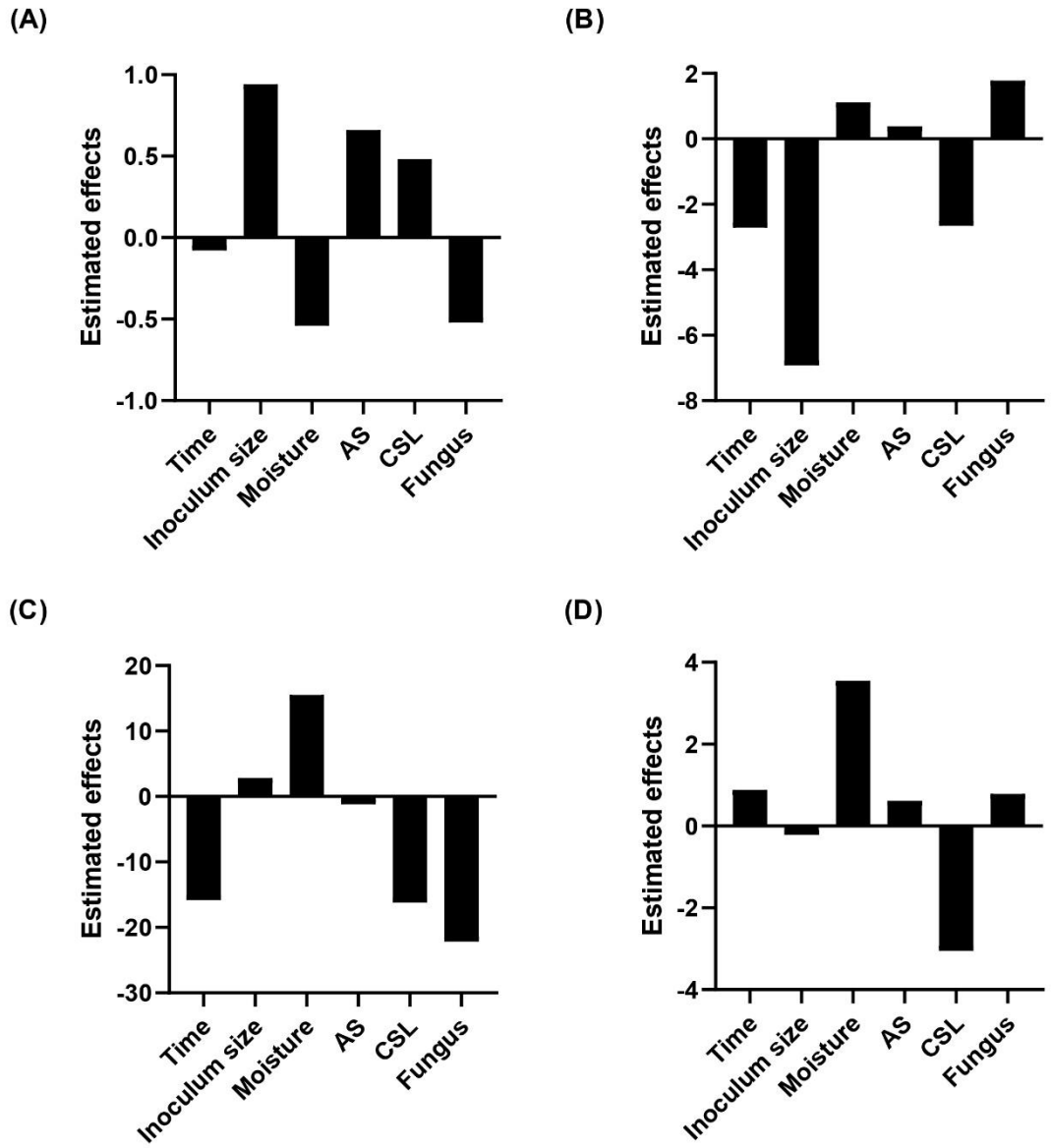

Figure 3.2. Estimated effects of several parameters (fermentation time, inoculum size, humidity of peels, ammonium sulfate and corn steep liquor supplementation and fungal species) on (A) total protein, (B) soluble sugars, (C) total phenols, and (D) antioxidant activity, obtained for solid-state fermentation of banana peels.

A positive effect of ammonium sulfate was observed for total protein, reducing sugars, and antioxidant activity, while a negative effect was observed for total phenols (Figure 3.2). This indicates that the

supplementation of banana peels with ammonium sulfate did not increase total phenols content. By contrast, the supplementation of banana peels with 0.01 g/g ammonium sulfate during SSF, favored the content of total protein, reducing sugars, and antioxidant activity. The highest protein content was obtained in run 4, which was supplemented with 0.01 g/g ammonium sulfate (Tables 2.1 and 3.6).

A negative effect of corn steep liquor (CSL) was observed for reducing sugars, total phenols, and antioxidant activity, while a positive effect was observed for total protein (Figure 3.2). This indicates that the supplementation of banana peels with 0.01 g/g CSL favored the protein content of banana peels after SSF. In fact, the highest content of reducing sugars, total phenols and antioxidant activity were obtained in runs 6, 1 and 12, respectively, which were not supplemented with CSL (Tables 2.1 and 3.6).

The level (+1) of fungal species had a positive effect on reducing sugars and antioxidant activity, while the level (-1) favored total protein and total phenols (Figure 3.2). This indicates that SSF of banana peels with *A. ibericus* favored protein and total phenols. By contrast, SSF of banana peels with *R. oryzae* favored reducing sugars and antioxidant activity. In fact, the highest concentration of antioxidant activity was obtained in run 12, which was inoculated with *R. oryzae* (Tables 2.1 and 3.6).

Schmidt and Furlong (2012) concluded that supplementation with ammonium sulfate was not a significative factor for protein production from rice bran under semi-SSF with *Rhizopus oryzae*, but it was significative for total phenols increase. Particle size was also significative for total protein and total phenols increase. Olorunnisola *et al.* (2018), however, reported a positive effect on protein production by ammonia supplementation (with ammonium phosphate), in sequential SSF of banana peels with *Phanerochaete chrysosporium* and *Candida utilis*. Kamal et al (2019) reported that fermentation time was a significative factor, with positive effect (in the range of 1 day – 5 days), for protein production from banana peels with *Aspergillus niger*, under SmF.

3.2.2. Variable optimization with Box-Behnken design

Since the results of regression coefficients obtained in the Plackett-Burman design were not significant, and also considering information reported in the literature, moisture content, and ammonium sulfate and corn steep liquor concentration were considered as three substantial factors for further SSF experiments. A Box-Behnken experimental design was used to optimize the level of each factor in order to attain a maximum content of total protein.

Table 3.9 summarizes the total protein content obtained in fermented orange peels (OP) and fermented banana peels (BP) in the Box-Behnken experimental matrix. The total protein content varied from 7.32 % (run 5) to 16.29 % (run 6) in fermented OP and from 8.35 % (run 14) to 17.67 % (run 2) in fermented BP. In general, the total protein content increased in fruit peels after SSF, relatively to unfermented peels. In SSF of orange peels, the lowest protein content was obtained in the run 5, which had as conditions 50 % moisture content, 0.005 g/g ammonium sulfate and no CSL. By contrast, the highest protein content was achieved in run 6, which had as conditions 70 % moisture content, 0.005 g/g ammonium sulfate and no CSL. The second highest protein content (13.49 %) was obtained in run 8, which had the same level of moisture and ammonium sulfate of run 6, but 0.01 g/g CSL. These results suggest that high level of moisture content favored protein production in SSF of orange peels, while CSL supplementation may had a negative effect in protein production. This was confirmed in Table 3.10, with the estimated factor coefficients by the quadratic model obtained by response surface methodology. Moisture content and the quadratic term of CSL were considered significant factors, with positive effect, for protein production ($p<0.05$). The individual effect of CSL, even though not significant ($p > 0.05$), was negative for protein production in SSF from orange peels.

Table 3.9. Experimental results of total protein content in fermented orange peels (OP) and in fermented banana peels (BP), obtained for Box-Behnken optimization analysis

Run	Total protein (%) in fermented OP	Total protein (%) in fermented BP
1	7.89	10.67
2	9.78	17.67
3	8.18	11.92
4	12.74	10.75
5	7.32	10.39
6	16.29	11.63
7	10.41	10.19
8	13.49	10.96
9	13.31	10.65
10	11.96	13.65
11	12.53	9.94
12	11.78	12.92
13	10.32	8.72

Table 3.9. Experimental results of total protein content in fermented orange peels (OP) and in fermented banana peels (BP), obtained for Box-Behnken optimization analysis (*continued*)

Run	Total protein (%) in fermented OP	Total protein (%) in fermented BP
14	10.64	8.35
15	10.68	9.09

Table 3.10. ANOVA results of the quadratic models obtained for protein production under solid-state fermentation (SSF) of orange and banana peels using a Box-Behnken experimental design. Significant factors are identified with an asterisk

	SSF of orange peels			SSF of banana peels		
Factor	Coefficient estimate	Standard error	*p*-value	Coefficient estimate	Standard error	*p*-value
Intercept	10.55	0.7198	-	8.72	1.13	-
A (Moisture)	2.31	0.4408	0.0033*	0.9800	0.6911	0.2154
B (SA)	0.1437	0.4408	0.7575	0.0387	0.6911	0.9575
C (CSL)	-0.0838	0.4408	0.8568	-0.2888	0.6911	0.6934
AB	0.6675	0.6233	0.3332	-2.04	0.9773	0.0909
AC	-1.47	0.6233	0.0646	-0.1175	0.9773	0.9090
BC	0.1500	0.6233	0.8194	-0.0050	0.9773	0.9961
A^2	-0.7083	0.6488	0.3247	1.52	1.02	0.1960
B^2	-0.1908	0.6488	0.7805	2.52	1.02	0.0564
C^2	2.04	0.6488	0.0256*	0.5550	1.02	0.6088
R^2		90.26 %			74.30 %	
Adjusted R^2		72.72 %			28.05 %	
Model *p*-value		0.0430*			0.3128	
Residual *p*-value		0.0150*			0.0214*	

In SSF of banana peels, the lowest protein content was obtained in the run 14, which was a central point for the experimental design and had as conditions 60 % moisture content, 0.005 g/g ammonium sulfate and 0.005 g/g CSL. By contrast, the highest protein content was attained in run 2, which had as

conditions 70 % moisture content, no ammonium sulfate and 0.005 g/g CSL. The second highest total protein content obtained in fermented banana peels (13.65 %, run 10) was performed with 60 % moisture content, 0.01 g/g ammonium sulfate and no CSL. The total protein content results for fermented banana peels could not be adjusted with a significant model, since many results with different operational conditions resulted in very similar total protein values (around 10 % total protein), specifically runs 1, 4, 5, 7, 8, 9, and 11 (Table 3.9). Consequently, it was not possible to determine the significancy of any factors or interactions between them (Table 3.10), in the range of operational conditions studied.

The quadratic models for the relation among protein production and moisture content (%), supplementation with ammonium sulfate (g/g), and supplementation with CSL (g/g), from SSF in orange (OP) and banana (BP) peels are the following equations:

Total Protein (%) in fermented OP = - 31.72 + 1.16175×Moisture − 725.91667×SA + 904.58333×CSL + 13.35×Moisture×SA − 29.45×Moisture×CSL + 6000×SA×CSL − 0.007083×Moisture^2 − 7633.33333×SA^2 + 81566.66667×CSL^2

Total Protein (%) in fermented BP = 47.82500 − 1.50700×Moisture + 1453.75000×SA − 137.75×CSL − 40.85000×Moisture×SA − 2.35×Moisture×CSL − 200×SA×CSL + 0.015175×Moisture^2 + 100600×SA^2 + 22200×CSL^2

These models predicted a maximum total protein of 16.22 % (with 0.992 desirability) in fermented orange peels at the conditions 70 % moisture, 0.01 g/g ammonium sulfate and without CSL. For banana peels, the quadratic model predicted a maximum total protein of 16.69 % (with 0.895 desirability) at the conditions 70 % moisture content, and without ammonium sulfate and CSL (Figure 3.3). Additionally, the 95 % confidence level intervals for these total protein content points were wide (12.43 % - 20.00 %, OP; 10.76 % - 22.63 %, BP).

The 3D surface plots for total protein content in fermented fruit peels are depicted in Figure 3.4. It can be observed that neither for orange peels nor for banana peels the plots are completely concave down surfaces. In fact, for SSF of orange peels, the plot for ammonium sulfate vs moisture (Fig. 3.4A1) is a concave down surface but has no maximum point; the plot for CSL vs moisture (Fig. 3.4A2) is a curved surface with inflexion after which the concave surface becomes a convex surface; and the plot for CSL vs ammonium sulfate (Fig. 3.4A3) is a concave up (convex) surface. For SSF of banana peels, all plots of moisture, ammonium sulfate and CSL are convex surfaces. This means that, although the quadratic

models predicted maximum total protein content points for the operational conditions of SSF of banana peels, it was not possible to predict, in the range studied, the optimal conditions for protein production.

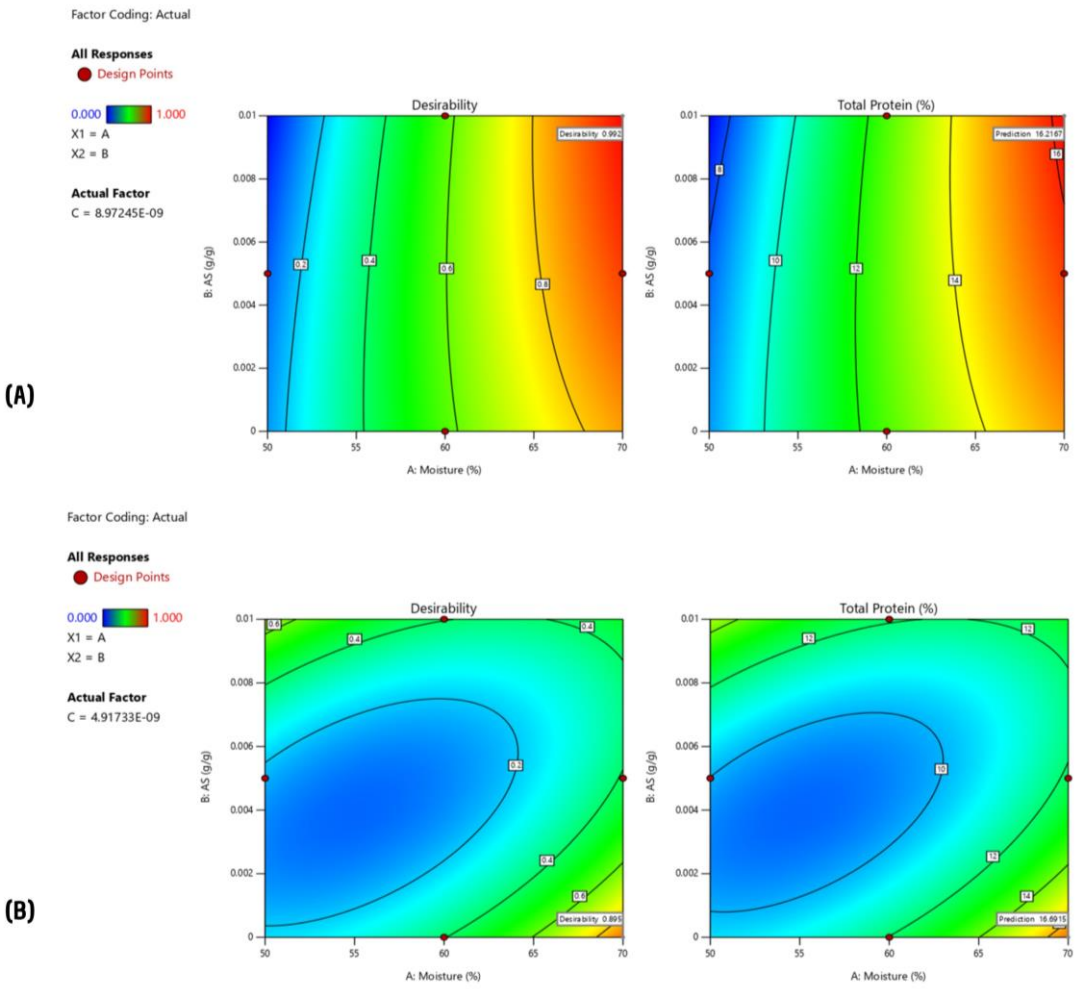

Figure 3.3. Plots of predicted desirability and total protein responses for solid-state fermentation of (A) orange peels and (B) banana peels, according to moisture and ammonium sulfate (SA) levels, maintaining CSL at 0.

Since the Box-Behnken optimization design did not predict optimal points for protein production and also due to the residual significance of the models obtained (Table 3.10), the confirmation of the maximum points predicted by the quadratic models was not performed. Instead, the fermented peels with the highest protein content (run 6 for OP; run 2, for BP) were further characterized for carbohydrates, reducing sugars, crude fiber, pectin, total lipids and antioxidant activity. SSF controls for these experimental runs were also characterized (Table 3.11).

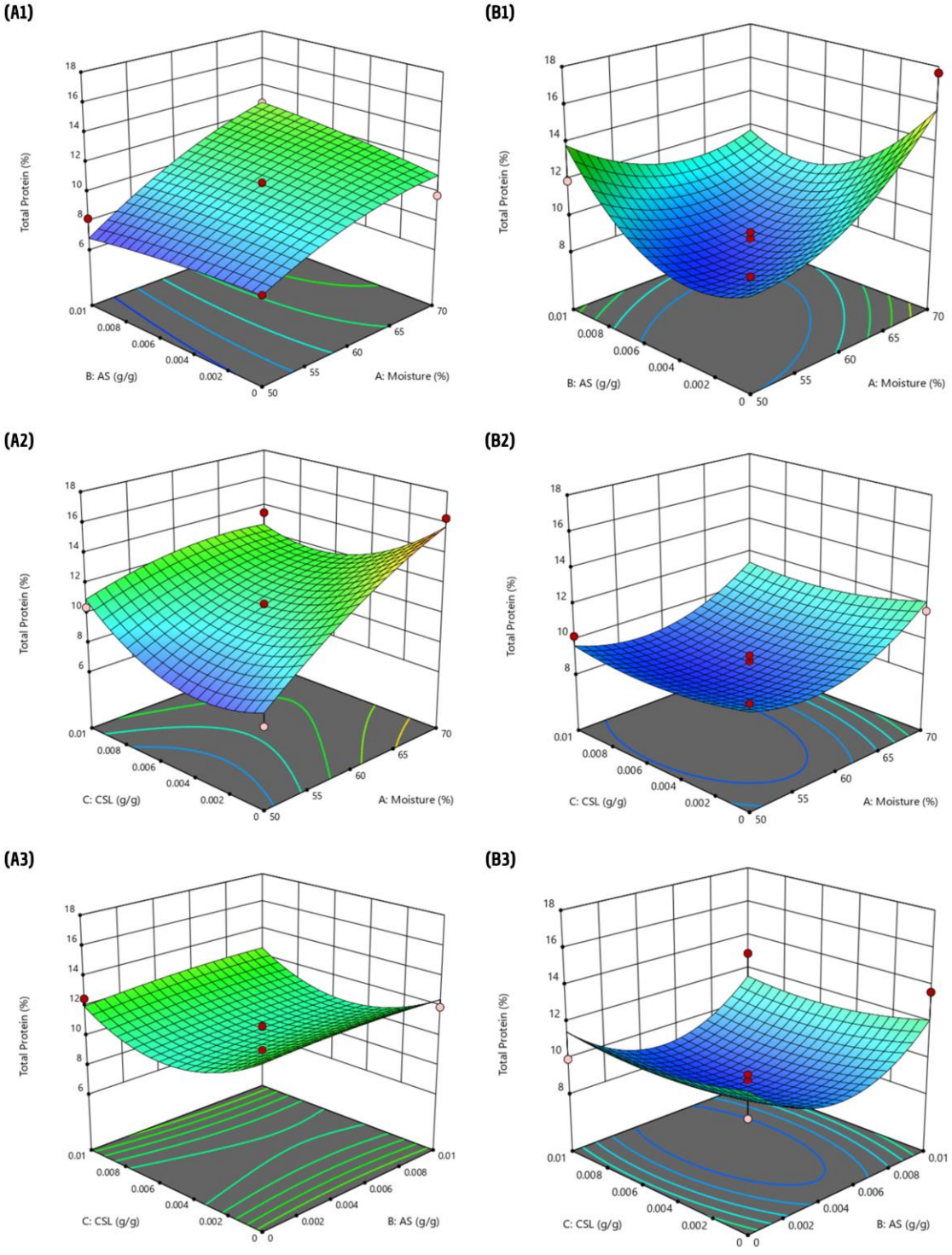

Figure 3.4. 3D surface plots indicating the effects of moisture vs ammonium sulfate (AS) (A1, B1), moisture vs corn steep liquor (CSL) (A2, B2), and ammonium sulfate vs corn steep liquor (A3, B3) on protein content obtained in experiments with orange peels (A1-A3) and banana peels (B1-B3).

Table 3.11. Composition of fermented orange peels (OP) and fermented banana peels (BP) obtained under solid-state fermentation at 25 °C, for 7 days with *Aspergillus ibericus* (initial concentration of 5×10^7 spores/g), at 70 % moisture content. Supplementation for OP was 0.005 g/g ammonium sulfate, and supplementation for BP was 0.005 g/g CSL. Data (w/w, in dry basis) were presented as average ± standard deviation of 2 independent analysis

Component	OP control	Fermented OP	BP control	Fermented BP
Carbohydrates (%)	32.63 ± 3.91	11.98 ± 1.18	20.22 ± 2.40	16.91 ± 1.87
Reducing sugars (%)	24.34 ± 3.02	1.72 ± 0.17	12.31 ± 2.92	4.03 ± 0.14
Crude fiber (%)	10.42 ± 0.24	26.86 ± 0.94	24.48 ± 3.69	23.88 ± 4.12
Pectin (%)	20.88 ± 2.07	3.81 ± 1.10	5.28 ± 0.14	3.76 ± 0.07
Total protein (%)	9.96 ± 0.20	16.23 ± 0.43	8.68 ± 0.45	17.49 ± 0.78
Total lipids (%)	1.67 ± 0.03	4.22 ± 0.11	8.35 ± 0.27	9.41 ± 0.60
Potassium (mg/g)	1.108 ± 0.112	1.973 ± 0.172	3.188 ± 0.147	4.817 ± 1.677
Calcium (mg/g)	1.157 ± 0.136	1.882 ± 0.156	0.482 ± 0.102	0.812 ± 0.403
Sodium (mg/g)	0.046 ± 0.004	0.075 ± 0.005	0.038 ± 0.006	0.048 ± 0.013
Phosphorous (mg/g)	0.144 ± 0.011	0.243 ± 0.006	0.252 ± 0.090	0.380 ± 0.146
Magnesium (mg/g)	0.115 ± 0.010	0.182 ± 0.015	0.132 ± 0.020	0.200 ± 0.074
Iron (μg/g)	0.038 ± 0.012	0.098 ± 0.035	0.071 ± 0.014	0.107 ± 0.046
Copper (μg/g)	0.006 ± 0.001	0.012 ± 0.001	0.010 ± 0.004	0.014 ± 0.005
Zinc (μg/g)	0.018 ± 0.002	0.026 ± 0.001	0.040 ± 0.002	0.035 ± 0.002
Manganese (μg/g)	0.010 ± 0.001	0.016 ± 0.001	0.056 ± 0.022	0.086 ± 0.033
Strontium (μg/g)	0.106 ± 0.006	0.163 ± 0.008	0.048 ± 0.021	0.078 ± 0.034
Nickel (μg/g)	0.001 ± 0.000	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Barium (μg/g)	0.011 ± 0.002	0.019 ± 0.001	0.016 ± 0.002	0.025 ± 0.010
Antioxidant activity (μmol/g)	9.79 ± 0.49	20.02 ± 1.26	8.91 ± 0.30	9.75 ± 0.56

Control experiments (fruit peels without inoculation) are important to evaluate if changes in fruit peels composition occurred due to solid-state fermentation with *A. ibericus*, or to time or addition of supplements. In both control experiments (orange and banana peels), the content of carbohydrates, reducing sugars, total protein, crude fiber, pectin, and total lipids is similar to those for natural peels (Tables 3.1, 3.2 and 3.11). The composition of macrominerals (potassium, calcium, sodium, phosphorous, and magnesium) was similar in natural fruit peels and in control experiments, however the composition of microminerals and trace elements (iron, copper, zinc, manganese, strontium, nickel, and

barium) decreased significantly (Tables 3.3, 3.4 and 3.11). This was probably due to differences in the procedures of characterization method: control experiments and fermented fruit peels were digested with a high moisture content, while the 2nd batch of orange peels was dried prior to digestion, thereafter, leading to an underestimation of minerals concentration in the controls and fermented fruit peels as a result of a decrease in the mass of dried sample/volume of nitric acid digestion ratio. Finally, antioxidant activities increased in the control fruit peels extracts (from 5.91 μmol/g to 9.79 μmol/g (OP; Tables 3.1 and 3.11) and from 2.91 μmol/g to 8.91 μmol/g (BP; Tables 3.2 and 3.11)). This increase may be explained due to the addition of nitrogen supplements or chemical transformations in the antioxidant compounds initially present in fruit peels. It could also be a result of loss of activity of polyphenol oxidase. This enzyme, present in many fruits, is responsible for the loss of antioxidant activity and their browning. As the fruit matures, enzyme loses its activity, therefore antioxidant activity may increase, however, its presence is more commonly associated with bananas, rather than oranges (Sui *et al.*, 2023; Ottaviani *et al.*, 2023).

Solid-state fermentation increased the nutritional value of fruit peels relative to the control experiments and natural fruit peels. Firstly, the carbohydrate decreased by 63.3 % in orange peels, from 32.63 % to 11.98 %, and by 16.4 % in banana peels from 20.22 % to 16.91 %, (Table 3.11). The reducing sugars content also changed from 24.34 % to 1.72 % (decrease of 92.9 %) in orange peels, and from 12.31 % to 4.03 % (decrease of 67.3 %) in banana peels. In contrast, the pectin content exhibited a considerable decrease, declining from 20.88 % to 3.81 % (an 81.8 % reduction) in orange peels and from 5.28 % to 3.76 % (a 28.8 % decrease) in banana peels. Ultimately, the final pectin content was found to be similar in both orange and banana peels (Table 3.11). These fluctuations observed in carbohydrates and soluble fiber suggest the possibility of *Aspergillus ibericus* species having secreted hydrolytic enzymes into the fruit peels medium to hydrolyze complex carbon sources into simple sugars and galacturonic acid for their metabolic activities, particularly in the breakdown of complex carbohydrates and pectin (Mahesh *et al.*, 2016; Biz *et al.*, 2016).

The crude fiber content, in the other hand, increased by 157.8 % in orange peels from 10.42 % to 26.86 %, and did not change in banana peels (final composition of 23.88 %) (Table 3.11). Additionally, protein production was confirmed, since relatively to the control experiments, the total protein content increased by 63.0 % (from 9.96 % to 16.23 %) in orange peels and by 101.5 % (from 8.68 % to 17.49 %) in banana peels (Table 3.11). Additionally, the lipids content was slightly enhanced in fruit peels (from 1.67 % to 4.22 % in orange peels, and from 8.35 % to 9.41 % in banana peels (Table 3.11)), likely due to microbial growth and the lipidic composition of microbial membranes (Rella *et al.*, 2016). Antioxidant activity also

increased by 104.5 % in orange peels, from 9.79 μmol/g to 20.02 μmol/g. However, it did not vary significantly in fermented banana peels, but also increased from 8.91 μmol/g to 9.75 μmol/g (Table 3.11). These results are in accordance with the literature, since SSF of fruit peels and other fruit by-products by filamentous fungi (particularly *Rhizopus* sp. and *Aspergillus* sp.) has been reported to increase their contents of protein, fiber, and lipids, as well as their antioxidant activity, and also to decrease reducing sugars content (Ibarruri *et al.*, 2021a, 2021b; Dulf *et al.*, 2016).

Relatively to micronutrient value, the mineral composition of fruit peels increased significantly with solid-state fermentation in all studied elements in both orange and banana peels, except for zinc and nickel in banana peels, which practically did not vary (see Table 3.11). In orange peels, all increases in mineral concentration were higher than 40 %, the biggest increase being of iron content (increase by 157.9 %, from 0.038 μg/g to 0.098 μg/g). In banana peels, all increases were higher than 25 %, the biggest increase being of calcium content (increase by 68.5 %, from 0.482 mg/g to 0.812 mg/g) (Table 3.11). The increase in nutrient value of fruit peels via increase of mineral content is also supported by the literature since this increase is due to microbial protein (and biomass) production. Single-cell microorganisms are a source of minerals, among other components (Sharif *et al.*, 2021).

To summarize, nutritional value of orange and banana peels increased with solid-state fermentation by filamentous fungi.

CHAPTER 4. CONCLUSIONS AND FUTURE PERSPECTIVES

The effect of fermentation time, inoculum size, moisture content, nitrogen supplementation (ammonium sulfate and corn steep liquor (CSL)), and fungal species on total protein, reducing sugars, total phenols, and antioxidant activity of orange and banana peels were evaluated by a Plackett-Burman variable screening analysis in solid-state fermentation (SSF). Moisture content, and concentration of ammonium sulfate and CSL were then studied with a Box-Behnken optimization design, with the objective of upgrading the nutritional composition of fruit peels, mainly by increasing their total protein content, by SSF with *A. ibericus* at 25 °C, with an initial concentration of 5×10^7 spores/g for 7 days. The highest protein content in SSF of banana peels was obtained in the experiments carried out with 70 % moisture content, no supplementation with ammonium sulfate and supplementation with 0.005 g/g CSL. The highest protein content in SSF of orange peels was obtained in the experiments carried out with 70 % moisture content, 0.005 g/g ammonium sulfate and without CSL.

It was demonstrated that SSF with *A. ibericus* improved the nutritional value of fruit peels. Specifically, the content of total protein increased by 63.0 % and 101.5 %, the content of carbohydrates decreased by 63.3 % and 16.4 % and the reducing sugars content decreased by 92.9 % and 67.3 % in orange and banana peels, respectively. Additionally, the content of lipids increased to 4.22 % in orange peels and to 9.41 % in banana peels, while crude fiber content and the antioxidant activity increased in orange peels, by 157.8 % and by 104.5 %, respectively.

Based on these results, microbial protein produced by filamentous fungi, particularly A. ibericus and R. oryzae, under SSF from orange and banana peels have the potential to be used as food, due to its rich nutritional composition. This would alleviate the pressure on conventional food systems while simultaneously adding value to by-products of large food industries.

In future experiments, it would be interesting to study other factors with the Box-Behnken design, such as inoculum size, fermentation time, and higher levels for moisture, to further maximize protein content and antioxidant activity of fruit peels. Other factors, such as pH and particle size, could also be studied. To further assess the potential of fermented fruit peels for human food applications, antinutrients and mycotoxins profile essays should be carried out. Moreover, to evaluate the value of protein, the amino acids profile should be analyzed.

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