POLITECNICO DI TORINO

College of Chemical and Materials Engineering

Master's Degree
in Chemical and Sustainable Processes Engineering

Master Thesis

Preliminary tests for the valorisation of food by-products - Brewer's Spent Grains (BSG) and Apple Pomace (AP) - through biotechnological approaches

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Sommario

Nel corso degli anni, la popolazione mondiale ha registrato un aumento significativo e si stima che supererà i 9 miliardi entro il 2050, portando conseguentemente a un aumento consistente della domanda di cibo. Soddisfare questa domanda comporterà a sua volta l'intensificazione di tutti quei processi atti a trasformare la materia prima in prodotto finito a livello di consumatore, con un consequenziale aumento dei quantitativi di rifiuti alimentari generati lungo tutto la filiera produttiva. Tale scenario viene ulteriormente esacerbato dai drastici cambiamenti climatici e dal calo della produttività agricola, per via dell'aumento dell'aridità dei suoli e di una scarsa disponibilità di fertilizzanti.

Oggi, i rifiuti alimentari o correlati al cibo originariamente destinati al consumo umano (FLW) sono stimati in 1,3 miliardi di tonnellate all'anno a livello globale e costituiscono il 25-70% dei rifiuti solidi urbani (MSW), stimati nel 2011 in circa 2 miliardi di tonnellate all'anno in tutto il mondo. La continua crescita degli FLW non rappresenta solo un'importante fonte di inquinamento con un significativo impatto ambientale, ma costituisce anche una fonte molto interessante di prodotti ad alto valore aggiunto, ampiamente disponibili e a basso costo.

I processi e le tecnologie che si pongono come obiettivo la valorizzazione di questa biomassa utilizzano e combinano diverse tecniche, che includono quelle biologiche (come la digestione anaerobica e la fermentazione), quelle biochimiche (come l'idrolisi enzimatica), quelle termochimiche (come l'incenerimento, la pirolisi e la gassificazione) e quelle termofisiche (come l'ultrafiltrazione e l'estrazione a ultrasuoni). Molti di questi processi risultano tuttavia solo su scala di laboratorio, la ricerca condotta in questo proposito sta quindi crescendo nel tempo per ottenere in futuro non solo un significativo potenziamento della valorizzazione, applicabile in particolar modo su scala industriale, ma anche un maggior inquadramento in un quadro di sostenibilità.

Il potenziale tecnologico degli FLW ha portato a una revisione e a un adattamento della gerarchia dei rifiuti solidi urbani ai rifiuti alimentari, mettendo in risaldo la necessità di attuare strategie che, con il minor impatto ambientale possibile, mirino a soddisfare il fabbisogno alimentare mondiale valorizzando tutti i sottoprodotti dell'industria alimentare come nuovi alimenti per il consumo umano e non solo come mangimi o fonti per la produzione di energia.

Fra gli FLW ritroviamo i sotto prodotti due importi filiere alimentari: le trebbie esauste della birra (BSG) e la sansa di mele (AP). La BSG costituisce circa l'85% in peso dei sottoprodotti totali generati dalla produzione di birra e nella sola Unione europea (UE) vengono prodotti circa 3,4 milioni di tonnellate ogni anno. A qualificare BSG come ingrediente alimentare funzionale è il suo alto valore nutrizionale - BSG è ricco di cellulosa e polisaccaridi non cellulosici, lignina e proteine - così come il suo basso costo e l'alta disponibilità. La sansa di mele (AP) è un sottoprodotto dell'industria dei succhi di frutta e rappresenta circa il 25% del frutto, mentre circa il 75% del peso del frutto viene estratto come succo. Ogni anno, in tutto il mondo, se ne producono diversi milioni di tonnellate. Solo in India, che è il nono produttore mondiale di mele, si producono annualmente 1 milione di tonnellate di AP. Utilizzata principalmente come mangime per il bestiame, la polpa di mela è una fonte economica e prontamente disponibile di alto valore nutrizionale - AP è ricca di fibre alimentari, polifenoli e composti bioattivi e povera di grassi e proteine - ed è quindi un sottoprodotto prezioso

da riutilizzare in nuove linee di produzione innovative.

Lo studio di tesi si pone come obiettivo quello di effettuare prove sperimentali per il recupero e la valorizzazione di questi sottoprodotti due importanti filiere alimentari, BSG e AP. L'approccio scelto è di tipo biotecnologico, per valutare preliminarmente la compatibilità tra queste matrici e alcuni microrganismi (di livello di biosicurezza 1 - BSL1- come *Rhizopous oligosporus*, *Chlorella vulgaris* e *Lentinula edodes*) ampiamente utilizzati nell'industria alimentare su matrici di simile composizione. Gli obiettivi della tesi riguardano lo sviluppo e il mantenimento di ciascun (micro)organismo (in base alle esigenze specifiche di ognuno), prove di fermentazione esplorative (in condizioni sommerse e allo stato solido) e l'acquisizione di conoscenze su possibili effetti sinergici derivanti dalla co-coltura di questi microrganismi per applicazioni simili.

Le prime prove respirometriche preliminari di *R. oligosporus* su ceci (come matrice libera o intrappolata) in microcosmi, come riportato nei grafici che seguono (Fig. 48 e Fig. 47) hanno dato 1,01 ± 0,07 mg/gTS con poche differenze per i sistemi in co-coltura con *C. vulgaris*. Infatti, se si considera il valore massimo di anidride carbonica prodotta, espresso in milligrammi per grammo di solido totale della matrice di ceci, indicato dal parametro a e la velocità con cui questo valore viene raggiunto, indicata dal parametro b, dal trattamento statistico (t – test) effettuato su tali parametri, riportati nelle tabelle 8 e 7, i sistemi, con matrice di ceci libera o intrappolata, in presenza e in assenza della microalga, per entrambi i parametri presentano statisticamente parlando un comportamento simile.

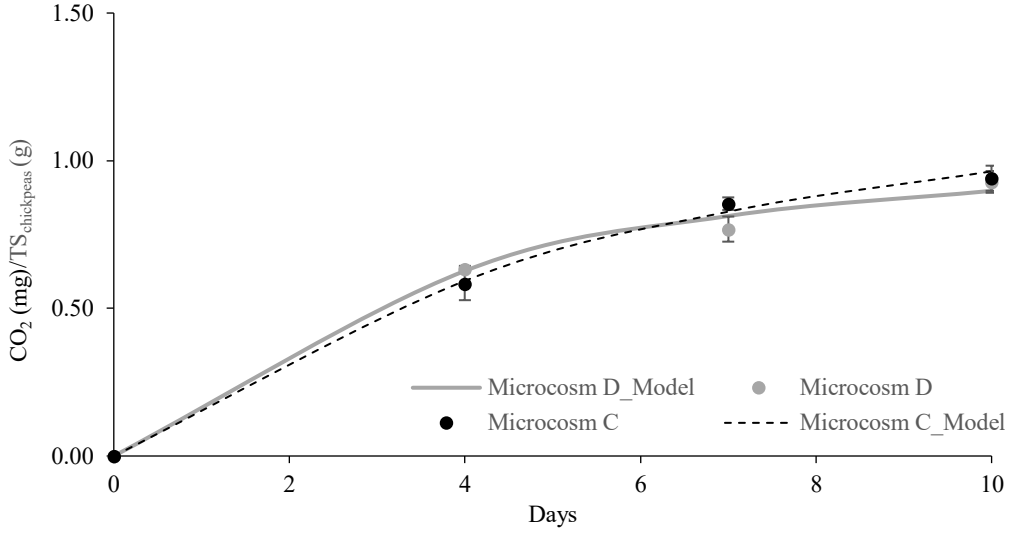

Fig. 48 Curve cumulative $CO_2/TS_{chickpeas}$ prodotte nei sistemi con matrice di ceci libera.

Tabella 8. Parametri della funzione del primo ordine con cui sono stati parametrizzati i valori di respirometria, normalizzati per $T_{Schickpeas}$, dei sistemi con matrice di ceci libera.

	Microcosm_C	**SD**	**Microcosm_D**	**SD**
a	1.15*	0.04	0.97*	0.03
b	0.18*	0.01	0.26*	0.03

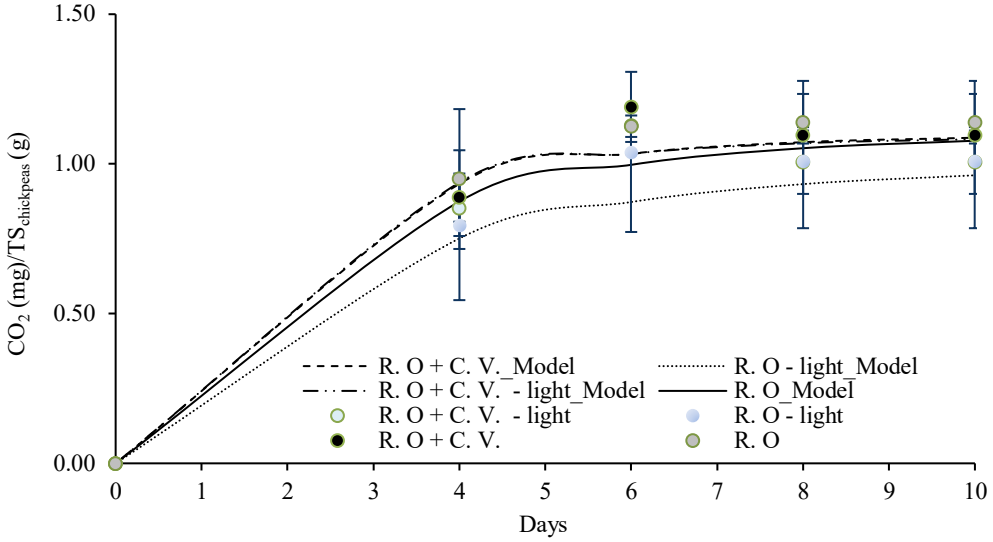

Fig. 47 Curve cumulative $CO_2/TS_{chickpeas}$ prodotte nei sistemi con matrice di ceci intrappolata.

Tabella 7. Parametri della funzione del primo ordine con cui sono stati parametrizzati i valori di respirometria, normalizzati per $T_{Schickpeas}$, dei sistemi con matrice di ceci intrappolata.

	R. o. - Light	**SD**	**R. o. - Dark**	**SD**	**R. o. + C. v. - Light**	**SD**	**R. o. + C. v. - Dark**	**SD**
a	0.99*	0.10	1.10*	0.03	1.09*	0.05	1.10*	0.01
b	0.36*	0.05	0.40*	0.02	0.49*	0.00	0.48*	0.02

Un'idea di prospettiva futura potrebbe essere quella di allestire microcosmi sempre di co-cultura di *R. oligosporus* e microsfere di *C. vulgaris*, in cui migliorare alcuni parametri legati all'intrappolamento della microalga, come il rapporto di volume alginato/microalghe e la concentrazione di ioni Ca^{2+}, o legati alla co-cultura, come il fotoperiodo adottato. Accertare se una condizione ottimale di *C. vulgaris,* intrappolata nell'alginato, consenta una sinergia significativa con *R. oligosporus* sarebbe di grande aiuto per sviluppare applicazioni innovative basate su tale sinergia. Ad esempio, si potrebbe provare a produrre un tempeh innovativo, il cui miglioramento produttivo trova la sua base in questa sinergia e il cui miglioramento nutrizionale trova la sua base nell'adozione di un altro microrganismo importante dal punto di vista nutrizionale, come *C. vulgaris*.

Nello sviluppo e nel mantenimento della crescita fungina di *L. edodes* in piastre di Petri in condizioni sia di buio che di illuminazione (luce rossa e blu, 100 lx), i risultati ottenuti, relativi alla crescita del micelio nel tempo, riportati nei grafici che seguono (Fig. 50 e Fig. 51), portano a concludere che il substrato di cellulosa micro fibrillata (MFC) rappresenta una valida alternativa al classico substrato MEA, sia in termini di crescita e velocità di crescita del micelio nel tempo che di fase di ritardo con cui tale crescita si verifica. Tale aspetto ha ritrovato riscontro anche nel trattamento statistico condotto sui parametri, riportati nelle tabelle 9 e 10, indicanti il raggio medio miceliare massimo ottenibile (r_{MAX}), la velocità massima con cui si raggiunge (V_{MAX}) e il tempo

di ritardo con cui si verifica (λ). La possibilità di utilizzare la MFC risulta particolarmente rilevante soprattutto considerato il basso costo attribuibile a tale substrato.

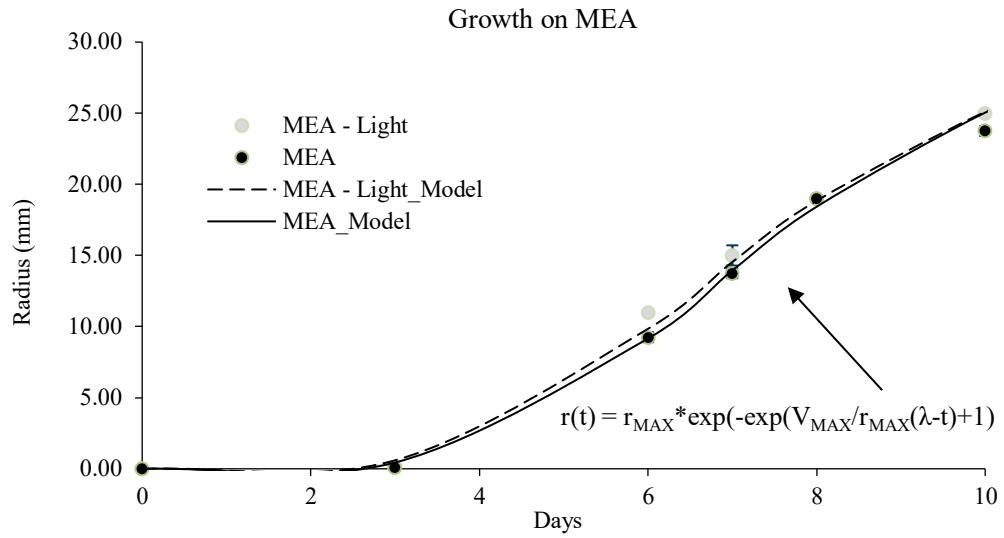

Fig. 50 Curve di estensione del micelio di *Lentinula edodes* su MEA.

Tabella 9. Parametri della funzione di Gompertz utilizzata per parametrizzare il tasso di estensione del micelio su MEA.

	MEA - Dark	**SD**	**MEA - Light**	**SD**
r_{MAX}	31.48*	1.24	31.86*	0.11
V_{MAX}	28.48*	4.17	28.37*	0.40
λ	2.28*	0.18	2.19*	0.02

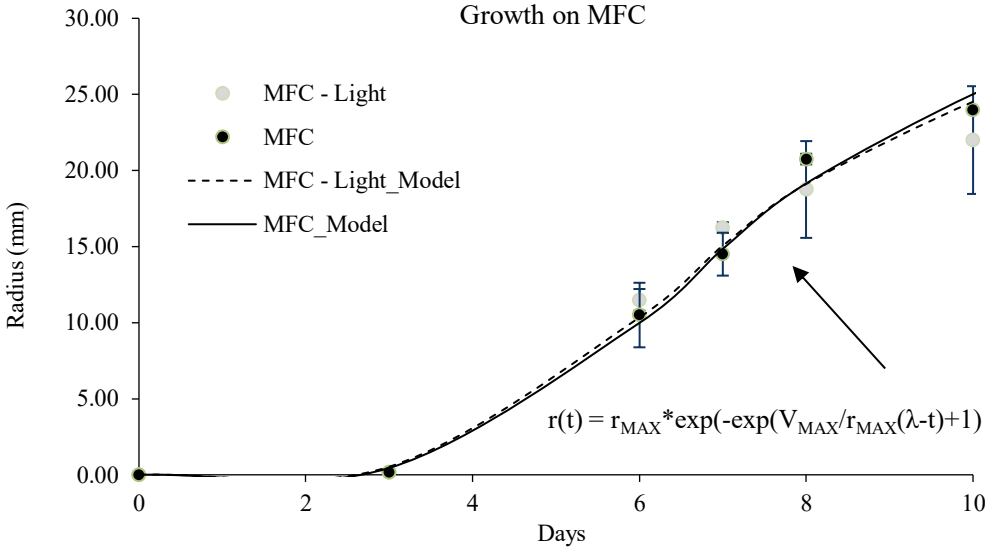

Fig. 51 Curve di estensione del micelio di *Lentinula edodes* su MFC.

Tabella 10. Parametri della funzione di Gompertz utilizzata per parametrizzare il tasso di estensione del micelio su MFC.

	MFC - Dark	SD	MFC - Light	SD
r_{MAX}	30.12*	0.13	29.04*	0.91
V_{MAX}	24.82*	0.80	23.61*	1.16
λ	2.33*	0.04	2.29*	0.04

Ulteriori indagini, riguardanti una fonte di illuminazione alternativa e una diversa intensità di tale illuminazione, potrebbero essere condotte per valutare la possibilità di utilizzare MFC come substrato non tanto alternativo quanto preferibile a MEA in tali condizioni ottimali.

Nelle prove di coltura sommersa in fiasca (slurry al 10% w_{TS}/w), *R. oligosporus* e *L. edodes* tendevano ad acidificare le matrici (da 5,90 ± 0,01 a 3,84 ± 0,25 e da 5,90 ± 0,01 a 3,87 ± 0,22 rispettivamente in presenza di BSG; da 3,72 ± 0,01 a 1,61 ± 0,10 e da 3,72 ± 0,01 a 1,59 ± 0,19 rispettivamente in presenza di AP) e presentavano un'attività proteolitica che, come mostrato nel grafico riportato di seguito (Fig. 59), portava ad ottenere titoli almeno due volte superiori negli omogenati finali rispetto alle matrici di partenza.

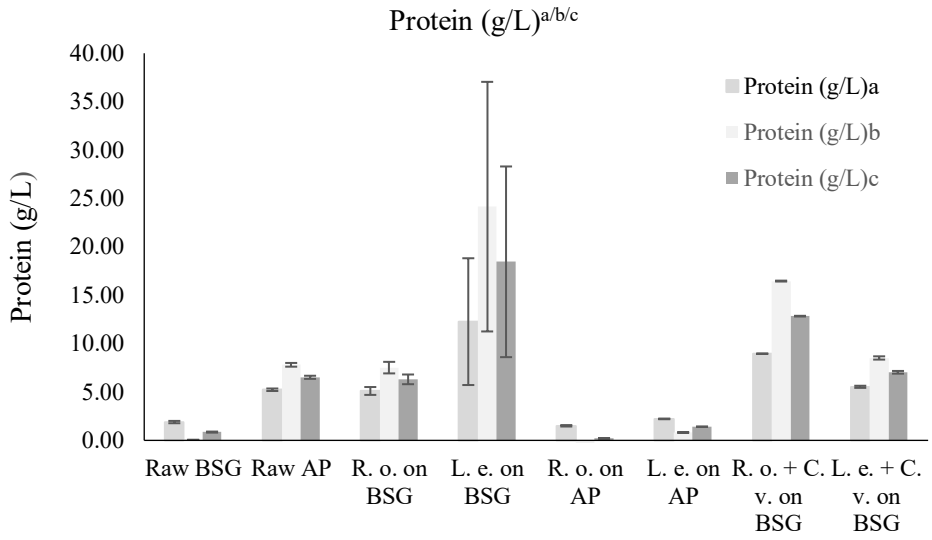

Fig. 59 Confronto della quantità di proteine nelle colture sommerse.

([a] retta di calibrazione della BSA; [b] retta di calibrazione delle proteine del siero di latte (WPI) alla vaniglia; [c] retta di calibrazione delle proteine del siero del latte (WPI) del cioccolato).

Un aspetto su cui porre attenzione, verificatosi indipendentemente dallo standard proteico considerato, è l'ottenimento di valori con una notevole deviazione standard per le colture sommerse di *L. edodes* su BSG. Una possibile giustificazione di questo fenomeno può essere ricercata nella possibile interferenza tra alcuni elementi - saccarosio, lipidi, tamponi fosfatici, monosaccaridi ed esoammine - presenti in questi campioni e la sensibilità del metodo utilizzato per analizzarli.

Per quanto riguarda i test allo stato solido, il pH aumenta nei sistemi (da 2,18 ± 0,08 a

4,84 ± 0,2 per i sistemi posti in condizioni di oscurità, da 2,18 ± 0,08 a 4,94 ± 0,39 per i sistemi posti in condizioni di luce), accompagnato da una diminuzione dell'acidità titolabile (i volumi di NaOH utilizzati per titolare i campioni di tempeh sono inferiori del 60.32% rispetto al volume utilizzato per titolare il campione della matrice di partenza). Inoltre, in tali sistemi, come è possibile evincere dal grafico sottostante (Fig. 65), si è registrata una diminuzione del contenuto proteico (da 100,76 ± 0,05 (g/L) a 21,70 ± 0,18 (g/L) per i sistemi in condizioni di buio, da 100,76 ± 0,05 (g/L) a 21,16 ± 0,15 (g/L) per i sistemi in condizioni di luce), il che potrebbe indicare una maggiore preferenza per la crescita della biomassa in questa configurazione.

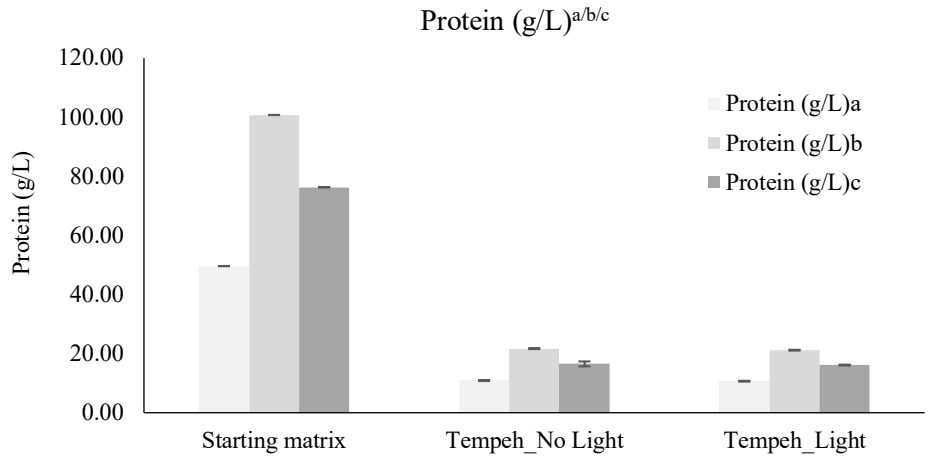

Fig. 65 Confronto della quantità di proteine nelle colture allo stato solido.

([a] retta di calibrazione della BSA; [b] retta di calibrazione delle proteine del siero di latte (WPI) alla vaniglia; [c] retta di calibrazione delle proteine del siero del latte (WPI) del cioccolato).

In generale, i risultati suggeriscono che le matrici BSG e AP sono compatibili per coltivare le specie fungine esaminate (fungendo da terreni complessi). Nelle configurazioni sommerse si osserva una maggiore attività fermentativa (con produzione di acidi organici) con conseguente incremento dell'attività proteolitica, che genera una maggiore disponibilità di proteine idrolizzate in fase liquida. Per le colture allo stato solido, si registra una maggiore crescita della biomassa, con consumo di acidi organici e utilizzo delle proteine di partenza misurate delle matrici.

In conclusione, è possibile affermare che risultano necessari ulteriori test per valutare la sicurezza dei prodotti ottenuti durante le prove esplorative, per analizzarne più dettagliatamente le proprietà, al fine di comprenderne e valutare la reale applicabilità di questi prodotti come cibo/integratore dell'alimentazione umana e animale. Il tutto tenendo presente non solo il valore nutrizionale aggiunto, ma anche l'impronta ambientale e i costi associati a questi tipi di processi produttivi nelle successive fasi di scale - up della strategia di valorizzazione.

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1. Introduction

1.1. General Overview

Over the years, the world population has significantly grown, it will continue to grow and it is estimated to exceed 9 billion by 2050 (FAO 2009; (Alexandratos, 2009)). This will be linked to a significant increase in the demand for food. Meeting this global food demand will in turn entail the intensification of all those processes, and higher consumption of required feedstock to transform raw materials into finished products at the consumer level. Further exacerbating this scenario are the drastic effects of climate changes and declining agricultural productivity due to increasingly arid soils and poor fertiliser availability (Fig.1) (Roth, M. et al., 2019).

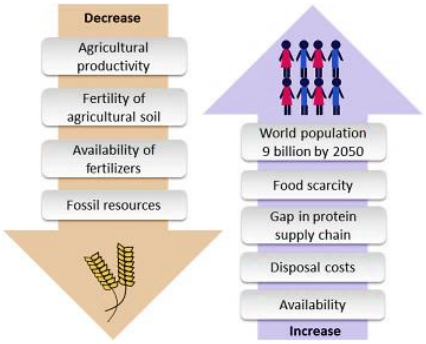

Fig. 1 The reasons for the inevitability of worsening the already drastic scenario in the grain sector (adapted from (Roth, Jekle and Becker, 2019)).

Today, food or food-related waste originally intended for human consumption (FLW) is estimated at 1.3 billion tons per year globally (FAO, 2011; (Blakeney, 2019)) and constitutes 25-70% of municipal solid waste (MSW), estimated in 2011 at about 2 billion tons per year worldwide (Fig. 2) (Pham *et al.*, 2015).

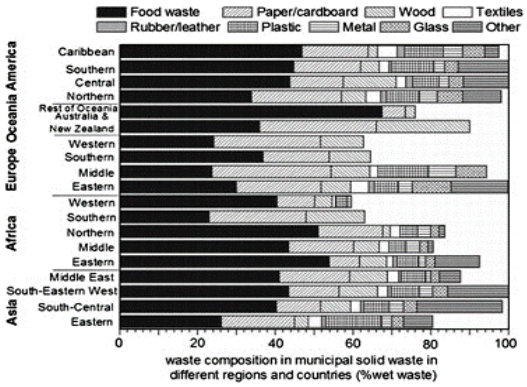

Fig. 2 The percentage of different waste types in municipal solid waste in different regions and countries (adapted from (Pham *et al.*, 2015)).

FLWs are generated throughout the production chain, starting with the production of raw materials and ending with consumption by producers (Fig. 3) (European Commission, 2019).

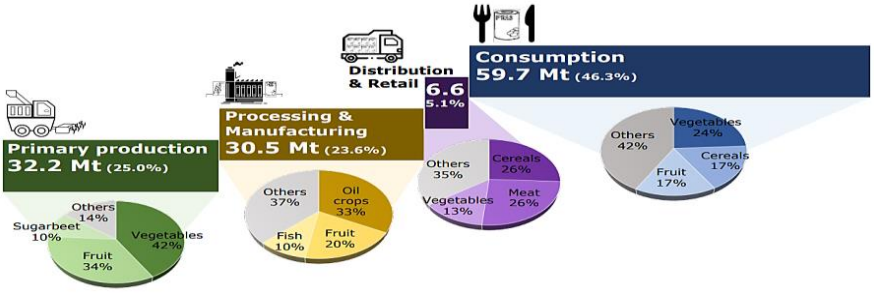

Fig. 3 Amount of food waste (in fresh weight) generated in Europe in 2019 during the different stages of the food supply chain (bars) and breakdown by main food groups (pie charts) (adapted from (European Commission, 2019)).

The continued growth of FLW not only represents a major source of pollution with significant environmental impact but also constitutes a very attractive source of widely available, low-cost, high-value-added products for various sectors. To date, processes and technologies used for the valorization of this biomass utilize and combine several techniques. These include biological ones (such as anaerobic digestion and fermentation); biochemical ones (such as enzymatic hydrolysis); thermochemical ones (such as incineration, pyrolysis, and gasification); and thermophysical ones (such as ultrafiltration and ultrasonic extraction) ((Pham *et al.*, 2015); (European Commission, 2019)). Although several processes and technologies are already in use, many of them are still on a laboratory scale. The research being conducted in this regard is therefore growing over time, to achieve in the future not only a significant enhancement of valorisation, applicable mainly on an industrial scale but also a greater framing in a sustainability framework (Nzihou, 2010).

The technological and economic potential of FLWs has led to a revision of the municipal solid waste hierarchy. Conceived in the 1970s, the municipal solid waste hierarchy was born and developed to prioritize the strategies to be adopted in waste management. The revision adopted consists of its adaptation to food waste (Fig. 4); as one moves down the pyramid, one moves from more preferable to more avoidable management than the previous ones (European Commission, 2019).

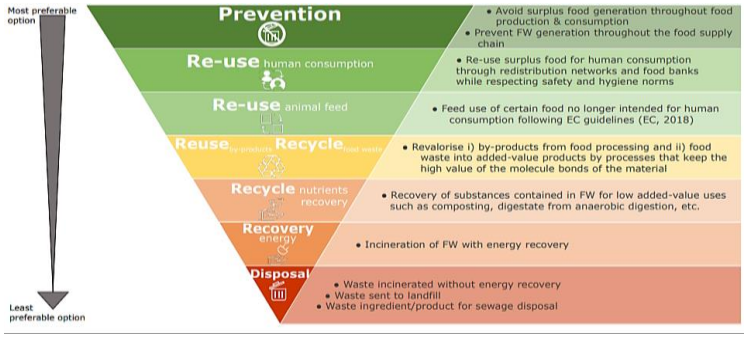

Fig. 4 Hierarchy for prioritisation of food surplus, by-products and food waste (FW) prevention strategies (adapted from (European Commission, 2019)).

It is clear, then, that there is an increasing emphasis on the need to meet the world's food needs by moving to re-evaluate food waste, with a focus on reducing additional costs and losses to businesses, as well as the environmental impact of various production processes. There is thus a need, which is emerging with increasing force, to find alternative uses and thus a way to valorize all by-products of the food industry to obtain new food for human consumption and not just feed or sources for energy production.

1.2. Motivation

Grains, cultivated since prehistoric times, are the most nutritious source of food among all edible plants. The main cereals are wheat, rice, maize, barley, millet and oats. Together with maize and rice, wheat constitutes about 90% in weight of the world's cereal production (Blakeney, 2019).

The key feedstock for the production chains of various industries, cereals undergo different treatments depending on the product of interest. Processing the raw cereals feedstock results in different by-product, which depend on the type of production and they vary on the composition and specific properties. Cereal by-products (CBP) are increasingly available and, in addition to representing an oversupply in the market, are an underutilized source of nutrient fractions. One of the typical end-of-life scenario for CBPs mainly is as animal feed since CBPs have high amounts of dietary fibre, protein and phenolic compounds. These properties render CBPs as suitable candidate to be valorised and reused within the food production cycle for food applications (that could eventually result in new value-added products). The most important cereal by-products from a quantitative point of view are BSG and distillation grains (DG) (Roth, Jekle and Becker, 2019).

BSG constitutes about 85% (*w/w*) of the total by-products generated by beer production. In the European Union (EU) alone, about 87 million tonnes are produced annually (Fig. 5) ((Sganzerla *et al.*, 2021); (Mussatto, Dragone and Roberto, 2006)). What makes BSG a functional food ingredient is its high nutritional value - BSG is rich in cellulose and non-cellulosic polysaccharides, lignin and protein - as well as its low cost and high availability.

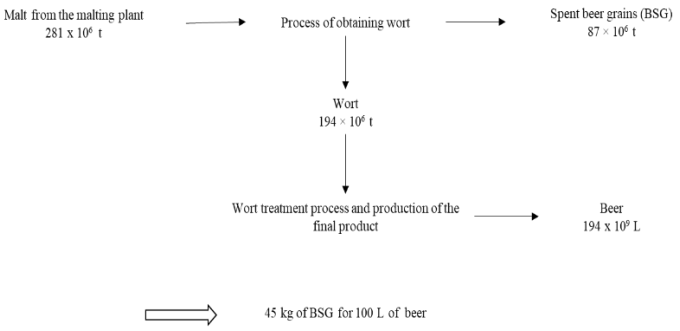

Fig. 5 Mass balance of BSG produced in one year in EU.

Fruit, an essential component of healthy and balanced nutrition, represents a food whose demand is expected to grow over time, not only because of increased consumer awareness but also because of new lifestyles and higher incomes. The supply of fruit on the market is not proportional to actual demand due to low consumption. This implies a progressive and timely adaptation of the fruit supply chains to the needs of the end consumer, going to research and implement supply chain changes, which see fruit as the protagonist as a fresh or processed product (Verdouw *et al.*, 2010).

Apples are one of the main fruits consumed worldwide and their production has increased significantly over the years. According to the Food and Agriculture Organisation of the United Nations (FAO), world apple production in 2018 was 86.14 million tonnes (fig.) ((Duan *et al.*, 2021); (Dhillon, Kaur and Brar, 2013)). The 25 – 30% in weight of the cultivated apples, not suitable for fresh consumption, is industrially processed to produce juices, flavourings and concentrates. Apple processing industries generate millions of tonnes of solid (25-30%) and liquid (5-10%) waste. Given the high biodegradability of this waste, due to the high values of moinsture content, biological oxidation demand (BOD) and chemical oxidation demand (COD) (for example AP has a moisture content about 75% w.t. and a COD of 250 – 300 g/kg), its disposal is not only a serious problem but also a real challenge. Often only 20% is recovered as animal feed, while the remaining 80% ends up in landfills, is incinerated or sent to composting sites (Dhillon, Kaur and Brar, 2013).

Apple pomace (AP) is a by-product of the fruit juice industry and accounts for about 25% of the fruit, while about 75% of the fruit weight is extracted as juice. Every year, several million tones are produced worldwide. In India alone, which is the world's ninth largest producer of apples, 1 million tonnes of AP are produced annually, of which only about 10,000 tonnes are reused (Fig. 6) (Shalini and Gupta, 2010).

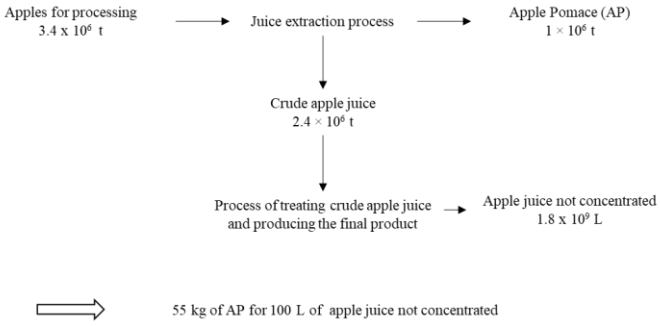

Fig. 6 Mass balance of AP produced in one year in India.

Used mainly as cattle feed, apple pomace is a cheap and readily available source of high nutritional value - AP is rich in dietary fibre, polyphenols and bioactive compounds and low in fat and protein - and is, therefore, a valuable by-product to be reused in a new innovative production lines.

1.3. Objective

This thesis aims to carry out experimental tests for the recovery of two key by-products in food supply chains. These matrices are Brewer's used grains (BSG) and apple pomace (AP). The chosen approach is biotechnological, to preliminarily evaluate the compatibility between these matrices and certain microorganisms (biosafety level 1 - BSL1- such as *Rhizopous oligosporus*, *Chlorella vulgaris* and *Lentinula edodes*) widely used in the food industry on similarly-composed matrices. The objectives of the thesis concern the development and maintenance of each (micro)organism (according to the specific needs of each one), exploratory fermentation tests (in submerged and in solid state conditions) and to gain insights on possible synergistic effects resulting from the co-cultivation of these microorganisms for similar applications.

Specific objectives of the present thesis work are:

- To perform respirometry tests on microcosm configuration using chickpeas as model matrix and inoculated with *Rhizopus oligosporus* and *Chlorella vulgaris*.
- To propagate mycelium culture of *Lentilula edodes* on malt agar extract (MEA) and micro fibrillated cellulose (MFC) petri dishes and compare their performance.
- To perform a submerged culture on a complex matrix (BSG/AP) of *Rhizopus oligosporus* and *Lentinula edodes* in single or co-culture with *Chlorella vulgaris*.
- The fermentation as tempeh of *Rhizopus oligosporus* on a complex matrix (BSG and AP).
- The cultivation of *Lentinula edodes* on BSG in race – tubes.

Through the adoption of microcosms and the respirometry test, a possible synergy between *Rhizopus oligosoprus* and *Chlorella vulgaris* under dark or illuminated conditions was investigated, which can be exploited in possible future applications.

The growth of *Lentinula edodes* in Petri dishes was used to evaluate the possibility of using MFC, a novel and inexpensive substrate, as an alternative to common MEA in the development and maintenance of this fungal growth line.

The submerged configuration was employed to understand particular difficulties in growing *Rhizopus oligosporus* and *Lentinula edodes*, on complex matrices such as BSG and AP. Both, single cultures and co-cultures with *Chlorella vulgaris* were tested. In addition, special attention was paid to the protein content obtainable in these culture types, again with a view to understanding the possibility of exploiting these cultures in

future applications focused on obtaining a novel protein source.

Tempeh, commonly obtained through the fermentation of *Rhizopus oligosporus* on a legume matrix, has already been experimentally obtained in the past on a waste matrix such as BSG (see (Gómez-Camacho *et al.*, 2022)). In this study, we will modify the culturing matrix by adding AP, with the idea that the peculiarities of these two matrices can improve the nutritional and especially protein content of the entire product.

Commonly known as shiitake, *Lentinula edodes* is already the protagonist of several experimental ideas aimed at expanding the cultivation on rural and agro-industrial residues (see (Philippoussis, Diamantopoulou and Zervakis, 2002)). In the present case study, the aim is to obtain a mushroom that is highly rich in vitamin D_2 and a culture medium consisting of BSG that constitutes a source of nutrients even at the end of the fermentation period and, as such, can be reused. So as to investigate the possibility of new and innovative production of this improved mushroom.

2. Theoretical Background

2.1. Brewers' spent grain (BSG)

2.1.1. Description

The BSG represents the first solid waste of the brewing line, it is obtained after the mashing phase and is recovered after the first filtration process. After the mashing phase inside the tank, there is a residual solid fraction and a liquid fraction. The solid fraction, which plays the role of a filter cake, constitutes what is called BSG once it is recovered. The liquid fraction, called sweet wort, once recovered, is fermented to produce beer (Fig. 7) (Mussatto, Dragone and Roberto, 2006).

Fig. 7 Scheme of the brewing process and consecutive production of BSG (adapted from (Bianco *et al.*, 2020)).

The structure and chemical composition of BSG vary depending on the type of beer to be produced and the type of grains. The factors involved are the types of crops used as raw materials, the possible addition of cereals (unmalted sources of fermentable sugars such as wheat, rice or maize) and the type of malting process conducted (Mussatto,

Dragone and Roberto, 2006).

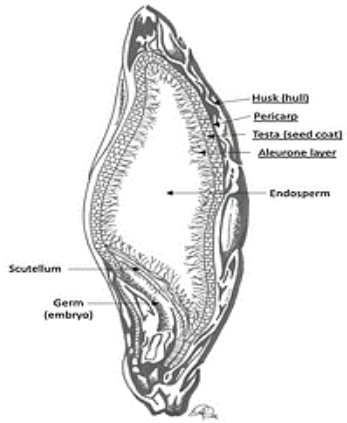

Fig. 8 Cross section of a barley grain
(adapted from (Lynch, Steffen and Arendt, 2016)).

However, in terms of BSG structure, there is the husk, pericarp and seed tegument of the grain, with residual amounts of endosperm and aleurone layer (Fig. 8) ((Lynch, Steffen and Arendt, 2016); (Mussatto, Dragone and Roberto, 2006)). Also in terms of composition, although with different percentages, it is common to find varied elements between the different types of BSG. It is a lignocellulosic material with a high fibre content - cellulose, hemicellulose and lignin - of up to 70% on a dry basis and a protein content of 20-30% on a dry basis (Fig. 9) ((Steiner, Procopio and Becker, 2015); (Lynch, Steffen and Arendt, 2016)).

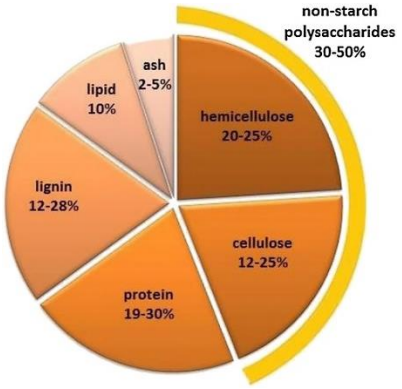

Fig. 9 Composition of BSG **(**adapted from (Lynch, Steffen and Arendt, 2016)**).**

The nutritional value of BSG comprehends an important fraction of minerals - including calcium, iron, magnesium, manganese, phosphorus and potassium - vitamins - such as choline, folic acid, pantothenic acid, riboflavin, thiamin and pyridoxine - free essential amino acids - leucine, valine, threonine and lysine - and non-essential amino acids - including serine, glycine, glutamic acid, aspartic acid and tyrosine - (Bonifácio-Lopes, Teixeira and Pintado, 2020).

Due to its high moisture content (70-80%) and the presence of polysaccharides and proteins, BSG constitutes a breeding ground for microbial populations and is easily perishable, the reference period lies between two to seven days ((Lynch, Steffen and Arendt, 2016); (Bianco *et al.*, 2020)).

2.1.2. Applications

Over the years, several methods have been developed and analysed to address or slow down the deterioration of BSG, thus favouring longer storage times, all while trying to preserve its organoleptic and nutritional characteristics. In the past, the method of choice was direct drum drying or condensed drying in two stages (the first method reduced the moisture content by 60% through pressing, and the second drying approach can lead to the remaining moisture content of less than 10%). Both involved a reduction in volume and a consequent reduction in storage and transport costs. However, these drying options tend to be were energy-intensive (Salihu Aliyu and Muntari Bala, 2010). Alternative methods have therefore been evaluated over the years. Such as oven-drying, freeze-drying or freezing (Bartolomeé *et al.*, 2002). The latter options also present problems, e.g. the costs of freeze-drying were decidedly unsustainable for the company while freezing involved the need to store huge volumes, with a consequent increase in storage costs, and the possibility of product spoilage (Mussatto, Dragone and Roberto, 2006). Considering that the search for alternative methods to stabilise and prolong the shelf life of BSG, which entail the least economic expenditure for companies, is still ongoing, it is not difficult to understand how this product is still widely promptly supplied to local farmers where it is used for animal feed, particularly cattle.

The search for new preservation methods is accompanied by the search for new uses and revaluation technologies. In a wide variety of sectors, there are numerous patents in which this product is biotransformed with the help of microorganisms. These range from human and animal nutrition (highly nutritious flours and baked goods), to the energy sector, where it represents a source of energy and heat production. Different approaches have tried valorise BSG for cosmetics and pharmaceuticals, where it is used to produce enzymes or as a matrix for the immobilisation of cells. (Fig 10). ((Bianco *et al.*, 2020); (Bonifácio-Lopes, Teixeira and Pintado, 2020); (Mussatto, Dragone and Roberto, 2006)).

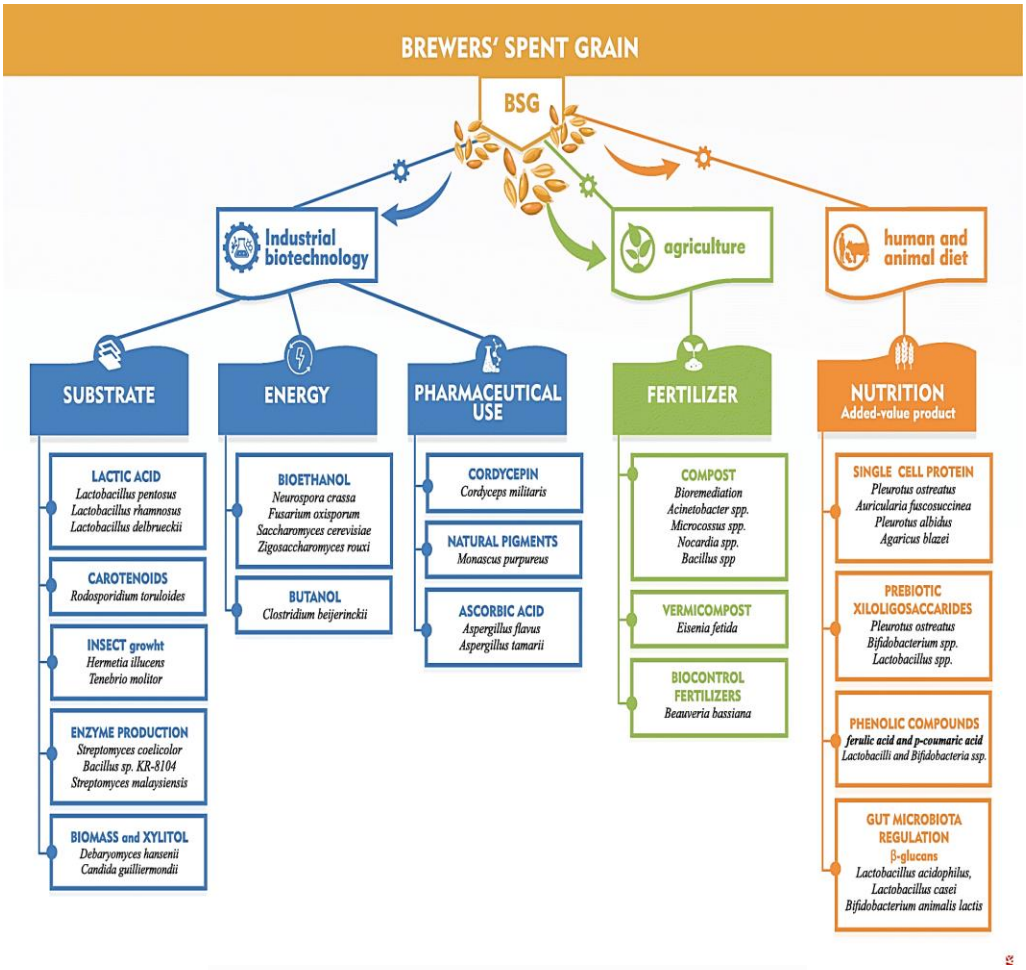

Fig. 10 The role of microorganisms on biotransformation of BSG (adapted from (Bianco *et al.*, 2020)).

2.2. Apple pomace (AP)

2.2.1. Description

The AP is the first solid residue obtained after the pressing of apple pulp during juice and cider production (Fig. 11). Depending on the type of production line, pectolytic enzymes are often added to the pulp during the pressing process to further break down the cell structure and increase the yield of the process. At this stage, a solid residue is obtained, which constitutes the apple pomace, and a liquid residue, which is further processed to obtain the finished juice for the trade ((Álvarez *et al.*, 2000); (Downing, 1989)).

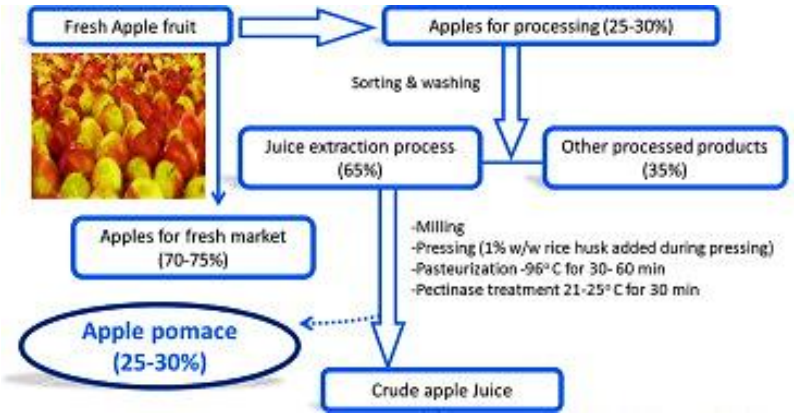

Fig. 11 Flowchart showing the production of AP during processing of apples in the juice industry (adapted from (Dhillon, Kaur and Brar, 2013)).

AP is a heterogeneous mixture in which we find fragmented skin and pulp (95%), seeds (2-4%) and stems (1%) (Fig. 12) (Sobczak *et al.*, 2022). Both, the percentage of the individual fractions and their nutritive value depend on the natural variability in the apples themselves, agricultural practices, the degree of ripeness of the fruit and the extraction process used to produce the juice (Pirmohammadi *et al.*, 2006).

Fig. 12 Apple Pomace (AP) [https://waste-management-world.com/artikel/scientists-in-new-zealand-find-more-sustainable-way-to-treat-apple-waste/]

However, in terms of composition, it has been reported that AP contains valuable nutrients, such as saccharides, proteins, minerals, pectin, dietary fibre, polyphenols, flavonoids, carotenes, organic acids and vitamins (including vitamin C) ((Sobczak *et al.*, 2022); (Sudha, 2011)). Literature references on the composition of the AP can be found in Tables 1 and 2 (Antonic *et al.*, 2020).

Table 1. Nutrient composition of dried AP (Antonic *et al.*, 2020)

Compounds	Quantity (g/100 g)	Compounds	Quantity (g/100 g)
Moisture	3.97 to 9.75	Sodium	2 to 200
Fat	0.26 to 8.49	Potassium	449
Protein	1.2 to 6.91	Calcium	50 to 150
Fructose	11.5 to 49.8	Phosphorus	50 to 950
Glucose	2.5 to 22.7	Magnesium	20 to 45
TDF (Total Dietary Fibres)	26.8 to 82.0	Iron	2.4 to 23
Pectin	3.5 to 14.32	Zinc	0.22 to 1.5
Total polyphenolics	0.17 to 0.99	Copper	0.11 to 0.22
Malic acid	0.05 to 3.28	Manganese	0.61 to 0.9
Ash	0.5 to 4.29		

Table 2. The main polyphenol compounds found in AP (Antonic *et al.*, 2020)

Main polyphenols detected in apple pomace	Content in mg/kg of dry weight of apple pomace
Phlorizin	8 – 1435.4
Catechin	1 – 127
Epicatechin	4.2 – 640
Chlorogenic acid	26 – 2298
Caffeic acid	3 – 280
Procyanidin B2	48.8 – 590.2
Hyperin	65.5 – 564.2
Quercitrin	69 – 373.8

Due to its high water content (>70%), apple pomace is very prone to microbial spoilage. In addition, this product quickly undergoes oxidation of polyphenols and rapid depolymerisation and de-esterification of pectins ((Sudha, 2011); (Antonic *et al.*, 2020)).

2.2.2. Applications

Due to the rapid deterioration, further operations are needed to stabilise apple pomace, facilitating its storage and transport. The operation commonly performed is drying, less frequently freezing is used (Sobczak *et al.*, 2022). Lately, the possibility of ensiling the AP is being considered, due to the low pH of this matrix (between 3.2 and 4.1) (Pirmohammadi *et al.*, 2006). The obtained product, particularly dried or frozen, becomes raw material for other production lines, often in different plants. It should be noted that the additional required operations entail an energy and economic outlay which, added to that resulting from the production lines, make these methods uneconomic and apple pomace a product still mainly intended for composting or animal feed (Sobczak *et al.*, 2022).

The research conducted over the years, which is constantly evolving, does not only concern stabilisation and preservation methods for this product. AP is increasingly the focus of innovative research projects in various fields (Fig. 13 and Fig. 14). Commonly used as enriched animal feed (Dhillon, Kaur and Brar, 2013), apple pomace is finding wide use in the food industry (Antonic *et al.*, 2020). It could potentially become a source of fibre and sugars, polyphenols and pectin (Kammerer *et al.*, 2014). In this regard, it is used for the production of a variety of products, such as jams and marmalades, alcoholic beverages and vinegar, colourings and fruit infusions (Sobczak *et al.*, 2022), as well as baked goods (Sudha, 2011). More innovative ideas see it as a potential substrate for the production of edible mushrooms with high protein and vitamin content (Dhillon, Kaur and Brar, 2013). Apple pomace is also enjoying great success in the bioenergy sector, where over the years the possibility of transforming

this product, through anaerobic digestion, into biogas has been explored, successfully replacing natural gas ((Sudha, 2011); (Sobczak *et al.*, 2022)). For bio-based materials production, and due to its high sugar content, it can be moulded and reformed at high temperatures into plastics, biofilms and 3D shapes (Gustafsson and Landberg, 2018). Furthermore, due to its composition, AP possesses essential chemical elements for the growth of microorganisms, making it an ideal substrate that could help limit the cost of macro and micronutrient supplementation for fermentation media (Dhillon, Kaur and Brar, 2013).

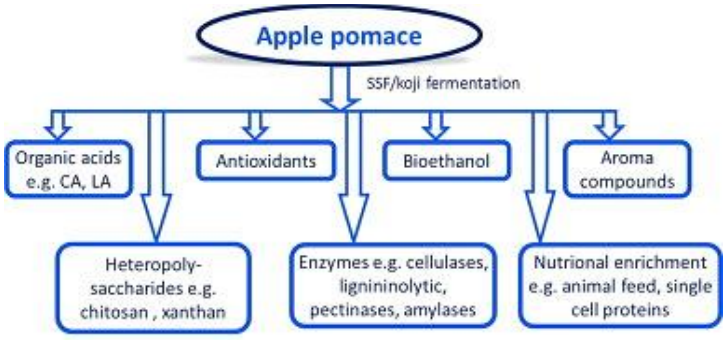

Fig. 13 Flowchart showing bioprocessing of AP for various value added products (adapted from (Dhillon, Kaur and Brar, 2013)).

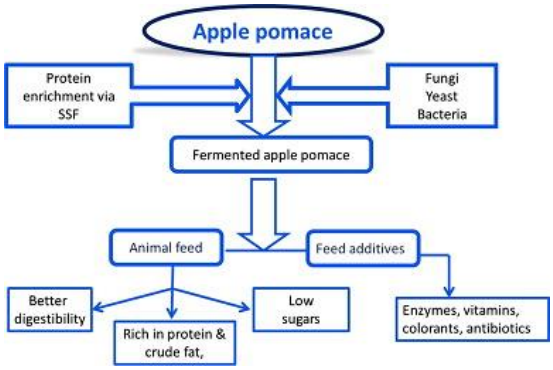

Fig. 14 Diagrammatic representation of biological protein enrichment of apple pomace via SSF with microorganisms (adapted from (Dhillon, Kaur and Brar, 2013)).

2.3. *Rhizopus oligosporus*

2.3.1. Description

The ubiquitous *Rhizopus* genus comprises ten species and each of these has several varieties. Among these species is the *microsporus*, which has *oligosporus* among its varieties (Dolatabadi *et al.*, 2014). *Rhizopus oligosporus*, like all other fungi belonging to the genus *Rhizopus*, belongs to the class of zygomycetes. Aerobic and saprotrophic, it reproduces asexually via sporangiospores. Furthermore, belonging to the Mucoraceae family, it is characterised by a plurinucleate, non-segmented thallus, which enables it to anchor itself to the substrate, on which it grows via rhizoids (Fig.

15) (Jennessen *et al.*, 2008).

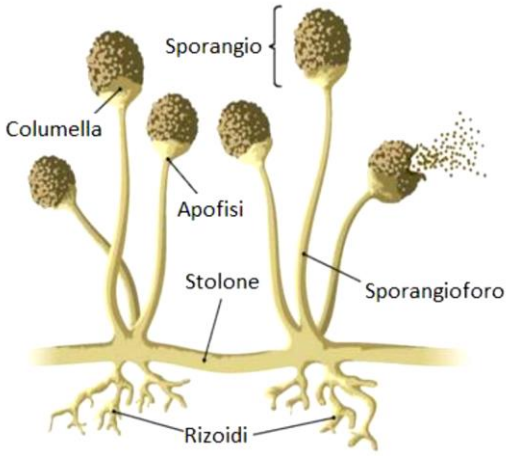

Fig. 15 Structure diagram of *Rhizopus oligosporus* (adapted from (Manzini, 2021)**).**

Like all saprotrophic fungi, it degrades many types of organic substances, actively participating in biodegradation (Oliveira *et al.*, 2013). This variety of *Rhizopus* does not appear to be pathogenic to humans (Ribes, Vanover-Sams and Baker, 2000)however, special care must be taken during its use, as it releases sporangiospores (spores) into the air during growth, which could easily contaminate the work area or adjacent speces (Jennessen *et al.*, 2008).

2.3.2. Applications

R. oligosporus is a fungus that is widely consumed worldwide and it is used as model microorganisms for different innovative studies. Used for the production of industrial enzymes and the treatment of waste and wastewater (Jennessen *et al.*, 2008), due to its ability to produce enzymes capable of hydrolysing proteins, lipids and starch (Handoyo and Morita, 2006) and the absence of harmful secondary metabolites (Jennessen *et al.*, 2008), this fungus is widely used in the production of a wide variety of high-value food/integrator products.

Fig. 16 Soy-based tempeh (adapted from (Manzini, 2021)).

In Indonesia, *R. oligosporus* has been used for the production of soy tempe (tempe

kedele) since ancient times. Fresh tempe or tempeh is a compact, sliceable cake in which the white mycelium of the fungus, covering and penetrating the cooked soybean matrix, keeps it bound, giving it its classic shape (Fig. 16) ((Dolatabadi *et al.*, 2014); (Dinesh Babu, Bhakyaraj and Vidhyalakshmi, 2009)). Soy is not the only type of legume that can be used, a viable alternative is chickpeas (Erkan *et al.*, 2019), and cereals and food by-products (Jennessen *et al.*, 2008) can also be used. During this type of solid-state fermentation, proteolytic enzymes are produced that hydrolyse high molecular weight proteins, including albumin and globulin, into amino acids and peptides, thus increasing the easily digestible protein content available in the food. In addition, there is an increase in free fatty acids (Handoyo and Morita, 2006) and in the composition of essential, semi-essential and non-essential (Wong *et al.*, 2021) amino acids. Taking all these aspects into account, it is not difficult to see how tempeh can be a viable vegan protein source and how research on it has increased considerably over time. In recent years, many studies have focused not only on improving its nutritional and organoleptic properties but also on better understanding its rheological and structural properties to improve its production process (Handoyo and Morita, 2006).

2.4. *Lentinula edodes*

2.4.1. Description

Lentinula edodes is a Basidomycota fungus, belonging to the genus *Lentinula*. Like all Basidiomycota fungi, it has dikaryotic hyphal structures, which are the dominant form throughout its life cycle. Basidiomycota cells appear as dikaryon, whose two nuclei exist separately and divide in a coordinated manner. Knowledge of this characteristic is crucial, since it has implications for fermentation times (for example in submerged cultivations), which are longer than in other fungi. The main features of the *Lentinula* life cycle are shown in figure 17, note that from a structural point of view, the fruiting body, which is widely-marketed and consumed, is the reproductive organ in the life cycle for the release of the spores, which are protected by the cap (Fazenda *et al.*, 2008).

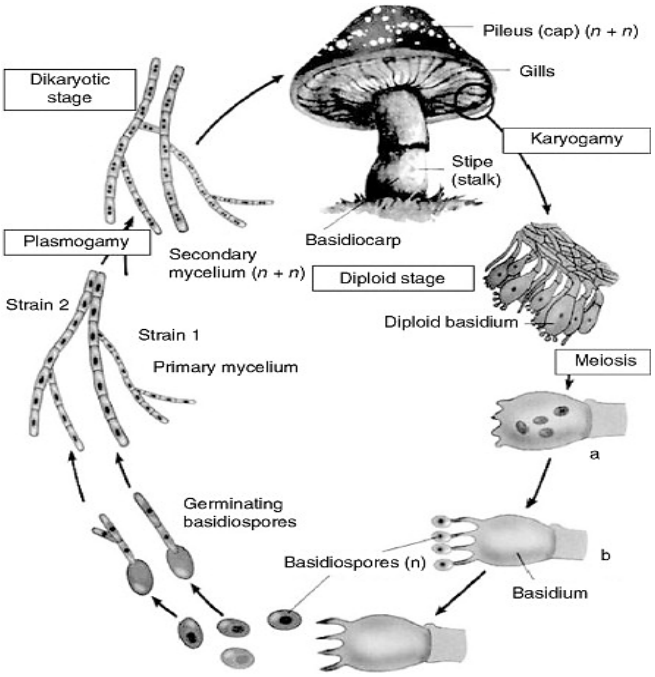

Fig. 17 Life cycle of typical basidiomycete (adapted from (Fazenda *et al.*, 2008)).

Lentinuna edodes is the most widely used species for biotech crops, the most studied and the best-selling of its genus, and, like all other fungi of this genus, grows gregarious on the fallen trunks of various types of deciduous trees. Native to South-East Asia, it is widely cultivated throughout the world (adapted from (Finimundy *et al.*, 2014)).

2.4.2. Applications

Lentinula edodes, rich in vitamins, proteins, mineral, fiber, polysaccharides, terpenoids, sterols and lipids, is one of many medicinal mushrooms that have been used for medicinal purposes since ancient times and on which extensive research has been conducted over the years to obtain new natural medicines. In the past, medicinal mushrooms were used as decoctions and essences in many countries, such as Korea, China, Japan and eastern Russia. The validity of these mushrooms as true means of healing lies particularly in the polysaccharides that characterise their entire composition. Polysaccharides, which are still widely studied today, have anti-cancer effects and promote an immune response. Those isolated from basidiomycetes, including *Lentinula edodes*, occur as homo- and heteropolymers, particularly as β-configuration glucans (Finimundy *et al.*, 2014).

Among the nutrients in the composition of *Lentinula edodes*, and particularly among vitamins, mushrooms are an important source of vitamin D_2. The precursor of this vitamin is ergosterol, which is widely found within fungal cell membranes. Ergosterol can be converted to vitamin D_2 through a series of photochemical/thermal reactions, which in nature occur through exposure to sunlight and, during cultivation, through

exposure to UV light (if used). Depending on the type of UV light they are exposed to, the surface area exposed and the duration of exposure, a certain amount of vitamin can be matured from the ergosterol present in the treated mushroom. Among commercialised mushrooms, shiitake is characterised by a high ergosterol content from which a high amount of vitamin D_2 can also be obtained. Table 3 gives an overview of the vitamin D content in UV-treated commercial mushrooms and other natural food sources of this vitamin (Simon *et al.*, 2013).

Table 3. Vitamin D: comparison to wild mushrooms and other natural food sources of vitamin (Simon *et al.*, 2013).

Food	Vitamin D content µg/100 g serving[a]	Vitamin D content IU/100 g serving[a]	References
Commercial UV processed mushroom			
Portobello (*Agaricus bisporus*)	2.36–20.9	134–836	Phillips et al. (2011)
Wild mushrooms			
Maitake	0.08–63.2	3.2–2528	Phillips et al. (2011)
Wild Chanterelle (*Cantharellus tubaeformis*)	29.8	1192	Mattila et al. (1994)
Wild Chanterelle (*Cantharellus tubaeformis*)	23.4	936	Outila et al. (1999)
Wild Chanterelle (*Cantharellus cibarius*)	10.7–21	428–840	Teichmann et al. (2007)
Chanterelle (*Cantharellus californicus and Cantharellus cibarius*)	2.18–8.41	112–336	Phillips et al. (2011)
Morel (*Morchella esculenta*)	4.39–6.26	175–250	Phillips et al. (2011)
Wild Porcini (*Boletus edulis*)	58.7	2348	Teichmann et al. (2007)
Sun-dried Shitake (*Lentinula edodes*)	40	1600	Holick (2007)
Sun-dried Shitake (*Lentinula edodes*)	42	1666	Health Canada (2012) (CNF)
Oyster (*Pleurotus ostreatus*)	0.07–2.59	2.8–104	Phillips et al. (2011)
Wild button (*Agaricus bisporus*)	0.7–2.3	28–92	Kristensen et al. (2012)
Sea food			
Filefish	69	2760	JFCD (2004)
Cod Liver Oil	55.4	2217	USDA SR21
Wild Atlantic Salmon	51.5	2061	USDA SR21
Canned Brisling	25–75	1000–3000	Lunde et al. (1937)
Mackerel	1–50	40–2000	Ostermeyer and Schmidt (2006)
Sardine	1.2–135	48–5400	Ostermeyer and Schmidt (2006)
Other			
Tofu	14.5	581	USDA SR21
Egg yolk	0.5	20	Holick (2007)
Sunlight exposure (5–10 min)[b]	75	3000	Holick (2007)
Rhizopus Oligosporus	Ergosterol content µg/100 g biomass dry: 20-240		Nout *et al*. (1987) https://doi.org/10.1007/BF00253532

[a]: USDA SR21 (2008) estimates are expressed as quantity of vitamin D per 200 cal serving;
[b]: Erythemal dose of ultraviolet B radiation would be absorbed after an average of 5–10 min of exposure (depending on season, weather, skin type and geographical).

2.5. *Chlorella vulgaris*

2.5.1. Description

Chlorella vulgaris, discovered in 1890 and defined as the first microalgae with a defined nucleus by Dutch researcher Willem Beijierinck; it is a Cholorellaceae, belonging to the genus *Chlorella*. Present on Earth for 2.5 billion years, it is a spherical unicellular microalga (2-10 µm in diameter) with plant-like structural elements (Fig. 18). It has a myxotrophic (heterotrophic/autotrophic) metabolism and it reproduces rapidly and asexually. Under favourable conditions, within 24 hours, four daughter cells are produced for each mother cell by autosporulation.

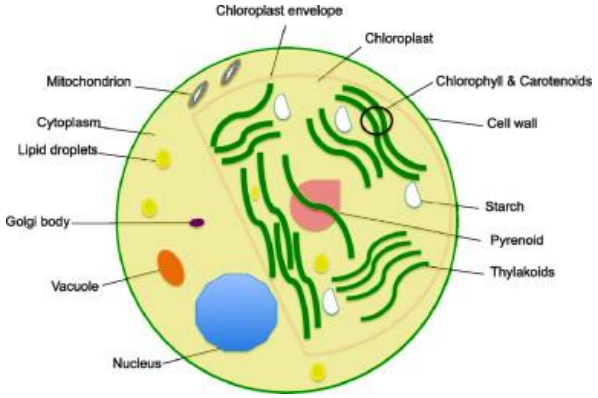

Fig. 18 Schematic ultrastructure of *C. vulgaris* (adapted from (Safi *et al.*, 2014)).

Due to its cell wall reaching 17-21 nm in mature conditions, depending on environmental and growth conditions, *Chlorella vulgaris* shows remarkable resistance to harsh conditions and invaders, making it an excellent microalga for cultivation. (Safi *et al.*, 2014).

2.5.2. Applications

A source of minerals, lipids, fatty acids, proteins, carbohydrates and pigments, it is cultivated using techniques that are still perfected today, to obtain the most favourable conditions not only for biomass productivity but also for optimising its nutrients content. Consumed as a food source since ancient times, the first are believed to be the Aztecs, today it is sold in the form of capsules, tablets, extracts and powders and is increasingly used in the production of high value-added products for human (Fradique *et al.*, 2010) and animal nutrition. It is of great interest to the pharmaceutical industry, especially for its medicinal and anti-cancer properties, and to sectors such as cosmetics and the dye industry (Safi *et al.*, 2014). Due to its ability to store large quantities of lipids and its fatty acid profile, it is has been hypothesized as an alternative and renewable raw material for the production of biodiesel (Lardon *et al.*, 2009) and other biofuels. Its remarkable ability to fix carbon dioxide (up to 74% when grown in a

photobioreactor) and absorb nitrogen (4%5 to 97%), phosphorus (26% to 96%), reduce COD (61% to 86%), and especially remove ammonium (under immobilized conditions and in the presence of growth-promoting bacteria it can remove up to 100%), makes it one of the best microalgae for wastewater bioremediation (Safi *et al.*, 2014).

2.6. Submerged fermentation (SmF) vs solid-state fermentation (SSF)

Solid-state fermentation (SSF) and submerged fermentation (SmF) are two types of cultivation types that are not always interchangeable in terms of culturing a certain microorganism or obtaining a certain product of interest. The choice between the two types of fermentation is not only made in relation to these two conditions, but also taking into account factors such as product quality, government regulations for the disposal of solid/liquid waste, as well as the costs of implementing and managing the entire production process (Singhania *et al.*, 2010).

2.6.1. Submerged fermentation (SmF)

The first attempts at submerged fermentation (SmF) started in the 1950s and concerned the cultivation of lower fungi. Over the years, the use of this culture technique has gradually increased, also concerning different types of higher fungi. SmF refers to a microbial culture that ferments in the presence of high levels of free water ($0<a_w<1$), within a nutrient-rich culture broth placed under agitation (Bakratsas *et al.*, 2021). Particularly in the presence of filamentous fungi, problems can occur of a certain significance, related to the rheology of the fermentation broth. In these cases, the growth of mycelia leading to the formation of biofilms or pellets, hence an increase in viscosity, resulting in problems with the functioning of the bioreactor. In particular, as viscosity increases, the difficulty of homogenising the culture broth increases, favoring the formation of stagnation zones. The difficulties and associated costs are proportional to the size of the bioprocess, so the implementation of this type of fermentation on an industrial scale would not only entail problems that are difficult to manage and solve but would also be characterised by particularly high costs (Gibbs, Seviour and Schmid, 2000). However, although the possibility of such problems exists, in general, SmF is a type of fermentation that allows good mass transfer of heat and oxygen and better homogeneity within the culture broth. Both on an industrial and laboratory scale, it is easier than SSF to monitor and control all process variables, thus intervening in physical factors (such as temperature, aeration and agitation), chemical factors (such as pH and composition of the culture medium) and biological factors (such as morphology of the fungus and rheology of the culture). The possibility and ease of managing these factors make possible not only an improvement in the performance of the bioprocess, with a consequent increase in the yield of the product of interest but also its reproducibility and extension ((Bakratsas *et al.*, 2021); (Fazenda *et al.*, 2008)).

2.6.2. Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is an ancient technology that is still widely used today. Examples are the Koji processes used to produce soy sauce, miso, sake and tempeh, the processes used for compost production and mushroom cultivation. SSF are also used for the production of biopharmaceuticals, bioremediation and the biodegradation of hazardous compounds and agro-industrial residues (Fazenda *et al.*, 2008). SSF is defined as a microbial culture that ferments on the surface and within a solid matrix with low levels of free water ($a_w \approx 0$) (Barrios-González, 2012). This process is slow and limited to microorganisms that can tolerate $a_w \approx 0.60 – 0.66$. Furthermore, on an industrial level, SSF is even more complex to monitor, control and expand. In bioreactors in which SSF takes place, the transfer of heat and oxygen is limited, as is the homogeneity of the culture, with possible negative consequences on product quality ((Fazenda *et al.*, 2008); (Bakratsas *et al.*, 2021)). However, this culture method has some important advantages. One relates to the physiology of the fungi, referred to as 'solid medium physiology', such as high production of secondary metabolites or enzymes in relatively brief times. One reason for this improved physiology and metabolic efficiency is that fungi have soil as their natural environment, and over the years they have been artificially grown on moist solid substrates. Further reasons related to this aspect of improved physiology and metabolic efficiency are still being studied. Another important advantage is the greater economic feasibility on an industrial level compared to other cultivation methods (Barrios-González, 2012).

2.7. Microbial synergy

Over millions of years, a nonparasitic symbiotic link has developed in nature between ascomycete fungi and marine and terrestrial algae, leading to the formation of symbiotic organisms such as lichens (Yuan, Xiao and Taylor, 2005). This type of mutualistic interaction has inspired researchers over time to experiment with co-cultures of fungi and (micro)algae to obtain and replicate the synergy underlying this symbiotic interaction and exploit it in biotechnological applications, such as wastewater treatment (Leng *et al.*, 2021). The co-cultivation of these two types of microorganisms can present a mutualistic or antagonistic interaction, depending on the nature of the species involved and the cultivation conditions. In the case of mutualistic interaction, the synergy to be exploited is based on the exchange of the products of the two microorganisms. While the (micro)algae utilise the minerals and carbon dioxide produced by the fungi during their respiration/fermentation for their metabolic activities, the fungi, in turn, utilise the nitrogen and oxygen produced by the (micro)algae for their sustenance (Reyes *et al.*, 2022).

Although applications based on this type of cultivation are still limited and underdeveloped to date, given the excellent prospects and important development that this technology represents in various industrial sectors, especially in terms of environmental and economic benefits, the research conducted on the subject is rapidly increasing.

3. Materials and methods

The experimental part of this study began with the development and maintenance of the microorganisms of interest and ended with the verification of the growth of these microorganisms on the complex matrices to be valorise (BSG/AP). The adopted experimental approach, an outline of which is given below (Fig. 19), comprises three major macro-areas:

- Growth of single/combined microorganisms (*R. oligosporus, L. edodes* and *C. vulgaris*) on different defined/complex substrates under different illumination conditions (dark and illuminated conditions).
- Complex matrices analysis (Brewer's Spent Grains /Apple Pomace).
- Growth of a single microorganism (*R. oligosporus* and *L. edodes*) on a complex (BSG/AP) matrix under different illumination conditions (dark and illuminated with red and blue light, c. 100 lx).

In the first phase, the growth of microorganisms in the presence and absence of light is tested to determine whether this factor can significantly influence their growth. At this stage, it is also investigated whether the combination of fungi (*R. oligosporus/Lentinula edodes*) with a microalga (*C. vulgaris)* can favour the fungal growth, attempting to exploit a synergy based on the type of metabolism characterizing the two microorganisms. During their growth, aerobic fungi consume O_2 and produce CO_2 as one of their ultimate metabolic products. Microalgae present the capacity for photoautotrophic growth, using light and CO_2 as C-source and producing O_2. This generated oxygen can in turn be used by fungi in a synergistic cycle, which is one of the motivations of this thesis work.

In the second part, two widely produced food industry by-products are characterised. These matrices are the Brewer's Spent Grains (BSG) and the Apple Pomace (AP). This is a preliminary characterization, comprehending two types of analysis: a simplified proximity analysis and the pH assessment.

The last part presents an experimental campaign aiming at studying the growth of the aforementioned microorganisms on these complex substrates of interest (BSG/AP), again investigating the possible effect of light on their growth.

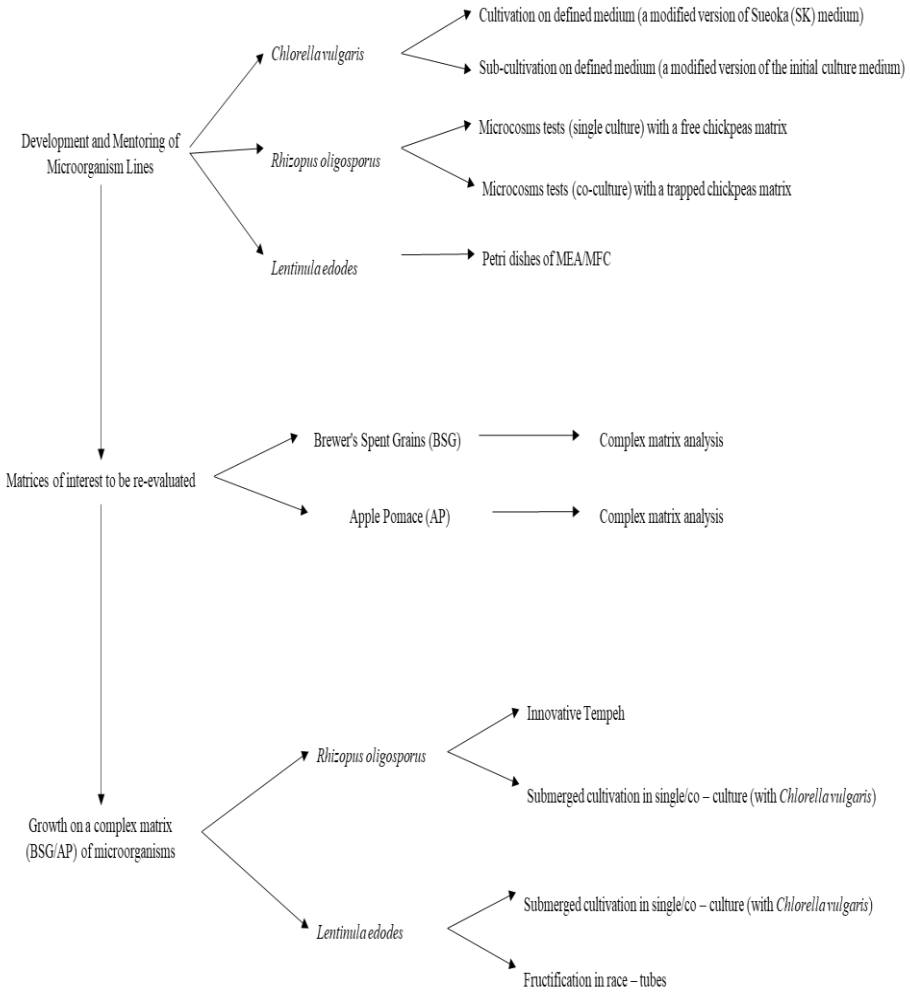

Fig. 19 Outline of the experimental approach adopted in the thesis.

The substrates used during these experiments are those commonly used for the growth of these microorganisms. These are defined culture media such as a modified version of the Sueka medium (SK) and Malt Extract Agar (MEA), and natural model complex substrates (such as chickpeas). The latter substrates are chosen because, as demonstrated in the literature, among the various leguminous plants these are those that most favour the growth of *Rhizopus oligosporus* (Erkan *et al.*, 2020). In addition to these, there is an innovative, natural and low-cost one medium, which comes from the laboratories of Empa (the Swiss Federal Laboratory for Materials Science and Technology), and it is micro fibrillated cellulose (MFC) (Siró and Plackett, 2010). The last listed substrates of natural origin, chickpeas and MFC, were chosen not only for the aspects mentioned above but also for their composition in terms of hemicellulose, cellulose and lignin, which is comparable to that characterizing the two retrieved substrates of interest in this study, BSG and AP. Going into detail, MFC is an aqueous mixture characterized by a weight percentage of cellulose of c. 3.00% (Siró and Plackett, 2010) while chickpeas, on a dry weight basis, present hemicellulose at 2.69%, cellulose at 3.70% and lignin at 2.17% (Vasishtha and Srivastava, 2013). On a dry weight basis, literature references suggest that AP is characterized by 12.00% hemicellulose, 31.00% cellulose and 15.00% lignin (Sudha, 2011) and BSG is characterized by 41.90% hemicellulose, 25.30% cellulose and 16.90% lignin (Silva *et al.*, 2004).

For a general overview, the light exposure conditions used in the experimental part of

this thesis are achieved by using a commercially available lamp (Yuymika plant lamp, Shenzen, China), placed at about 20 cm above the samples. At the experimental conditions, the illuminance was measured and maintained constant throughout the experiments at a luminous flux of approximately 100 lux. The lamp was set to irradiate in the mode that combines two spectra: blue and red (Fig. 20).

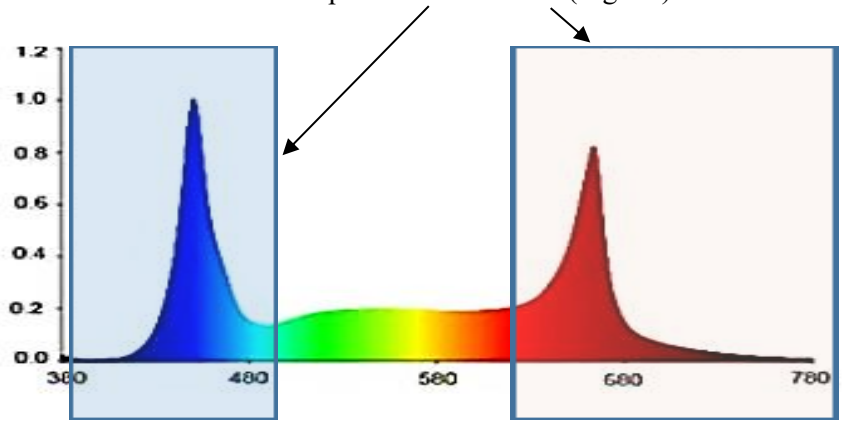

Fig. 20 YUYMIKA lamp spectrum used.

The methods of the experimental phases, relating to the three macro-areas into which the thesis can be divided, are described below.

3.1. Growth of single/combined microorganisms on different substrates under dark/light conditions

This section includes information on the first start-up of microalgae cultures (20 mL), their sub-cultivation at a larger scale (500 mL), as well as their entrapment in alginate for their use in latter co-culture experiments. Then, the microcosms setup are introduced, under the two modalities studied (with a free chickpeas matrix and chickpeas contained in perforated plastic bags), in addition to the CO_2 calculation method. After, the methods for petri dish growth of *Lentilula edodes* in two different media (MFC/MEA) are introduced under different illumination conditions.

3.1.1. Growth of *Chorella vulgaris* in liquid phase under natural light conditions

The culture of *Chlorella vulgaris* was initially conducted in the liquid phase on a defined medium under natural light conditions. Subcultures were subsequently obtained from the previously produced cultures on a modified version of the initial culture medium and again under daylight conditions. After reaching consistent volumes, a small aliquot of the subculture was pelletised with sodium alginate for use in subsequent experimental steps.

3.1.1.1. Initial inoculum growth on Sueoka medium

For the initial growth of the microalga on defined medium, 1 mL of inoculum of *Chlorella vulgaris* 9–88 (UAM 101) was equally divided into four 20 mL flasks (A, B, C and D), containing 10 mL of Sueoka (SK) medium (8.3 mM KH2PO4, 5.3 mM K_2HPO_4, 0.25 mM $MgSO_4 \cdot 7H_2O$, 88 µM $CaCl_2 \cdot H_2O$) and 1 mL of Hutner's trace metal solution (Hutner *et al.*, 1950) and 0.03 g of KNO_3. First, the control systems (A – B) were created: 20 mL of modified SK medium (free of trace metal) and 20 mL of sterile distilled water were mixed; this solution was then divided into two 100 mL sterilized flasks (20 mL for each flask). Using a graduated pipette, 0.1 mL of the trace metal solution and 0.2 mL of the starting algae culture were introduced into both flasks. Subsequently, the two replicates of the modified culture (C – D) were produced: 20 mL of modified SK medium (free of the metal trace) was mixed with 20 mL of a $CaCO_3$ solution (0.01 M); the resulting solution was again divided equally into two 100 mL sterilized flasks (20 mL for each flask). Using a graduated pipette, 0.1 mL of trace metal solution and 0.2 mL of the starting microalgae culture were added to each flask. The four systems were closed with cotton and parafilm, and they were allowed to grow in the laboratory at room temperature and pressure and under the natural alternation of light and dark

3.1.1.2. Sub-cultivation

Fifteen days after the preparation of the microalgae starter cultures (section 3.1.1.1.), a 30 mL aliquot was taken from flask C to prepare sub-cultures with a calcium carbonate medium to further increase the available microalgae biomass. The medium for the sub-cultivations was composed of 1 litre of $CaCO_3$ solution (0.01 M) and 3 mL of Hutner's trace metal solution. The medium was divided into 1 L flasks (approximately 500 mL in each) and 15 mL inoculum from the previous cultivation phase. The two prepared systems (A and B) were closed with parafilm wrapped around cotton plugs to allow gas exchange. The systems were then left at room temperature and pressure and under natural light, and they were manually stirred once a day.

3.1.1.2.1. Alginate beads preparation

From the last culture in flask A, an aliquot of 45 mL was taken and divided into three falcons of 15 mL each. These tubes were centrifuged for 5 minutes at 4000 rpm, and pellets of microalga were collected in each falcon tube, as shown in (Fig. 21).

Fig. 21 Falcon containing the sub-culture of microalgae after centrifugation.

After centrifugation, the supernatant was removed from all three falcons and algal biomass pellets were combined. These pellets were then re-suspended in 10 mL sterile distilled water. This concentrated algal suspension was poured into a 100 mL beaker, and 0.3 g of sodium alginate was added (hence, 0.3 % *w/w*). This mixture was placed under magnetic stirring so that it was homogeneously dispersed. A *simple direct gelation* procedure was chosen for the beds preparation, using a $CaCl_2$ solution (0.5% *w/w*) for the bath. Once both solutions were prepared, the suspension containing the alginate/algal biomass was dripped into the $CaCl_2$ bath, using a 10 mL syringe. The alginate suspension solidified rapidly as soon as it came in contact with the calcium chloride bath. Then, microspheres were left to solidify for 20-30 seconds in the $CaCl_2$ bath and then were carefully removed with a spatula and rinsed 10 times with distilled water in a beaker (Fig. 22). Approximately, 70 microspheres (c. 10.5 g) were obtained from the initial volume of microalgae suspension, which were stored in a 50 mL serum bottle under refrigerated conditions.

Fig. 22 *Chlorella vulgaris* entrapped in microspheres of sodium alginate.

3.1.2. Growth of *Rhizopus oligosporus* on a chickpeas matrix in different microcosms types in dark/light conditions

The growth of *Rhizopus oligosporus* on a chickpea matrix was analysed in the presence and absence of spherical microalgae and under light/dark conditions. The setup for these experiments was sealed microcosm configuration (hermetically closed glass jar with daily opening for air exchange and titration volume sampling for respirometric analyses), as shown in (Fig. 23).

The evaluation of respirometric activity within the systems was carried out by measuring the produced CO_2, using acid-base titrations. The milligrams of evolved gas are calculated using an empirical formula whose data are derived from the volumes of the acid/base solutions involved in the titrations. By comparing the CO_2 producing dynamics of each system, it was possible to ascertain which set-up resulted better-suited for fungal growth (based on the direct correlation between growth and carbon dioxide production for this type of microorganism).

Fig. 23 Set–up microcosms under the illuminated conditions using the red-blue light (entrapped chickpea matrix on plastic bags).

3.1.2.I.1. Respirometry

The microcosms setup allows to follow the dynamics of CO_2 evolution, since the produced CO_2 derived from the microbial growth remains in the jar and it is trapped by a high basic solution (trap solution). Periodic sampling of this trap solution and titration are performed, by taking aliquots to be titrated using an acid solution and typically precipitating agents are added as well as colour indicators to properly identify the final titration point. The microcosm setup is widely used in different fields of study such as ecology, limnology, microbiology, soil science (e.g., bioremediation and bioaccumulation), biochemistry and toxicology. There have long provided a platform for studying functional relationships (rates, fates, dynamics) within biological systems at a scale between the simplicity of laboratory and the complexity of natural ecosystems (Hlickabee, 1985).

In this study, the acid-base titration is carried out every 24 hours and for this, 10 mL of NaOH is taken from each microcosm and diluted in a beaker with 80 mL of distilled water, 500 µL of $BaCl_2$ (0.5 M) and 50 µL of phenolphthalein (0.1% *v/v* of a solution in 80% *v/v* ethanol). The resulting solution is shaken magnetically to homogenize it and then it is divided into three 100 mL flasks (30 mL each). These steps are quickly performed in a chemical fume hood to avoid the dispersion of carbon dioxide into the surrounding space. The three flasks are then titrated under magnetic stirring with an HCl solution (1.5 M), using a 25 mL burette (Fig. 24). The end-point during titrations is determined by means of the indicator colour change (phenolphthalein); in this case, an instantaneous colour change from pink to white is observed (Fig. 25). The volumes of HCl used for each sample are registered, and they are averaged (since measurements

are performed in triplicate).

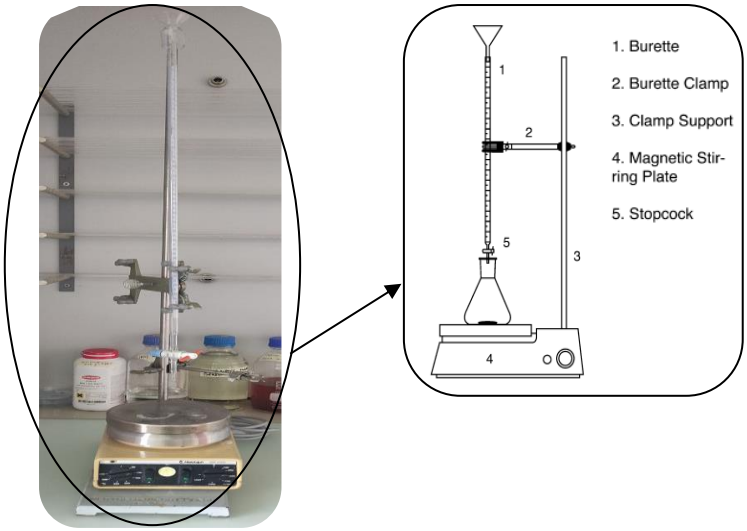

Fig. 24 Setup used for acid-base titration:
burette with magnetic stirrer Heidolph MR 2002 underneath.

Fig. 25 Left: not yet titrated solution; Right: titrated solution.

By using hermetically sealed abiotic control systems, it is possible to have an estimate of the amount of carbon dioxide in the air occupying the headspace which is not related to the biological activity of the microorganisms. The resulting titration values for these abiotic systems are fundamental references, as they allow the amount of CO_2 already present in the system but not related to the activity of the microorganism to be subtracted from the titrating volume of the biotic systems.

Thus, the average titrant volumes of the abiotic and biotic microcosms enable to estimate the produced mg of CO_2 (mg_{CO2}) in the biotic systems by using the following empirical correlation (1):

$$mg_{CO2} = (V_0 - V) * f \quad (1)$$

Where:

- mg_{CO2} = mg of CO_2 evolved due to biological activity in the microcosms;
- V_0 = mL of HCl used to titrate the NaOH solution in the abiotic system;
- V = mL of HCl used to titrate the NaOH solution in the biotic system;
- f = conversion factor derived from 22∗M and expressed in mg/mL;
- M = molarity of HCl solution used to titrate.

3.1.2.1. Microcosms tests (single culture) with a free chickpeas matrix (dark conditions)

The microcosms were set using 1 L hermetically sealed jars and placing approximately 100 g of pre-cooked canned chickpeas in the bottom, as well as a glass support in one corner of the jar (to support the CO_2-trap). The chickpeas layer was then at the same height as the glass support. The obtained systems were subsequently autoclaved (30 min, 121 °C, 2 bar). After this step, the CO_2 trap was placed on the glass supports inside each jar consisting in a 50 mL-filled beaker containing the NaOH solution (2 M). Four systems were prepared (A-D): A and B serving as abiotic controls and, C and D were inoculated with 3 mL of a suspension in water of 2.5 % *m/v* powdered commercial tempeh starter (Tempehtation, Edinburgh, United Kingdom) containing *Rhizopus oligosporus* spores and traces of organic rice flour (c. $7.5 \cdot 10^7$ spores/mL, see (Gómez-Camacho *et al.*, 2022)). The systems were incubated under dark conditions in a growing chamber under controlled temperature and humidity conditions (27 °C, 70% humidity).

3.1.2.2. Microcosms tests (single/co-culture) with contained chickpeas matrices (in perforated plastic bags) with/without entrapped microalgae (dark/light conditions)

For this experimental campaign, a design of experiments was carried (Tab. 4) out that sought to shed light on different process conditions. In particular, the aim was to tests the influence of illumination conditions (dark/light exposure) and the presence of alginate-entrapped *Chlorella vulgaris* on the growth of *Rhizopous oligosporus* under the microcosm setup.

Table 4. Table of the design of experiments.

	Solid Substrate: Chickpea	
	N° rep. in dark conditions	N° rep. in light conditions
Abiotic	1	1
Rhizopus oligosporus	2	2
Rhizopus oligosporus* + *Chlorella vulgaris	2	2

Ten systems were used for this purpose: two abiotic controls (I-II), four biotic systems without *Chlorella vulgaris* (two illuminated – III and IV – and two under dark conditions – V and VI –) and four systems with *Chlorella vulgaris* (two illuminated – VII and VIII – and two under dark conditions – IX and X–).

The microcosm setups are similar to the ones described in section 3.1.2.1. They consist of hermetically sealed 1 L jars, inside which perforated bags containing the matrix and the inocula (for biotic systems) were placed. The glass trap support and trap solution are the same. These microcosms were prepared as follows: first 500 g of pre-cooked

chickpeas were autoclaved (30 min, 121 °C, 2 bar), as well as the glass jars, the bags, the glass supports. The bags used (90x110 mm) are commercially available plastic bags with closures, which were perforated to maintain a distance of 1 cm^2 between each hole, resulting in a geometric grid with a total of fifteen holes (similar to reference (Gómez-Camacho *et al.*, 2022)). For all systems, the bags were filled with approximately 50 g of chickpeas each. These bags were placed inside the glass jar, for abiotic systems no further amendments were performed (I and II). For the biotic systems (III – X), chickpeas were inoculated as described in the section 3.1.2.1. and put into the plastic bags. For the systems containing the co-cultures (VII-X), 15 alginate beds of *Chlorella vulgaris* (approx. 2.26 g) were added in each. The systems were incubated in a growing chamber under controlled temperature and humidity conditions (27 °C, 70% humidity). Out of the ten systems, five were placed in the dark and five under illumination conditions (red and blue, 100 lx), ensuring that each type (abiotic, biotic without microalgae and biotic containing microalgae) was placed in both conditions for proper benchmarking.

3.1.3. Growth of *Lentinula edodes* in Petri dishes (MEA/MFC) in dark/light conditions

The experimental phase of this thesis also included the evaluation of the growth of *Lentinula edodes* in petri dishes. The parameters to be investigated in this phase are the effect of illumination during the growth of this microorganism, as well as the influence of the culture medium. Due to the difficulties of measuring biomass growth in fungal cultures, the mycelium (radial) extension rate method was used.

In brief, this method is suitable for evaluating the radial mycelium growth rate on solid media, by daily following the mycelium extension (in different points of the petri dish) and calculating also the growth rate as the difference between daily extensions. It is suitable for comparison purposes, such as the selectivity for the medium, the differences between strains, among others.

3.1.3.I.1. Mycelium extension rates on plates

Basidiomycetes fungi, such as *Lentinula edodes*, are higher fungi organisms that assume different forms during their life cycle, thus presenting a very complex morphology. The vegetative structure assumed during growth, which can be recognized as the basic one, is known as the hypha and consists of tubular filaments. These continue to grow by branching repeatedly and forming a dense mass called the mycelium, which can present itself in different morphological configurations (ranging from mycelial filaments dispersed to a densely intertwined hyphal network, called pellets). Several factors contribute to shape the specific morphology to be expressed over their life stages, such as the type of fungal species, the nature of the inoculum and the chemical (soil constituents) and physical (submerged or solid state conditions,

temperature, pH, mechanical forces) conditions of the culture.

It is not uncommon for one or more of these parameters to be modulated during the controlled culturing of these (micro)organisms to obtain a particular morphological form that maximizes biomass yield, as morphology influences not only nutrient consumption but can have a significant effect on the performance of the bioreactor, as it can affect the rheology of the fermentation broth, causing it to become highly viscous with non-Newtonian and pseudo-plastic flow behaviour. Intervening with the morphology of these microorganisms to make the most of their reproductive capacities is a real problem, on which scientists have been directing their attention and energies for years. For the same reasons mentioned above, the adoption of an appropriate strategy to accurately estimate the biomass growth is also a real problem. As far as this latter aspect is concerned, more and more often manual methods are being used, carried out under the microscope, by direct observation or photography, or based on direct measurement of the extension of the mycelium in Petri dishes with the aid of a ruler; these methods, although simplified and less accurate, nevertheless allow a fairly valid estimate of the parameter of interest to be obtained in a very simplified and reproducible manner, given the complexity of the systems to be analysed (Papagianni, 2004).

In this study, to assess fungal growth, one of these manual techniques is adopted to directly measure the extension of the mycelium over time and thus understand under which conditions *Lentinula* growth is most favoured. Specifically, during the time on all the Petri dishes, the radius value is assessed with the aid of a caliper; every 24 hours, from the day of inoculation until the day the mycelium reached the edges of the Petri dish, the radius was measured in four different directions for each Petri dish to obtain an average radial length value that was as representative as possible.

3.1.3.1. Petri dishes of MEA/MFC (dark/light conditions)

For these tests, petri dishes of 100 mm diameter were used. Two different media were prepared to fill the petri dishes: *i)* malt extract agar (MEA, 2% *w/w*) and ii) micro fibrillate cellulose (MFC + 4% *w/w* malt). Approximately, 25 g of medium was poured into the petri dishes (four replicates for each culture medium). Then, at the centre of each Petri dish the inoculation was performed under microbiological hood placing a mycelial plug of 0.8 mm^2 *of Lentinula edodes* (c. 0.172 g). This inoculum is derived from strain established in EMPA, which has been isolated from fruiting bodies of commercially available mushrooms, identified as *Lentinula edodes* (EMPA -100 G2). The eight Petri dishes obtained were left to grow in a temperature- and humidity-controlled chamber (27 °C, 70% humidity). Four of them were placed under illumination conditions (blue and red light, 100 lx), while the rest were left under dark conditions.

3.2. Complex (BSG/AP) matrices analysis

In this section, the approach for the characterisation of the complex matrices of interest, - Brewer's Spent Grains (BSG) and apple pomace (AP) - for this case study is described. These matrices present a complex and highly variable structure depending on their origin and the processes they undergo within the production line in which they are retrieved from.

The AP used in the experiments came from a local factory in St. Gallen (Switzerland). It is obtained as a by-product of the pressing stage, a characteristic step of apple juice and cider production. It was supplied as such, and it contained mainly the skin, pulp, seeds and stem residues of the fruit. The BSG used in the experiments came from a local brewery in St. Gallen (Switzerland). As it can be seen from the photo below (Fig. 26), it was supplied immediately after the mashing process, before the filtration process took place, which are characteristic steps in the wort production process.

Fig. 26 The fresh BSG.

Therefore, the first step is to perform an initial characterization in terms of the total solids content and corresponding moisture content, as well as its pH value (proximate analysis and pH assessment).

3.2.I.1. Moisture content (MC) and total solids (TS)

For estimating the moisture (MC) and total solids (TS) content of the BSG and AP matrices, a gravimetric/drying method was chosen. The analyses were carried out in triplicates for the fresh received biomasses. The samples were placed in an oven (Fig. 27) at 65 °C for 24 hours, inside typical porcelain crucibles which are used for analyses of this type. The weight of the samples is recorded before and after the oven-drying operation, and the difference can be used for calculating the TS and the MC.

The respective MC and TS percentages are calculated as follows:

$$MC\% = [(\text{fresh sample weight} - \text{dry sample weight}_{65\,^{\circ}C}) / \text{fresh sample weight}] \times 100 \quad (2)$$

Where:

- MC% = percentage of moisture content present in the BSG/AP sample;
- fresh sample weight = g of fresh BSG/AP sample;
- dry sample weight $_{65\,°C}$ = g of dry BSG/AP sample (65°C, 24h).

The complement of the MC represents the total solids weighed.

Fig. 27 Oven PANTATHERM D.

3.2.I.2. pH

The pH is another key parameter that can significantly influence (or even govern) microbial growth dynamics. Indeed, certain species are favoured within tight or wide pH ranges and, in biotechnological applications with retrieved biomasses, the pH is a fundamental factor for deciding valorisation strategies. Extreme value of pH, to which the biomasses would be exposed, would require prior pH amendments or controlled fermentations with pH control, which can influence the output of the process.

For both matrices (AP and BSG), 3 g of dried, crushed substance (filtered through a 1 mm^2 mesh filter) was placed in a beaker containing 14.50 mL of distilled water; the resulting solution was homogenized at 500 rpm and room temperature for two hours using a magnetic stirrer. After this step, the pH was then assessed with a pH-meter operated with a hand-held electrode (Fig. 28) in triplicates.

Fig. 28 Left: pH-meter wtw Inolab 730; Right: electrode Sentix 81.

3.3. Growth of a single microorganism on a complex (BSG/AP) matrix in dark/light conditions

This section first presents the methods by which the growth systems of individual microorganisms on a complex matrix (BSG/AP) were characterised under different lighting conditions. Next, the method by which these growth systems were obtained is described. Going into detail, first the cultivation of individual microorganisms and co-cultures in flasks is reported, then the method for the cultivation of *Rhizopus oligosporus* as tempeh, and finally the method for the cultivation of *Lentinula edodes* in race - tubes.

3.3.I.1. Moisture, total solids

The moisture content and the corresponding total solids content were calculated for the subculture samples (described in section 3.3.1.) and for the tempeh samples (described in section 3.3.2.) by gravimetric method (see section 3.1.I.1.).The sub-culture samples obtained as described in section 3.3.1.1. and the tempeh samples were placed inside common beakers and placed in an oven (Pantatherm D) at 65 °C for 24 hours. The weight of the samples was recorded before and after oven drying and the difference was used to calculate TS and MC; the respective percentages of MC and TS were instead calculated using the expression (2).

3.3.I.2. Electrical conductivity

The electrical conductivity was assessed in duplicate with the aid of a portable conductivity meter (Fig. 29). The procedure adopted for the subculture samples (described in section 3.3.1.1.) and the tempeh samples (described in section 3.3.2.1.) is very simple: under a microbiological fume hood, the conductivity meter was inserted into the beakers containing the samples, the value was allowed to stand for a few moments and the measurement was reported in ppm.

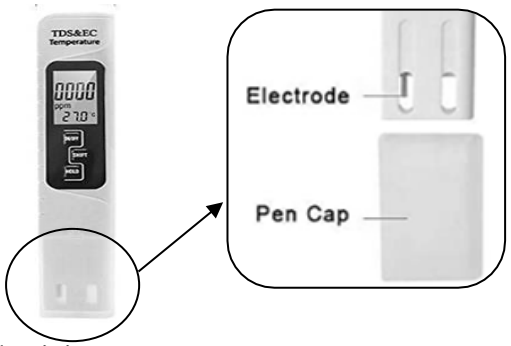

Fig. 29 Conductivity meter TDS&EC.

3.3.I.3. pH

The procedure adopted for subculture samples (described in section 3.3.1.1.) and for tempeh samples (described in section 3.3.2.1.) is based on the following steps: under a microbiological fume hood, the pH meter (Fig. 30) was inserted into the beakers containing the samples, the value was allowed to stand for a few moments and the pH value (-) was marked.

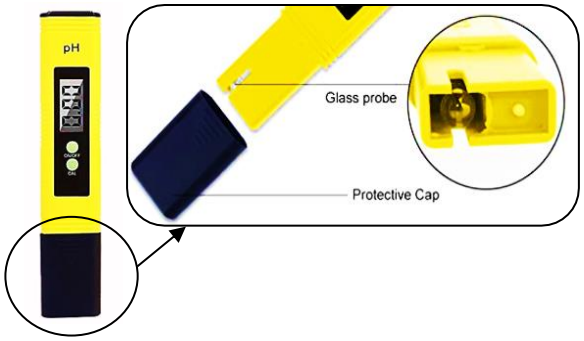

Fig. 30 pH-meter JIYISHIHOU.

3.3.I.4. Titratable acidity (TA)

Titratable acidity is used to estimate the total concentration of acids contained in food matrices (also known as total acidity). It is determined by neutralizing the acid present in a known quantity (weight or volume) of sample with a standard base, until reaching the end point of the titration constituted by a target pH or the colour change of a pH-sensitive substance; the greater the volume of the base solution used to reach this point, the greater the degree of acidity of the titrated system.

Acids in food are generally partially ionised organic acids - citric, malic, lactic, tartaric and acetic acids - whose presence and, above all, the concentrations in which they are present in different foods determine certain sensory and organoleptic characteristics. However, inorganic acids - phosphoric and carbonic (derived from carbon dioxide in solution) - can also be found, and these often play an important and even predominant role in the acidification of foods. It should be noted that while some properties of foods are only influenced by the ionized fraction of acid molecules other properties are influenced by the total acid content. For this reason, it is of fundamental importance to combine this type of analysis with pH measurement, which provides an estimate of the acid activity (also known as active acidity), in order to obtain a general and complete picture of the effects on food of the acids intrinsically present in it (Koyama *et al.*, 2015).

In the present study, it is the organic acids that are predominant and it is on these that the analysis of the total acid content in the samples of interest is based ((Castro and Colpini, 2021); (Antonic *et al.*, 2020)).

This type of analysis performed on the slurry samples (described in section 3.3.1.1.)

and on the homogenized samples after the tempeh solid-state fermentation (described in section 3.3.2.1.). The measurements were carried out in duplicate using NaOH (2 M) as titrant and a phenolphthalein solution as colour indicator (0.1% *v/v* in ethanol 80% *v/v*). From each previously prepared sample, 5 mL of sample was taken with a graduated pipette, diluted in a beaker with 15 mL of distilled water and 20 µL of phenolphthalein; the resulting solution was magnetically stirred to homogenize it as best as possible and then divided into two 100 mL flasks. The two flasks were titrated under magnetic stirring with a NaOH solution, using a 25 mL burette (Fig.31). When the solution is fully titrated, due to the presence of the colour indicator, it shows an instantaneous colour change from white to pink (Fig.32) the volume of NaOH used for each titration is noted and, as the analysis is in duplicate, the values are averaged.

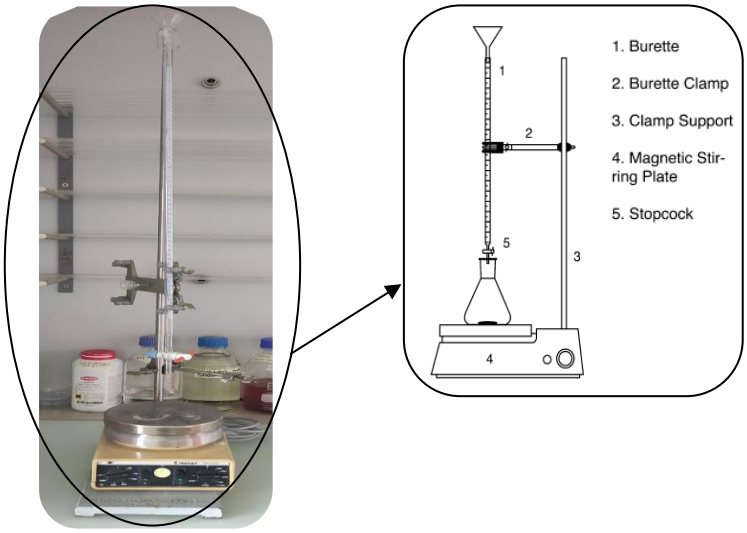

Fig. 31 Setup used for acid-base titration:
burette with magnetic stirrer Heidolph MR 2002 underneath.

Fig. 32 Left: not yet titrated solution; right: titrated solution.

3.3.I.5. Protein determination

Protein quantification is necessary to understand the total protein content in a sample or in formulated products; an accurate analysis is of paramount importance, especially if this property is a key parameter when comparing different samples or products to see which provides the optimal trade-off between cost and benefit.

Various techniques can be used to analyse the concentration of extracted proteins. They are based on different physicochemical principles and are characterized by a different sensitivity depending on the nature of the protein and its concentration, the accuracy can be influenced by the presence of possible interfering substances and a different speed of execution of the test.

Knowledge of these aspects plays a decisive role in the choice of method and the reliability of the results obtained (Martina and Vojtech, 2015).

A technique that is particularly used due to its ease of execution and good accuracy is spectrophotometry, which can be conducted either by directly analysing the sample (direct spectrophotometric method) or by analysing the sample following different preparing protocols (including the addition of reagents and dyes) known as colorimetric methods. Each of these latter methods is based on particular chemical mechanisms that are also subject to the interference of chemical substances typical of complex biological preparations and they are sensitive only to particular concentrations of proteins.

A panoramic of such characteristic aspects for methods such as Lowry, Biuret and Bradford is given in the table below (Tab. 5):

Table 5. Overview of methods (Martina and Vojtech, 2015).

Method	Sensitivity	Accuracy	Interference
Lowry	0−0.1 mg	Partially dependent on the amino acid composition	Acids, EDTA, DTT, phenol, $(NH_4)_2SO_4$
Biuret	0−1 mg	High, no depend on amino acid composition	Amino-group $[(NH_4)_2SO_4]$
Bradford	0−0.01 mg	Dependent on amino acid composition	Detergents (soap, SDS, Triton X-100)

The principle behind spectrophotometry is the ability of molecules to interact with electromagnetic waves. Depending on the nature and types of chemical bonds they form, molecules only have certain energy levels and are therefore only able to absorb light at specific wavelengths.

Proteins, due to the presence of three aromatic amino acids - tryptophan, phenylalanine and tyrosine - show this property in a very pronounced manner and are characterized by a peak of interaction with light at a wavelength of 280 nm, and it is this peculiarity that makes spectrophotometry an accurate technique in the determination of protein quantity (Piccolo, 2021).

3.3.I.5.1. Lowry Colorimetric Method

One of the standardized colorimetric methods is the Lowry method, developed by Oliver Lowry (1951), which is very sensitive and accurate and can detect proteins at low concentrations, in the 2-5 µg range.

This time-dependent spectroscopic procedure consists of two reactions. The first is the biuret reaction: by adding a copper solution to the protein solution under alkaline

conditions, the peptide bonds complex with the copper resulting in a purple-purple colour with maximum absorption at 540 nm. This is followed by the reduction of the Folin-Ciocalteu reactive (phosphomolybdic-phosphotungstic reactive) under alkaline conditions: the copper-protein complex causes a reduction of the reactive to tungsten blue and molybdenum blue, in particular Cu^{+} catalyses the reduction of the Folin reactive with the concomitant oxidation of the aromatic residues (tyrosine and tryptophan), the entire solution takes on a blue colour with a maximum absorption at 750 nm. The protocol adopted in this study is the 'Lowry Protein Assay Protocol (LOWRY *et al.*, 1951). In accordance with the protocol, the following solutions were prepared:

- 500 mL of solution A: 2% *m/v* Na_2CO_3 in NaOH (0.1 M);
- 20 mL of solution B: 1% *m/v* NaK tartrate in H_2O;
- 20 mL of solution C: 0.5% *m/v* $CuSO_4 \cdot 5H_2O$ in H_2O.

The above solutions were then used to produce the solution D required for the biuret reaction, consisting of 48 mL of solution A, 1 mL of solution B and 1 mL of solution C; they were stored in a 50 mL falcon in the refrigerator along with Folin reagent. Also according to the protocol, Lowry's analysis was performed on the liquid samples by performing the following steps:

1) 250 µL of sample is diluted with 1.6 mL of previously produced solution D in UV cuvettes;
2) the sample obtained is allowed to stand for 5 minutes;
3) 150 µL of Folin is added to the rested sample;
4) the new sample is allowed to stand in the dark for 30 minutes;
5) 750 nm absorbance readings are taken with a spectrophotometer and the corresponding values are reported.

In this study, the analysis was performed in duplicate on the submerged fermented samples (described in section 3.3.1.1.) and on the solid-state fermentation tempeh samples (described in section 3.3.2.1.). Before the Lowry analysis, a gentle centrifugation step was performed to sediment large particles and debris (with an Eppendorf centrifuge, Fig. 33), to avoid interference with the readings. For this, aliquots of 2 mL of each sample was taken with a graduated pipette and placed inside 2 mL eppendorf, which were weighed to balance the centrifuge core as correctly as possible and they were centrifuged for 5 minutes at 4000 rpm.

Fig. 33 Eppendorf centrifuges MiniSpin ® plus

After centrifugation, the protein content of the samples was assessed by spectrophotometer readings (Fig. 34) following the procedure described above. To ensure the linearity of the Lambert-Beer law, the 250 µL volume of sample to be analysed according to the protocol was obtained by diluting the liquid extracts of the homogenizations in the cuvettes at 2:3 ratio with distilled water.

Fig. 34 Spectrophotometer GENESYS 10S UV-Vis.

3.3.I.5.2. Construction of the calibration line

The amount of protein in the subculture/tempeh samples was estimated using calibration lines constructed from solutions of known protein concentration, using as standards bovine serum albumin (BSA) and two commercial whey protein mixtures (WPI) (vanilla and chocolate flavoured). The choice of using different standard proteins and protein mixtures is dictated by the diversity of proteins and amino acids present in the biomass matrices of interest (BSG and AP).

First, for each standard with known protein concentration, a 'stock' solution was prepared with a concentration of 5 g/L in distilled water, gently homogenized with a vortex mixer for 30 seconds, from which appropriate dilutions were then made to produce the corresponding calibration lines.

Dilutions of stock standards were made directly in the UV cuvettes from a volume of sample at the desired concentration; for all three stock solutions, six dilutions were made to the following concentrations: 0.625, 0.500, 0.375, 0.250, 0.125, 0.000 g sample/L in distilled water. Then, the Lowry's colorimetric method was then applied in duplicate to these dilutions and the absorbance readings obtained from the spectrophotometric at 750 nm were used to construct the calibration line (protein concentration [g/L] vs. absorbance [AU]) by linearly interpolating the obtained values. Once the calibration line was obtained, the unknown protein concentration of the samples was evaluated and, to this end, the absorbance values at 750 nm obtained from the spectrophotometric analysis of the samples were plotted on the concentration/absorbance graph and, with the help of the calibration line, it was possible to correlate the absorbance value of each sample with the corresponding protein concentration.

3.3.I.5.2.1. Serum Bovine Albumin (BSA)

Bovine Serum Albumin (BSA) is a large, stable, soluble, monomeric, globular protein which makes up about half of the total serum protein. Due to its binding and transport properties, BSA acts primarily as a carrier for hormones, fatty acids, trace minerals, vitamins and iron; it also plays a role in stabilizing extracellular fluid volume and maintaining osmotic balance, as well as binding harmful toxins and free radicals. Typically isolated by heat shock, it is suitable for use in a wide range of molecular biology applications, including the determination of the amount of protein in a sample, where it tends to serve as a reference protein.

The BSA used in this study is lyophilized bovine serum albumin powder, ≥96% (agarose gel electrophoresis) from Sigma-Aldrich. Using this BSA, a 5 g/L solution was produced, through which, following the procedure described above, the following calibration line was obtained (Fig 35.):

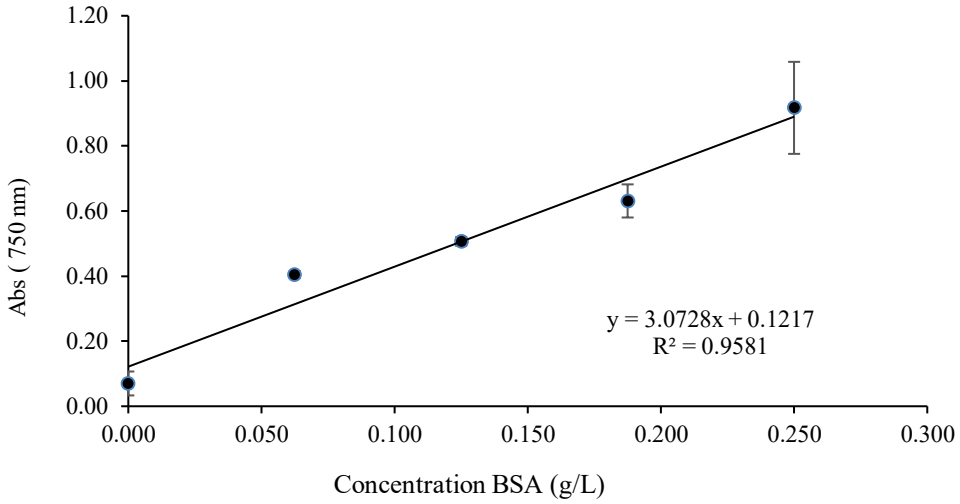

Fig. 35 BSA calibration line.

It should be noted that the absorbance readings obtained from the known concentrations of 0.625, 0.500 and 0.375 g BSA/L in distilled water lied beyond the linearity range of the lambert-beer law (absorbance values greater than 1). Hence, appropriate dilutions were performed to construct the calibration lines, using instead 0.188 and 0.063 g BSA/L in distilled water points to improve the fitting as much as possible.

3.3.I.5.2.2. Commercial WPI

Whey protein isolate (WPI) is a high-protein source whose protein content reaches over 90% by weight in its natural form. Almost free of carbohydrates and fat, they are rapidly digestible due to their bioavailability. WPIs are produced using techniques such as cross-flow microfiltration (MF) and ultrafiltration (UF), or combinations of these, to

produce an extremely pure protein powder and increase the percentage of protein available, which is usually around 75% by weight. Ideal for supporting lean muscle growth and post-workout recovery, WPIs can in some cases have adverse effects on the human body, such as causing allergies and intolerances. Widely marketed, they are available in a variety of flavoured versions, from the simplest to the most unusual tastes. The whey protein isolates (WPI) used in this study are of two types, one vanilla-flavoured and the other chocolate-flavoured, both with a moisture content of 10%, assessed by the gravimetric method after drying in an oven at 65°C for 24 hours; from both, 5 g/L stock solutions were produced, through which, according to the procedure described above, the following calibration lines were obtained respectively (Fig. 36 and Fig. 37):

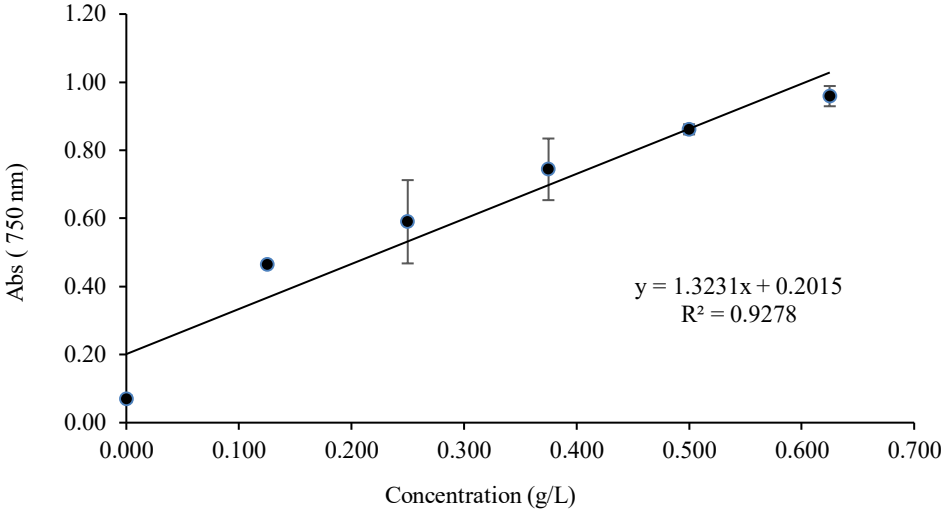

Fig. 36 Vanilla whey protein (WPI) calibration line.

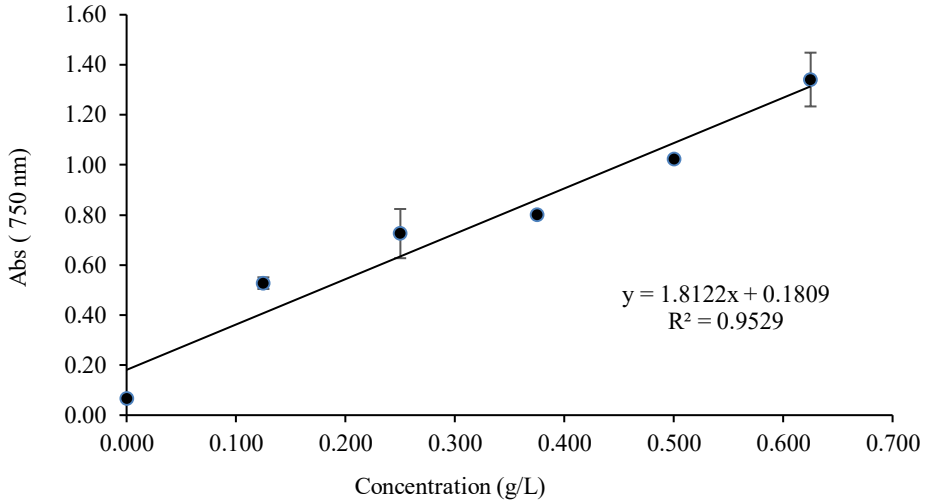

Fig. 37 Chocolate whey protein (WPI) calibration line.

3.3.1. Submerged cultivation of *Rhizopus oligosporus* and *Lentinula edodes* with and without *Chlorella vulgaris* (dark conditions)

In this section, a liquid fermentation approach for the valorisation of BSG and AP is tested. *Lentinula edodes* and *Rhizopus oligosporus* were separately inoculated on both matrices. For this, slurries were prepared (at a concentration of c. 10 % *w/w* of dry matter) after the mechanical pre-treatment of the matrices, they were inoculated and maintain under agitated conditions for two weeks. The raw slurries and the fermented samples were then analysed to gain insights into the compatibility between BSG and AP and the selected fungal species.

In six sterile 250 mL flasks, 58.5 mL of distilled water and 6.5 g of dry, crushed BSG (sieved through a 1 mm^2 mesh filter) were placed. In three of these, 3 mL of *Rhizopus oligosporus* (1.0% vol.) was inoculated, while in the other three, a mycelial plug of 0.8 mm^2 of *Lentinula edodes* (c. 0.172 g) from a Petri dish with MEA produced earlier (section 3.1.3.1.) was placed.

A further six sterile 250 mL flasks were filled with 58.5 mL of distilled water and 6.5 g of dry, crushed AP (sieved through a 1 mm^2 mesh filter). Again, in three of these, 3 mL of *Rhizopus oligosporus* (1.0% vol.) was inoculated, while in the other three, a mycelial plug of 0.8 mm^2 of *Lentinula edodes* (c. 0.172 g) from a Petri dish with MEA produced earlier (section 3.1.3.1.) was placed.

In addition, two subcultures of both fungal microorganisms were prepared but also contained entrapped microalgae. Two sterile 500 mL flasks were filled with 90 mL of distilled water and 10 g of dry, crushed BSG (sieved through a 1 mm^2 mesh filter). In one, 3 mL of *Rhizopus oligosporus* (1.0% vol.) and 3 microspheres of microalgae (0.452 g) were inoculated, in the other, a mycelial plug of 0.8 mm^2 of *Lentinula edodes* (c. 0.172 g) from a Petri dish with MEA produced earlier (section 3.1.3.1.) and 3 microspheres of microalgae (0.452 g) were inserted.

The systems of this submerged fermentation campaign were closed with cotton and parafilm, were they allowed to grow in an incubator shaker (ISF-1-V) at 25°C and 100 rpm.

3.3.1.1. Preparation of samples for the analysis

After thirteen days of fermentation in the incubator shaker, the flasks were poured into sterile glass bottles. The weight of the systems was also recorded for closing the mass balance with the initial values. Then, 50 mL of sterile distilled water was added to each flask to facilitate the preparation of homogenized samples and assess the final parameters of the systems.

3.3.2. Growth of *Rhizopus oligoporus* in perforated bags: innovative Tempeh (dark/light conditions)

The novel tempeh was formulated using the model microorganisms for this traditional food (*Rhizopus oligosporus)*, but using a combined matrix of BSG and AP. In addition, it was also investigated whether a light source could improve the growth of the biomass.

For this purpose, six sealed plastic bags (90x110 mm) were used, which after being perforated (1 cm distance between the holes, 20 holes in total per bag) were filled with 85 g of the substrate on which a 25 g inoculum (5% by weight) from the previously produced BSG and AP submerged cultures (section 3.3.1.) was added. The matrix, as mentioned, was composed of both matrices of interest (BSG/AP), specifically, the 85 g placed in each bag consisted of 60 g of fresh BSG as is and 25 g of dry, crushed AP (filtered through a 1 mm^2 mesh filter).

The prepared systems were left in the growing chamber at a controlled temperature and humidity (27 °C, 70% humidity), three of which were placed under illumination conditions (blue and red, 100 lx) and the rest were grown in dark conditions. For this, two plastic containers (8 L), each containing three systems placed on metallic support and a beaker containing 50 mL of distilled water. One plastic container placed under illuminated conditions, the other was left in the dark. Both containers were not completely closed to favour air recirculation (Fig. 38).

Fig. 38 Plastic containers photographed at time zero, before being placed in the appropriate chamber.

3.3.2.1. Preparation of samples for the analysis

After twenty days of fermentations, the systems were characterised. For each system, 10 g of sample were taken and placed in beakers (Fig. 39) in which they were subsequently mixed in a 1:3 *w/v* proportion with distilled water; the samples were then homogenised at room temperature using a magnetic stirrer for 1 h at 1000 rpm. While the samples were being homogenised, an abiotic sample was created by introducing 10 g of the starting matrix and 30 mL of sterile distilled water into a beaker (maintaining the 1:3 *w/v* dilution ratio).

Due to the high viscosity of the system, the abiotic system was further diluted with 20 mL of water before the acidity titration was carried out, resulting in a final dilution of 1:5; it should be noted that this dilution only occurred for this type of analysis, in all

other analyses this system maintained the dilution ratio of 1:3.

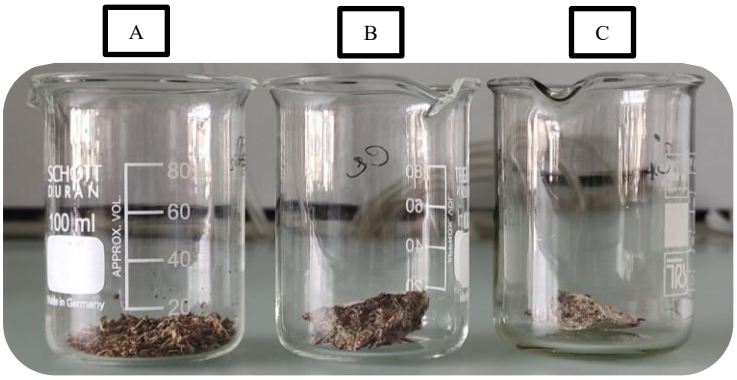

Fig. 39 A) Starting matrix; **B)/C)** Tempeh samples were fermented for 20 days**.**

3.3.3. Growth of *Lentinula edodes* in race – tubes (dark/light conditions)

Lentinula edodes was inoculated inside test tubes with the aim of fructification and obtaining the macrofungus (fruiting bodies) inside the tube (Fig. 40).

To this end, six test tubes (200x30 mm) were prepared, each containing approximately 46 g of BSG, dried and crushed (sieved through a 1 mm^2 mesh filter); the obtained systems were autoclaved and at the end of sterilization the substrate was rehydrated by inserting 70 mL of sterile distilled water in each race tube. Next, a small inoculum of *Lentinula edodes* (0.172 g) mycelium from the previously produced MEA Petri dish were inoculated into each tube (see section 3.1.3.1.2.). Each tube was then sealed with sterile cotton and parafilm, three of which were placed under illuminated conditions (red and blue light, 100 lx) and the other three were left in the dark, but all in temperature- and humidity-controlled chambers (27 °C, 70% humidity).

Fig. 40 *Lentinula edodes* fructified in race – tubes
(adapted from (Philippoussis, Diamantopoulou and Zervakis, 2002)).

4. Results and discussion

4.1.1. Growth of *Chorella vulgaris* in liquid phase in natural light conditions

4.1.1.1. Initial inoculum growth on Sueoka medium

The initial cultures of *Chlorella vulgaris* were allowed to grow to ambient temperature and pressure and under natural light. Over time, there was an increase in the intensity of the green colour of the cultures as is visible in the photos below (Fig. 41), as well as in the microscope images, shown below, obtained by analysing a sample from flask C (Fig. 42). From this factor, it was deduced that the cultivation conditions adopted were suitable for the growth of microalga biomass.

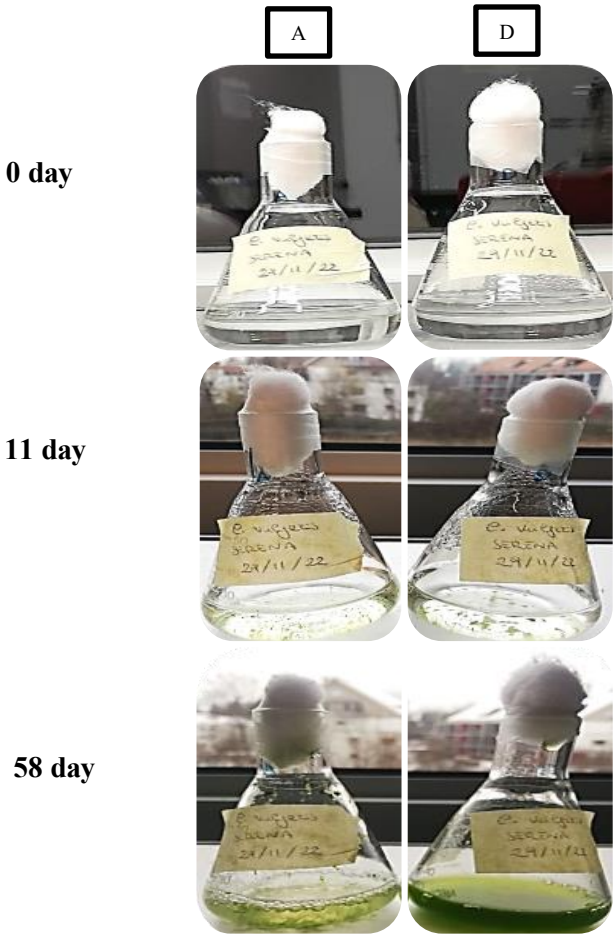

Fig. 41 A) System without modified medium (control system);
D) System with the modified medium.

0 day

41 day

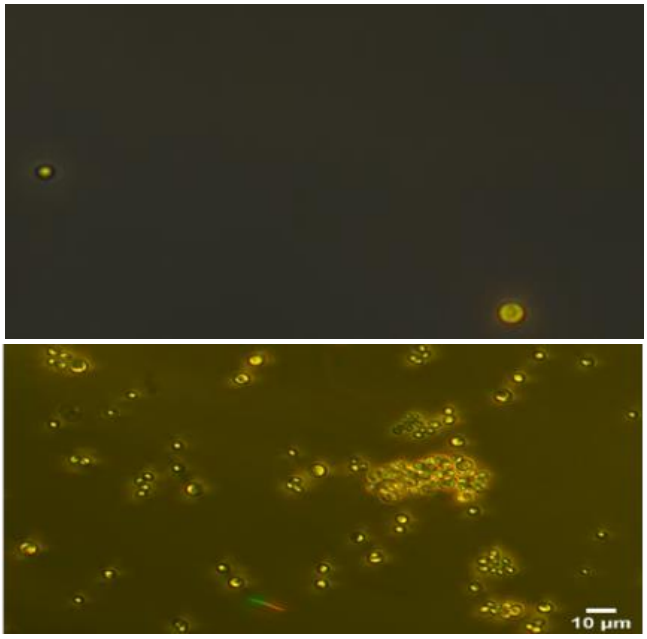

Fig. 42 Sample taken from the culture of flask C.

4.1.1.2. Sub-cultivation

The sub–cultures of *Chlorella vulgaris* were allowed to grow to ambient temperature and pressure and under natural light. Over time, there was significant increase in the intensity of the green colour, clearly visible in the photos below (Fig.43), from this factor, it was deduced that the cultivation conditions adopted were suitable for the growth of microalga biomass.

0 day

43 dav

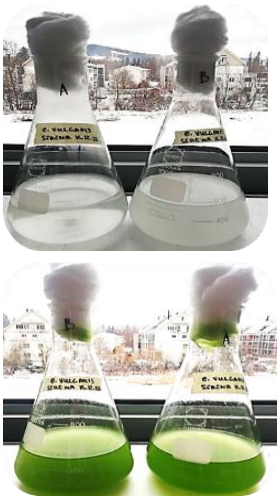

Fig. 43 Sub – cultures of *Chlorella vulgaris*.

4.1.2. Growth of *Rhizopus oligosporus* on a chickpeas matrix in different microcosms types in dark/light conditions

4.1.2.1. Respirometry in microcosms tests (single culture) with a free chickpeas matrix (dark conditions)

The first microcosm systems of *Rhizopus oligosporus* (single culture), were left to grow for 41 days. The growth of the fungal biomass over this period was visible as shown in the photos below (Fig. 44). It could be seen the mycelium extending and colonizing the matrix, which acquired the characteristic white colour, compared to the abiotic systems in which growth was not observed.

Fig. 44 Microcosms tests of *R. oligosporus* (single culture) with a free chickpea matrix.

To evaluate the fungal growth in the systems over time, respirometry within them was assessed by acid-base titration, stopped at 41 day because in this period the stationary state was. The results of the respirometry were normalized to the amount in grams of total solids (contained in the chickpea matrix), using as reference value of $TS_{fresh\ chickpeas}$=88.99% found in the literature (Amer Eissa *et al.*, 2010).

In order to better analyse the growing dynamics through the CO_2 measurements, the obtained respirometry data was fit to a first-order function. The experimental values and the fitted models are for the biotic systems C and D and their parameters are shown in the following graph (Fig. 45) and table (Tab. 6) respectively:

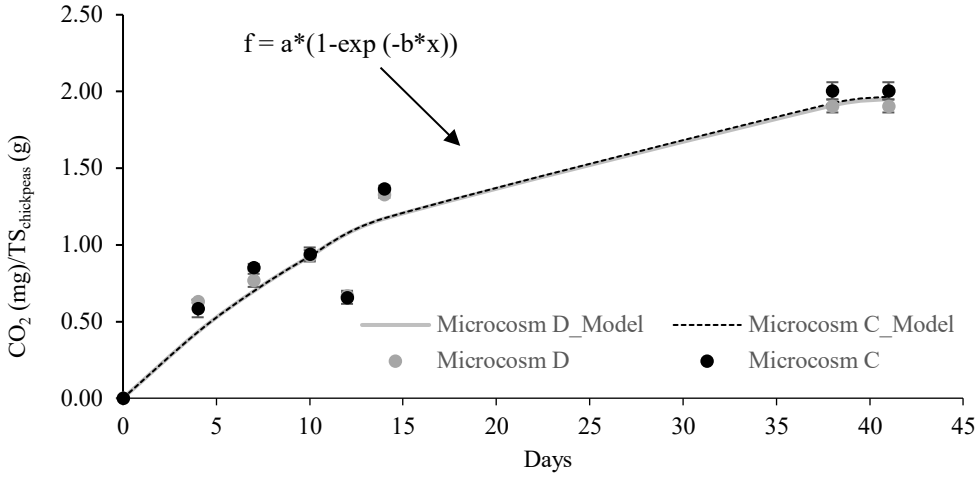

Fig. 45 Cumulative $CO_2/TS_{chickpeas}$ curves produced in systems C and D.

Table 6. Parameters of the first-order function with which the respirometry values, normalized for $TS_{chickpeas}$, of systems C and D were parameterized.

	Microcosm_C	**SD**	**Microcosm_D**	**SD**
a	2.21*	0.03	2.18*	0.03
b	0.05*	0.00	0.06*	0.00

The cumulative curves of the total amounts of $CO_2/TS_{chickpeas}$ (mg/g) produced in biotic systems C and D show a similar trend over time, in the first 40 days for both microcosms there is an exponential phase of CO_2 production, indicating exponential growth of the microorganism, followed by a stationary phase; the variation in the slope of the curve between days 12 and 14 could be attributable to the saturation of the NaOH trap solution present in the systems, which was then replenished.

The maximum carbon dioxide yield, expressed in milligrams per gram of total solid of the chickpea matrix is indicated by parameter a, while the rate at which this value is reached is indicated by parameter b. A t – test of the parameters a and b corresponding to the two systems was performed and, as can be seen from table 6 (see *), for both parameters the two systems present similar behaviour (statistically).

4.1.2.2. Respirometry in microcosms tests (single/co-culture) with contained chickpeas matrices (in perforated plastic bags) with/without entrapped microalgae (dark/light conditions)

The microcosm analysis systems of *Rhizopus oligosporus* (single culture) and *Rhizopus oligosporus* and *Chlorella vulgaris* (co – culture) were left to ferment in a temperature- and humidity-controlled chamber (27 °C, 70% humidity) in dark/light conditions for 10 days. The growth of the fungal biomass during this period was not very visible (contrary to the case with free matrix, section 4.1.1.1.), as shown in the photos below (Fig. 46). This can probably be due to the type of set-up used, which, despite

recirculating air every 24 or 48 h, did not allow good aeration of the systems and was not adequate to allow the fungal biomass to grow and completely colonize the matrix with mycelium (due to the presence of the additional barrier, the plastic bags where the substrate matrix is contained). For this reason, the experiment was stopped once the systems had reached a stationary state (in terms of CO_2 respirometry).

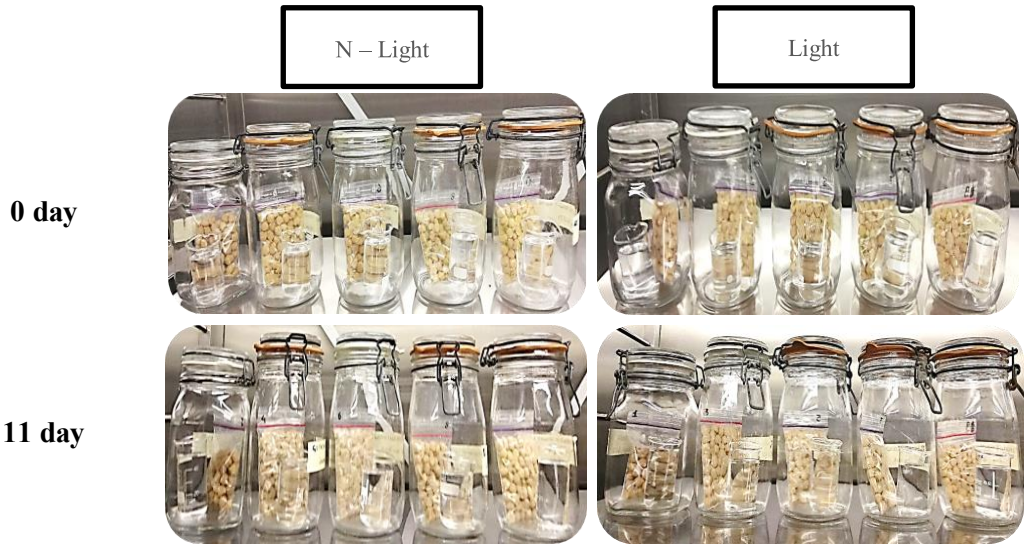

Fig. 46 Microcosms tests of *Rhizopus oligosporus* with contained chickpeas matrices (in perforated plastic bags) with/without entrapped microalgae (single/co-culture).

The respirometry results were also normalized to the amount in total solids of chickpea used (as in the previous case, see section 4.1.1.1.), considering a total solids (TS) a percentage of 88.99%, as reported in the literature (Amer Eissa *et al.*, 2010).

The obtained experimental values were fitted to a first-order function; the trends of the carbon dioxide production/TS of chickpeas (mg/g) for the systems and the parameters of these curves are shown in the following graph (Fig. 47) and table (Tab. 7), respectively:

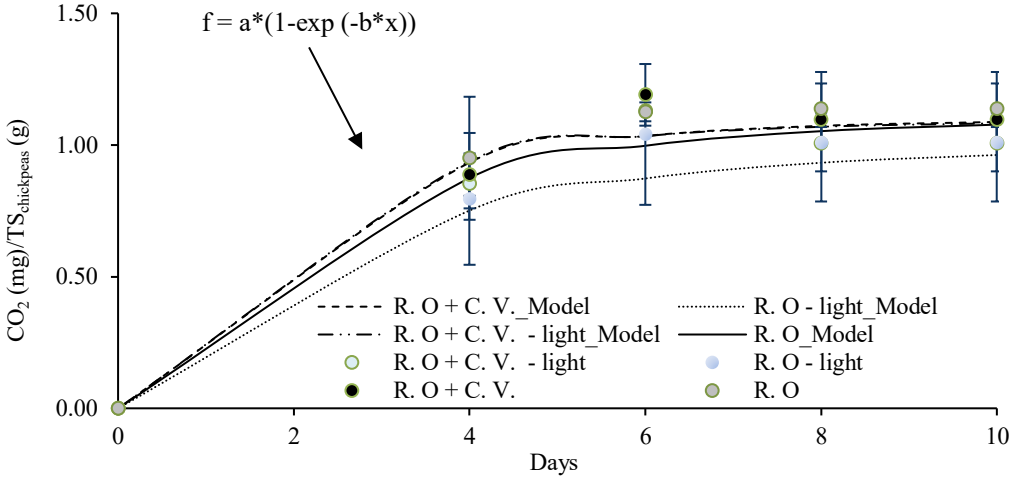

Fig. 47 Cumulative $CO_2/TS_{chickpeas}$ curves produced in systems.

Table 7. Parameters of the first-order function with which the respirometry values, normalized for $TS_{chickpeas,}$ of systems were parameterized.

	R. o. - Light	SD	R. o. - Dark	SD	R. o. + C. v. - Light	SD	R. o. + C. v. - Dark	SD
a	0.99*	0.10	1.10*	0.03	1.09*	0.05	1.10*	0.01
b	0.36*	0.05	0.40*	0.02	0.49*	0.00	0.48*	0.02

The cumulative curves of the total quantities of $CO_2/TS_{chickpeas}$ (mg/g) produced in the systems show a similar trend over time; in the first 4 days for all four types of microcosms, there is an exponential phase of gas production, from which it can be deduced that the microorganism grows exponentially in each condition in which it is placed, followed by a stationary phase.

Looking at the values of parameter a, which refer to stationary values, it can be seen that a very similar average value is reached in all four types of systems; slightly higher for microcosms in dark conditions where only the fungus is present. The average value of $CO_2/TS_{chickpeas}$ (mg/g) in these systems is 11.41% higher than that obtained for the same types of microcosms under light conditions, and with the same percentage difference, there is a higher carbon dioxide production for total chickpea solids than for systems also under light conditions but in which the microalga is also present.

It should also be emphasized that the light source might contribute to the growth of the microalga. It is then consistent to find, in co-culture microcosms placed under dark conditions, an average amount of carbon dioxide per total chickpea solids that is 8.19% higher than in the same types but placed under light conditions.

The CO_2 production rate, indicated by parameter b, is similar in all four types of systems, being slightly higher in systems in which both the fungus and the microalga are present and which are subjected to illumination conditions. The b-parameter obtained for these microcosms differs by a minimum value of 0.01 compared to the b-parameter obtained in systems of the same type but placed under dark conditions, and by a maximum value of 0.13 compared to that which characterizes, on average, microcosms with only the presence of the fungus placed under the same light conditions.

It can be deduced from these results that a good synergy between *Rhizopus oligosporus* and the entrapped microalgae is not established under these growth conditions. Even under the most favourable conditions for microalgae, i.e. those in which a light source is present. In fact, if one considers the microcosm under light conditions in which the microalgae is present and compares it with microcosms in which only *Rhizopus oligosporus* is present, both of which are present and not under the same light conditions, the percentage differences in carbon dioxide produced vary from a minimum of 0.00% to a maximum of 11.41% respectively. The effect of light was also negligible, as it seemed not to improve the growth of fungal biomass in the presence of the microalga but rather to restrict fungal growth in its absence, where a lower steady-state value was reached and at a slower rate than in the same types of microcosms but placed in dark conditions.

A static analysis of the corresponding parameters a and b of the systems was also performed, and as can be seen from table 7, for both parameters the systems do not appear to be statistically different from one another.

4.1.2.3. Microcosm types compared

The most important aspect to be considered when comparing the different microcosms is the different type of setup used to conduct the experimental trials. In the first experiments, the inoculated chickpea matrix was free (simply placed at the bottom of the jar), while for the second the contained matrix setup in plastic bags was evaluated. This aspect is extremely important because the different set-ups influence the output of the microcosm studies.

The free-matrix systems allowed the experimental test to be carried out for a much longer period than the others (as the growth was visually evident) and higher yields of $CO_2/TS_{chickpea}$ (mg/g) were obtained. However, it is interesting to note that after 10 days, as shown in the graph (Fig. 48) and table (Tab. 8) below and in the graph (Fig. 47) and table (Tab. 7) above, irrespective of the type of matrix (free or contained), a value of $CO_2/ TS_{chickpea}$ (mg/g) around unity is reached, which is slightly higher for the systems grouped under the 'trapped' matrix type. In fact, considering the systems with the highest yield of this parameter for both types, the system with the trapped matrix (microcosm under dark conditions and without entrapped microalgae) shows a value of this parameter 15.70% higher than that recorded by the system of the other type (microcosm C). The rate of CO_2 evolution is also similar between the two types of microcosms, being slightly lower for those with a 'free' matrix. In fact, if we consider the characteristic parameter b for the fastest system of this type (microcosm D), it is lower by a factor of 0.23 than that recorded for the fastest system of the other type (microcosm under light conditions and in the presence of entrapped microalgae).

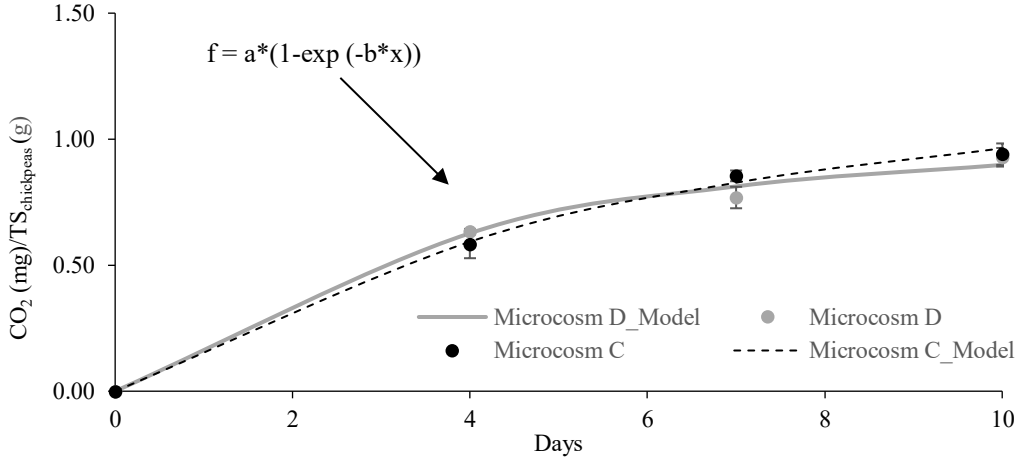

Fig. 48 Cumulative $CO_2/TS_{chickpeas}$ curves produced in systems C and D in the first 10 days.

Table 8. Parameters of the first-order function with which the respirometry values obtained in the first 10 days, normalized for $TS_{chickpeas}$, of systems C and D were parameterized.

	Microcosm_C	**SD**	**Microcosm_D**	**SD**
a	1.15*	0.04	0.97*	0.03
b	0.18*	0.01	0.26*	0.03

From these results, it is possible to hypothesise that although the two types of microcosms are characterised by configurations that allow for different aeration of the systems and thus different growth of the fungal biomass, the two types of microcosms have the same yield of carbon dioxide produced per gram of total chickpea solids used, with comparable rates of carbon dioxide production. This consideration is reflected in the statistical treatment carried out on the parameters a and b of the different systems, as shown in the tables 7 and 8, there is no statistical evidence (significance) that the systems exhibit different steady-state values and rates carbon dioxide production.

4.1.3. Growth of *Lentinula edodes* in Petri dishes (MEA/MFC) in dark/light conditions

4.1.3.1. Mycelium extension rate on plates (MEA/MFC)

Petri dishes (MEA/MFC) of *Lentinula edodes*, placed in a temperature- and humidity-controlled chamber (27 °C, 70% humidity) and under dark/light conditions, were analysed for 11 days. During this period, the mycelium gradually extended, as can be seen in the photos below (Fig. 49), before reaching the edges of the dishes.

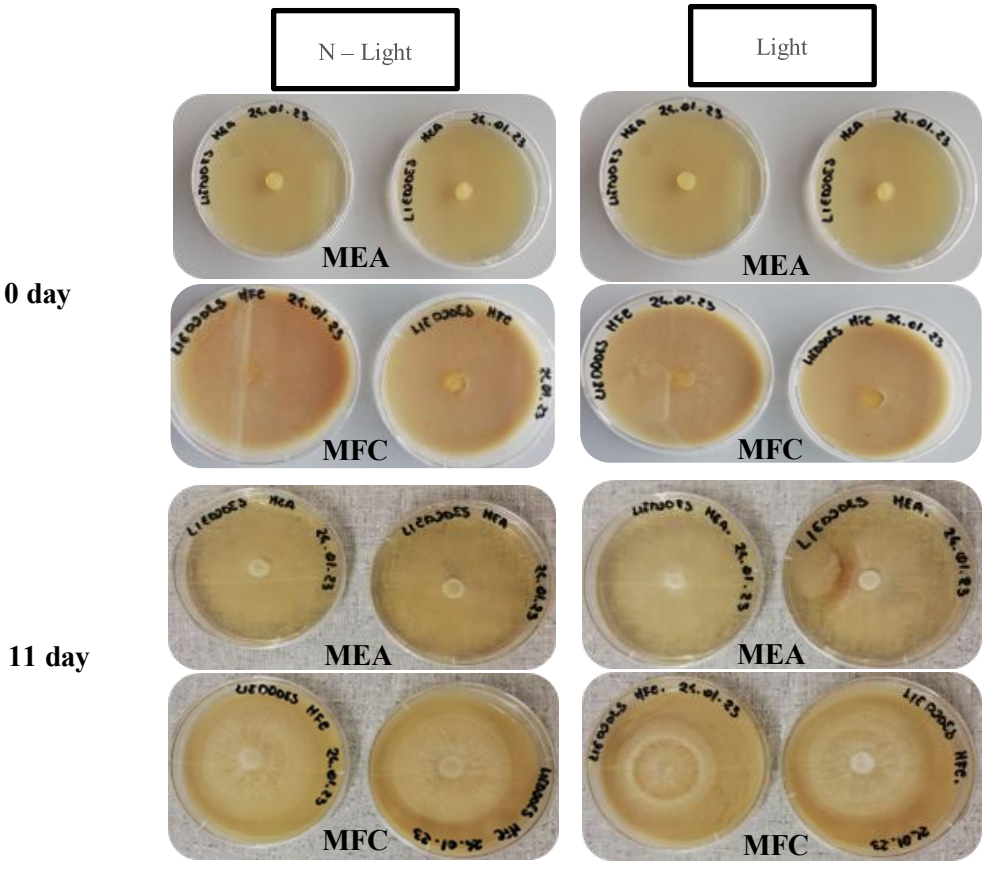

Fig. 49 *Lentinula edodes* in Petri dishes (MEA/MFC).

The average radius values obtained over time for the different systems were parameterized using a three-parameter Gompertz function; mycelium extension curves were then created for each system and the corresponding parameters were tabulated.
Considering the MEA medium, under dark/light conditions, the experimental radius values trend and the corresponding fitting are shown in the following graph (Fig.50) and table (Tab. 9):

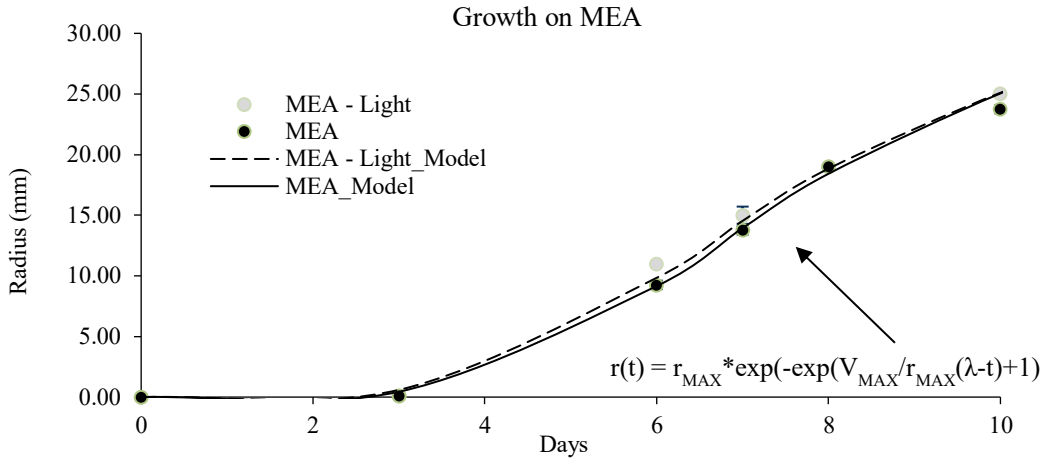

Fig. 50 Extension curves of the mycelium of *Lentinula edodes* on MEA.

Table 9. Parameters of the Gompertz function used to parameterize the rate of mycelium extension on MEA.

	MEA - Dark	**SD**	**MEA - Light**	**SD**
r_{MAX}	31.48*	1.24	31.86*	0.11
V_{MAX}	28.48*	4.17	28.37*	0.40
λ	2.28*	0.18	2.19*	0.02

On the MEA medium, under light conditions, the microorganism reached higher radius values than under dark conditions, although the difference is not very significant, it is only 0.08 mm. Furthermore, in the presence of light, the growth rate is slightly lower than in the absence of the light source and with a slightly reduced lag phase. The obtained parameters for both conditions, light and dark, were subjected to statistical treatment, from which the lack of significance between the two conditions is confirmed.

Considering instead the MFC medium, under dark/light conditions, the radius growth trend and the corresponding parameters are shown in the following graph (Fig. 51) and table (Tab. 10):

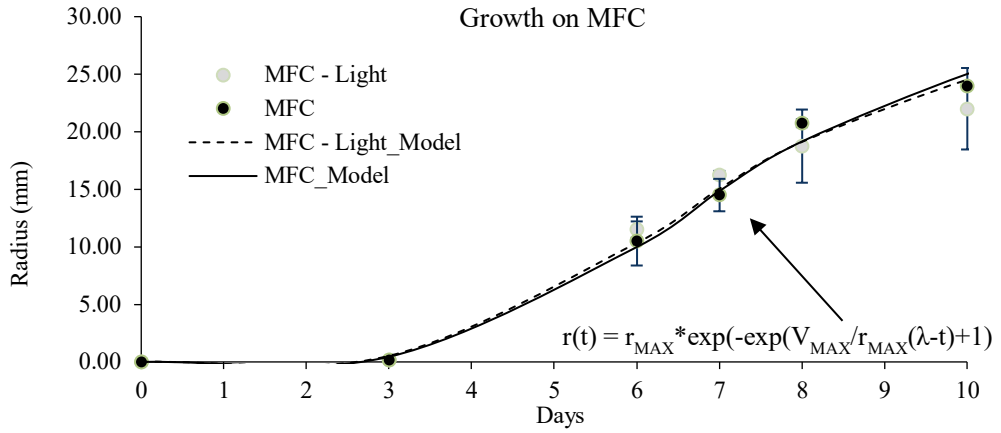

Fig. 51 Extension curves of the mycelium of *Lentinula edodes* on MFC.

Table 10. Parameters of the Gompertz function used to parameterize the rate of mycelium extension on MFC.

	MFC - Dark	**SD**	**MFC - Light**	**SD**
r_{MAX}	30.12*	0.13	29.04*	0.91
V_{MAX}	24.82*	0.80	23.61*	1.16
λ	2.33*	0.04	2.29*	0.04

On the MFC substrate, the effect of light is opposite to that in the presence of MEA concerning the maximum mean value obtained. In this case, the maximum mean value obtained under dark conditions is slightly higher than that obtained under light conditions, with a difference between the two values of 1.50 mm. As regards the rate of mycelium extension and the lag phase, the effect of light is similar to that recorded in the presence of MEA. In fact, under light conditions, the maximum value of the mean mycelial radius is reached with a slightly lower rate and lag phase than under dark conditions. Furthermore, the parameters obtained for both conditions, light and dark, were subjected to statistical treatment, from which the lack of significance between the two conditions is evident.

4.1.3.2. Comparison of the two growth media (MEA/MFC)

Comparing the MEA and MFC substrates and considering the best conditions for both types of substrate, the average radius obtained under dark conditions on MEA is only 3.36% higher than the average radius obtained, under the same conditions, on MFC. Furthermore, considering both conditions, light and dark, the two substrates exhibit comparable growth rates and lag phase. The lack of significance between the parameters of the different systems is evident from the statistical treatment carried out on them. As can be seen from the tables (Tab. 9 and Tab. 10) above, all the case studies are not statistically different from each other in terms of the average maximum radius obtained, the rate and the lag phase with which it is obtained that characterise each system.

4.2. Complex (BSG/AP) matrices analysis

4.2.1. Characterization of Brewers' spent grains (BSG)

In regard to the characterisation of the BSG used in the experiments, the moisture content and total solids obtained are given in table 11, while the pH value obtained from the analyses conducted is given in table 12 (see also (Manzini, 2021) and (Piccolo, 2021)).

Table 11. Results of the gravimetric method.

BSG	Total solids, TS (% w.t)	Moisture content, MC (% w.t)
Average	30.29	69.71
SD	2.10	2.10

Table 12. Results of the pH analysis.

Ground dry BSG	
pH	5.90
SD	0.05

4.2.2. Characterization of Apple pomace (AP)

The characterisation of the AP used in the experiments, the moisture content and total solids obtained are given in table 13, while the pH value obtained from the conducted analyses is given in table 14 (see also (Sato *et al.*, 2011)).

Table 13. Results of the gravimetric method.

AP	Total solids, TS (% w.t)	Moisture content, MC (% w.t)
Average	95.35	4.65
SD	1.65	1.65

However, it should be emphasized that the AP used had already been partially dried; as can be verified in the literature, the total solids content of fresh AP is about 20%, while the corresponding moisture content is 80% (Fernandes *et al.*, 2021).

Table 14. Results of the pH analysis.

Ground dry AP	
pH	3.72
SD	0.05

4.3.1. Submerged fermentation of AP and BSG with *Rhizopus oligosporus* and *Lentinula edodes* with and without *Chlorella vulgaris* (dark conditions)

The subcultures were left to grow in an incubator shaker (25°C, 100 rpm) for 13 days. As can be seen from the photos below (Fig. 52 and Fig. 53), at the end of this period, all systems showed an increase of fungal biomass and more or less extensive areas of mycelium that circularly covered the walls of the flasks, due to the agitation to which they had been subjected.

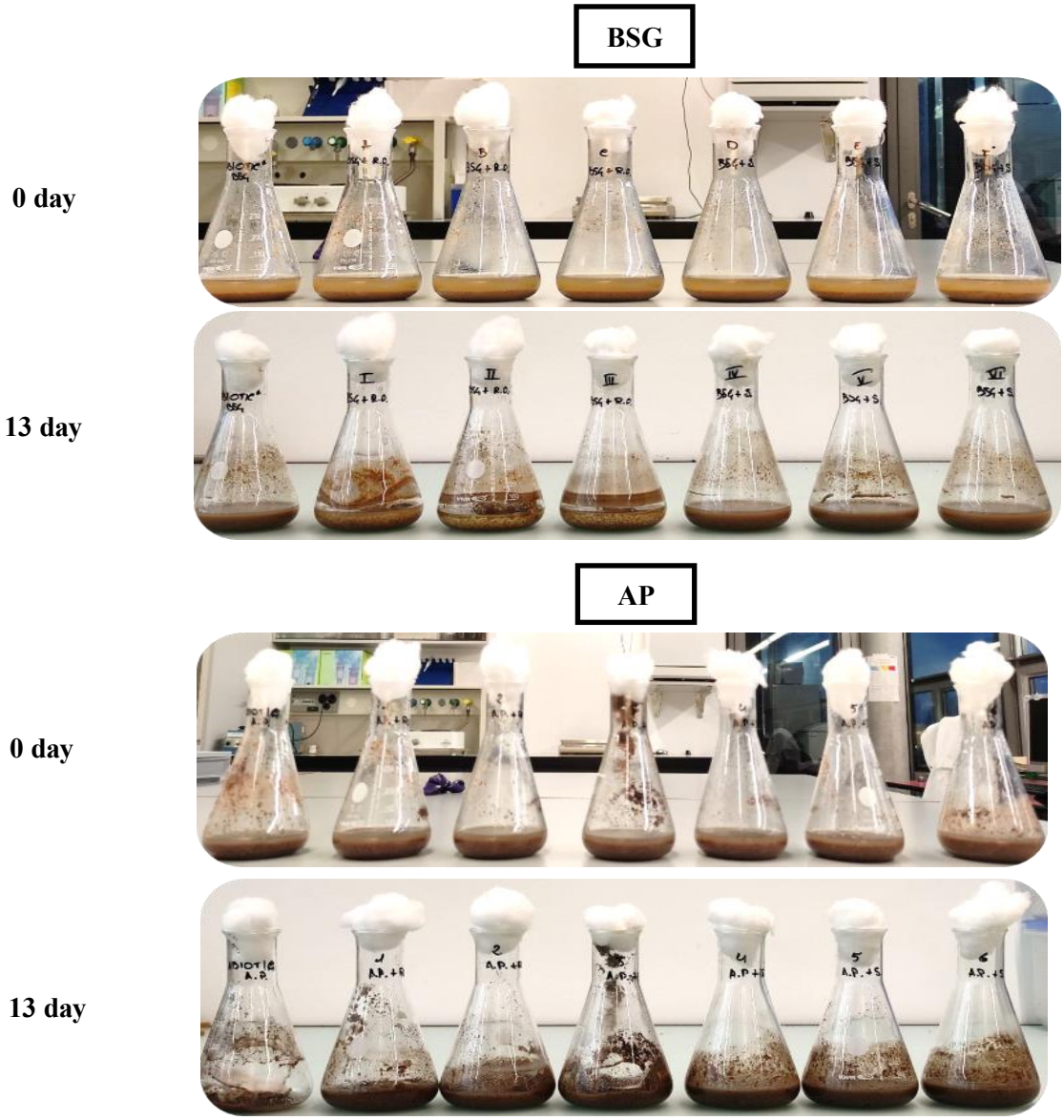

Fig. 52 Fungal subculture on BSG/AP.

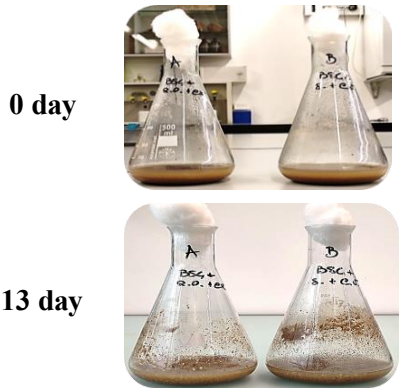

Fig. 53 Fungal subculture with the presence of entrapped microalgae on BSG.

Some of these subcultures were also observed under the microscope, including one of *Rhizopus oligosporus* on the BSG matrix and one of *Lentinula edodes* on BSG; photos taken of these two systems are shown below (Fig. 54):

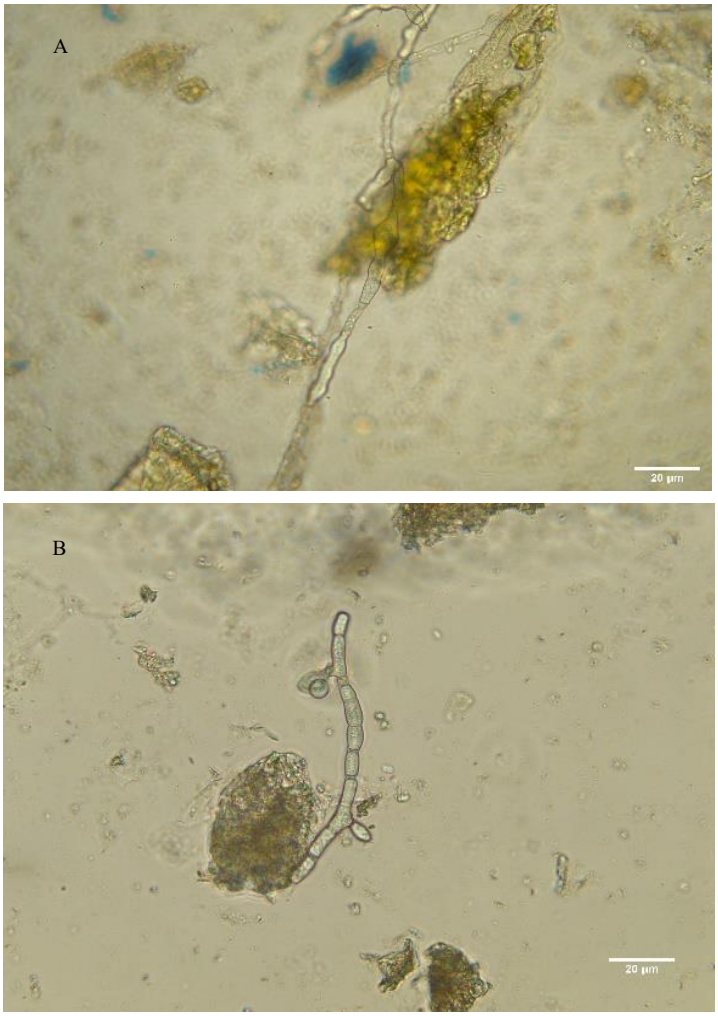

Fig. 54 A) *Rhizopus oligosporus* on BSG; B) *Lentinula edodes* on BSG.

4.3.1.1. Moisture, total solids, electrical conductivity

In this type of submerged fermentation, due to the non-hermetic nature of the systems and the agitation to which they have been subjected, as can be seen in the graph below (Fig. 55) evaporation tends to occur (McDaniel and Bailey, 1969).

In the case of *Rhizopus oligosporus* on BSG matrix, both in the presence and absence of microalgae beds, the total solids content in the culture broth decreases by an average of 4% compared to the abiotic broth consisting of the same starting matrix; the same phenomenon is found in the systems of *Lentinula edodes* on BSG, where the total solids content decreases by an average of 3%. The variation is also consistent with the fungal growth, which hydrolyses the substrate, consuming water, hydrolyses organic matter and releasing a fraction of it as CO_2.

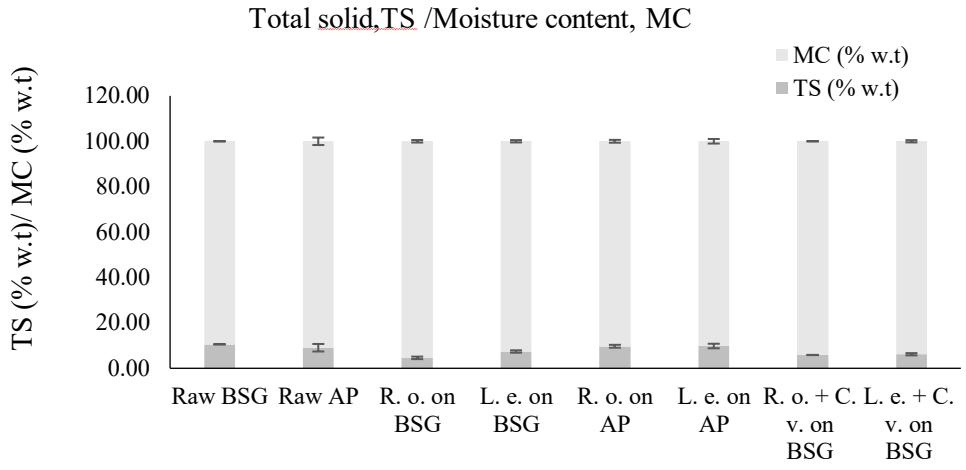

Fig. 55 Comparison of moisture content/total solids parameter in subcultures.

The rheological properties of the broths are determined in particular by the concentration and morphological state of the mycelium, which are in turn influenced by the operating conditions (Fazenda *et al.*, 2008).

The change in conductivity of the systems with respect to the starting matrix is shown in the following graph (Fig. 56). A slight decrease in this parameter is observed in each system, with small differences between the systems depending on the fungal inoculum and the matrix. While the growth of *Lentinula edodes* leads to a decrease in this parameter of 34% on the BSG matrix and 42.74% on the AP matrix, the growth of *Rhizopus oligosporus* leads to a similar percentage reduction of approximately 25% on both matrices. Even in the presence of microalgae beds, the two fungi lead to a different percentage change in conductivity, which is lower in the case of *Rhizopus*, 7.85%, and higher in the case of *Lentinula*, 27.95%.

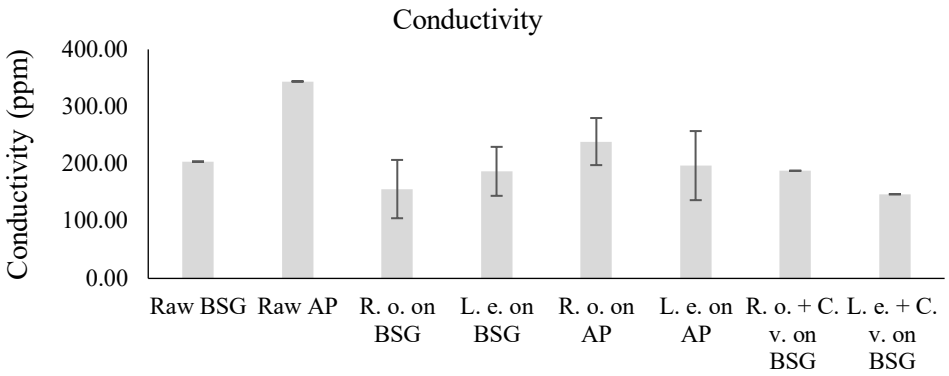

Fig. 56 Comparison of conductivity parameter in subcultures.

4.3.1.2. pH and titratable acidity measurements

As shown in the graph below (Fig. 57), all systems presented a pH decrease. A possible explanation for this increase in acidity can be found in the presence within the cultures of acidic compounds, such as organic and fatty acids, from the two matrices of BSG and AP used. ((Castro and Colpini, 2021); (Antonic *et al.*, 2020)). In systems with BSG, for *Rhizopus oligosporus* and *Lentinula edodes*, a decrease in pH relative to the starting matrix was obtained from 5,90 ± 0,01 to 3,84 ± 0,25 and from 5,90 ± 0,01 to 3,87 ± 0,22, respectively. While in the presence of AP, the pH value with respect to the starting matrix decreased from 3,72 ± 0,01 to 1,61 ± 0,10 and from 3,72 ± 0,01 to 1,59 ± 0,19 for *Rhizopus oligosporus* and *Lentinula edodes*, respectively. In the presence of entrapped microalgae, the pH decrease from 5,90 ± 0,01 to 4,78 ± 0,01 and from 5,90 ± 0,01 to 4,19 ± 0,01 in the presence of *Rhizopus* and *Lentinula*, respectively. The lower value of this parameter compared to that obtained for both microorganisms fermented on BSG, but in the absence of the microalgae, is probably justified by the lower presence of carbon dioxide within the systems in which the fungus and microalgae are in co-culture compared to those in which the fungus is in monoculture. The marked acidity found in AP systems compared to BSG systems may be because the initial pH of the AP matrix is also lower than that of the BSG.

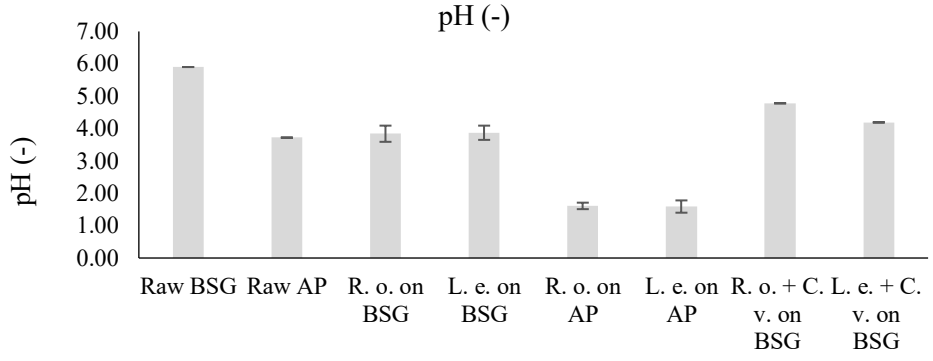

Fig. 57 Comparison of pH parameter in subcultures.

The titratable acidity values obtained for the systems, shown in the following graph (Fig. 58), are consistent with what was observed during the pH analysis. The systems in which the matrix AP was used had a more pronounced acidity than those for the BSG matrix; this is reflected in the volumes of basic solution used to titrate them. In fact, the volumes of NaOH (2 M) used to titrate the samples of the systems characterised by the AP matrix were approximately 33% higher than those used to titrate the samples that had BSG as the medium.

It should also be noted that the volumes of basic solution involved in the acid-base titration of each subculture are very small. They range from a minimum of 0.15 mL to a maximum of 0.3 mL of NaOH (2 M), which, as can also be seen from the graph (fig.), caused the results of some samples to show considerable standard deviations.

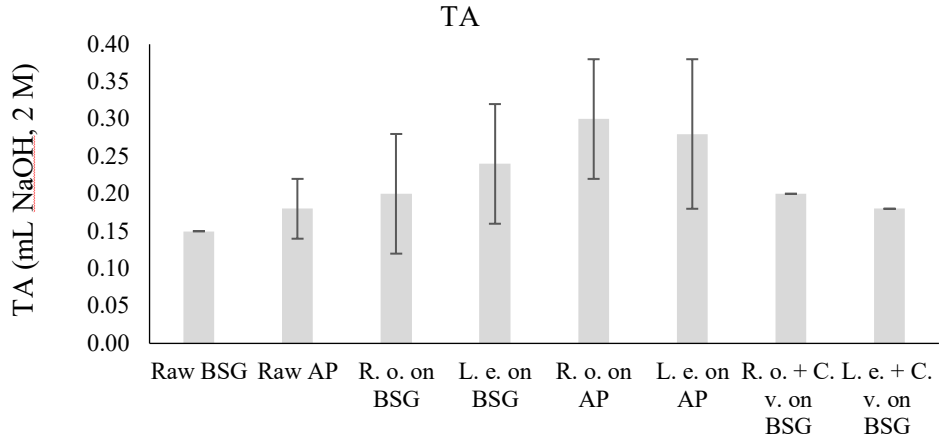

Fig. 58 Comparison of titratable acidity parameter in subcultures.

4.3.1.3. Protein determination

The amount of protein obtained for the submerged systems, shown in the following graph (Fig. 59), was evaluated with different protein standards and protein mixtures, for each of which, therefore, irrespective of the type of calibration line used, an increase in this parameter was observed in the presence of BSG as the culture medium and a decrease in the presence of AP. Regardless of the protein standard considered, values with a considerable standard deviation were obtained for subcultures of *Lentinula edodes* on BSG. A possible justification for this phenomenon can be sought in the possible interference between certain compounds - sucrose, lipids, phosphate buffers, monosaccharides and hexoamines - that are present in these samples and the sensitivity of the method used to analyse them.

Among the three protein standard used: bovine serum albumin (BSA), vanilla whey protein (v-WPI) and chocolate whey protein (c-WPI), the second of these is the one that detected higher amounts of protein in the analysed systems than the other mixtures, especially compared to those obtained using BSA. This is justified by the fact that the vanilla WPI blend, unlike the BSA, is a mixture of proteins and amino acids and

therefore allows a broader spectrum of proteins and amino acids to be detected in the samples, thus being more reliable than the other.

Analysing the results using the v-WPI standard, in the systems using BSG as the culture medium, the amount of measured protein increased from 0.05 ± 0.01 (g/L) to 7.52 ± 0.60 (g/L) in the case of *Rhizopus* and from 0.05 ± 0.01 (g/L) to 24.14 ± 12.98 (g/L) in the case of *Lentinula* compared to the non-fermented system. The protein content also increased in the co-cultures, the difference in the measured protein concentration, was 16.41 ± 0.04 (g/L) and 8.46 ± 0.16 (g/L) for *Rhizopus* and *Lentinula* respectively. Whereas, considering the systems in which AP is used as the culture medium, as mentioned above, there is a decrease in protein content, in fact, the 7.81 ± 0.19 (g/L) experimentally measured on the starting matrix decreases to 6.99 ± 0.18 (g/L) in the presence of *Lentinula* and 6.97 ± 0.15 (g/L) in the presence of *Rhizopus.*

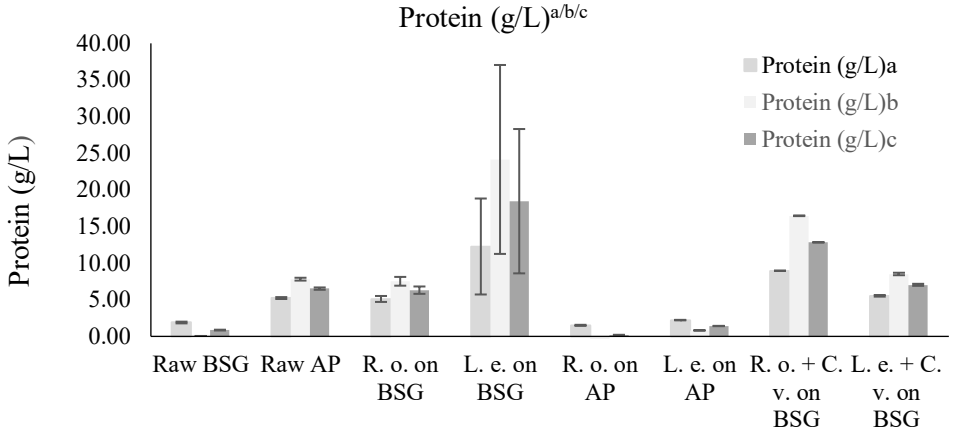

Fig. 59 Comparison of protein quantity in subcultures

([a] BSA calibration line; [b] vanilla whey protein (WPI) calibration line; [c] chocolate whey protein (WPI) calibration line).

4.3.2. Growth of *Rhizopus oligoporus* in perforated bags: innovative Tempeh (dark/light conditions)

The tempeh obtained were left for 15 days in a temperature- and humidity-controlled chamber (27 °C, 70 % humidity), under light or dark conditions. As is evident from the following photos (Fig. 60), for some systems the colonization of the matrix by the mycelium was not complete, even though the systems were incubated for a considerable long period. The reasons why the mycelium was unable to completely colonise the substrate on which it was left to grow, and the considerable time in which it was able to do so completely/partially, are probably to be found in the marked acidity of the AP that made up the matrix. Although representing only 30% by weight of the matrix, the quantity of AP present drastically lowered the pH of the starting matrix, we are talking about a very acidic pH, around 2, thus making the system challenging for the growth of *Rhizopus oligopsorus*. Other possible causes are the small amount of free water present in the matrix (see section 4.3.2.1.) and the small amount of space in the

bag that makes up the system.

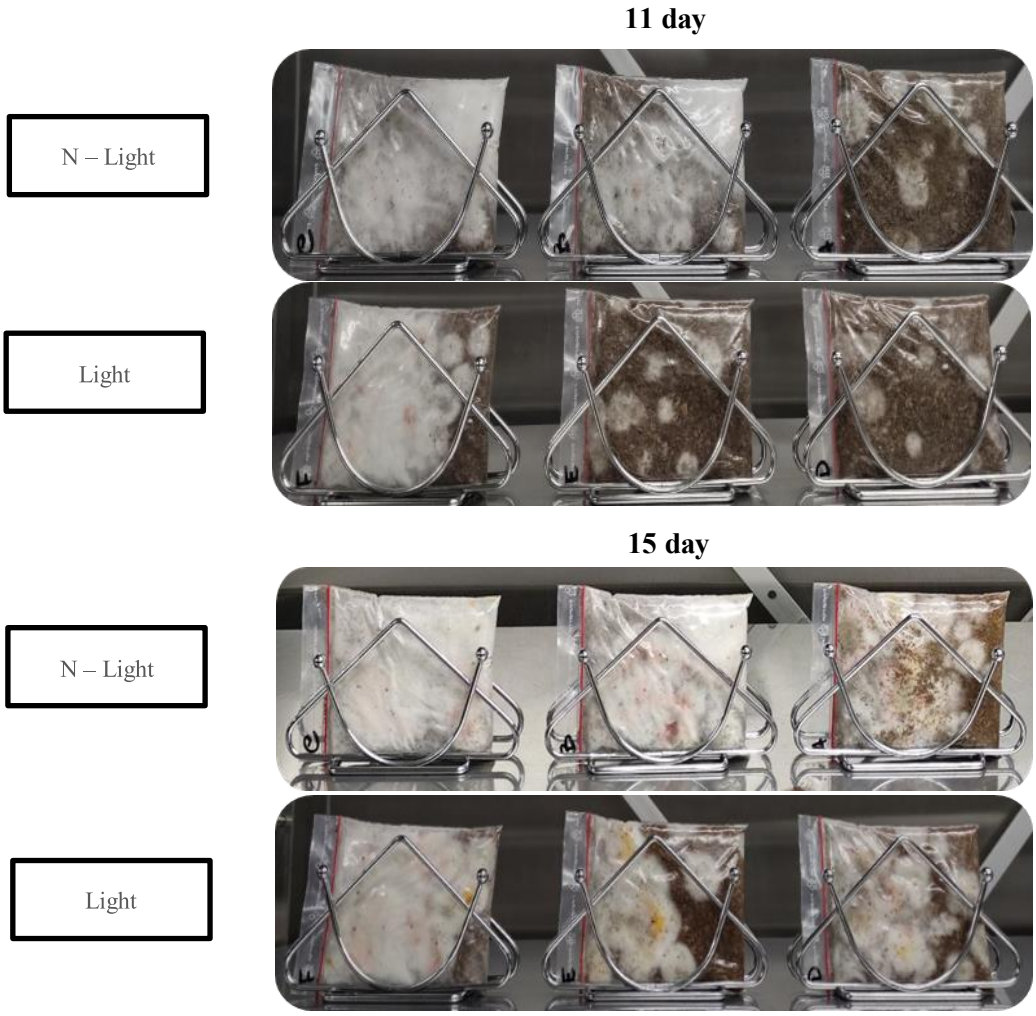

Fig. 60 Tempeh of *Rhizopus oligospur* on the complex matrix (BSG/AP).

4.3.2.1. Moisture, total solids, electrical conductivity

In both tempeh fermentation conditions (dark/light), as can be seen in the graph below (Fig. 61), the combined matrix fermentation with *Rhizopus oligosporus* led to a slight decrease in the percentage of total solids in the system. Starting from a percentage of total solids in the matrix of 75.32 ± 0.07 (% *w.t.*), this parameter decreased by 22.03% in the systems under dark conditions, while in those placed in the presence of the light source, the recorded decrease was slightly less, amounting to at 21.61% (see also (Manzini, 2021)).

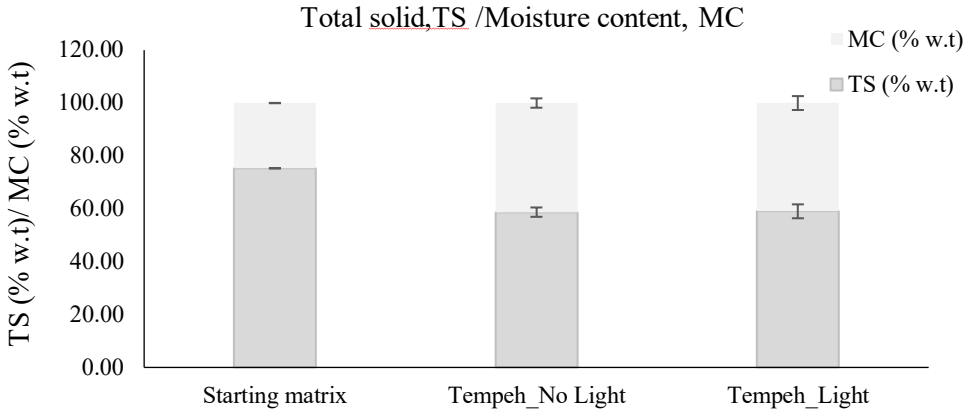

Fig. 61 Comparison of moisture content/total solids parameter in systems of tempeh.

The change in conductivity of the systems with respect to the starting matrix is shown in the following graph (Fig. 62). For both the systems placed in the dark and those left to ferment in the presence of a light source, there is a decrease in the conductivity, which in percentage terms is 39.56% and 40.19% respectively.

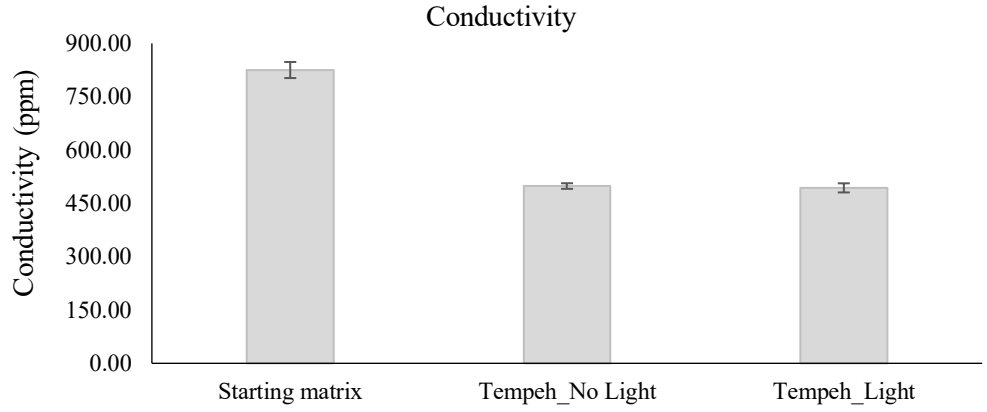

Fig. 62 Comparison of conductivity parameter in systems of tempeh.

4.3.2.2. pH and titratable acidity measurements

During the growth, a change in pH occurs in the systems, as can be seen from the graph below (Fig. 63). Under both conditions (dark/light), an increase of pH occurs (Sparringa *et al.*, 2002). Specifically, the pH rising from 2.18 ± 0.08 of the starting matrix to a final pH of 4.84 ± 0.2 for the systems placed to ferment under dark conditions and to a final pH of 4.94 ± 0.39 for those placed to ferment in the presence of the light source.

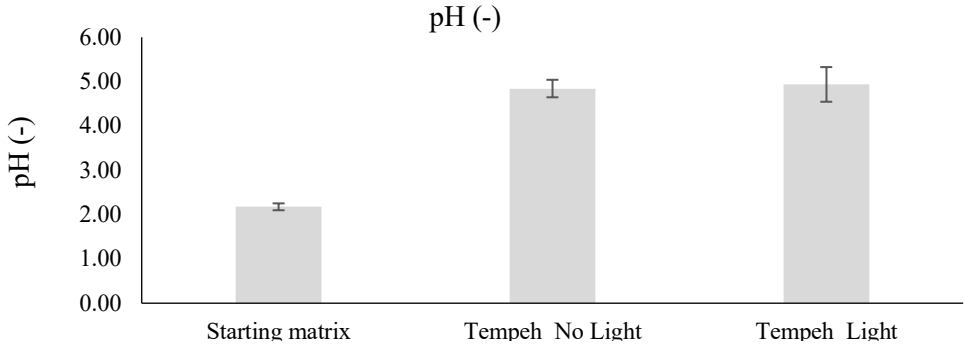

Fig. 63 Comparison of pH parameter in systems of tempeh.

The titratable acidity values found for the systems, shown in the following graph (Fig. 64), are consistent with the pH values obtained. After fermentation, both types of tempeh are characterised by an increase in pH compared to the starting system; this is reflected in the volumes of basic NaOH solution (2 M) involved, in fact the volumes of NaOH used to titrate the tempeh samples are 60.32% lower than the volume used to titrate the starting matrix sample.

It should be noted that also in this case, as with the acid-base titration of the subcultures (see section 4.3.1.2.), the volumes involved in the titration are very small. They ranged from a minimum of 0.15 mL to a maximum of 0.75 mL of NaOH (2 M), which, as can also be seen from the graph (Fig. 64), results in large standard deviation for the results of the starting matrix samples.

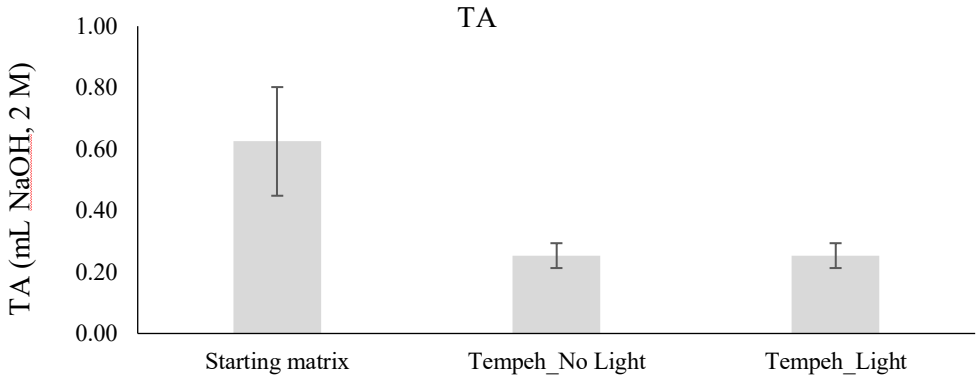

Fig. 64 Comparison of titratable acidity parameter in systems of tempeh.

4.3.2.3. Protein determination

The amount of protein obtained for tempeh, shown in the following graph (Fig. 65), was evaluated with different protein standards and protein mixtures, for each of which, therefore, irrespective of the type of calibration line used, a decrease in this parameter was observed under both fermentation conditions (dark/light conditions).

Of the three protein standard: bovine serum albumin (BSA), vanilla whey protein (v-WPI) and chocolate whey protein (c-WPI), as in the previous case, it was the vanilla whey protein (v-WPI) mixture that detected higher amounts of protein in the analysed systems than the other mixtures.

Taking this mixture as a reference standard, the amount of protein in the systems fermented under dark conditions decreased from 100.76 ± 0.05 (g/L) to 21.70 ± 0.18 (g/L), while under light conditions from 100.76 ± 0.05 (g/L) to 21.16 ± 0.15 (g/L).

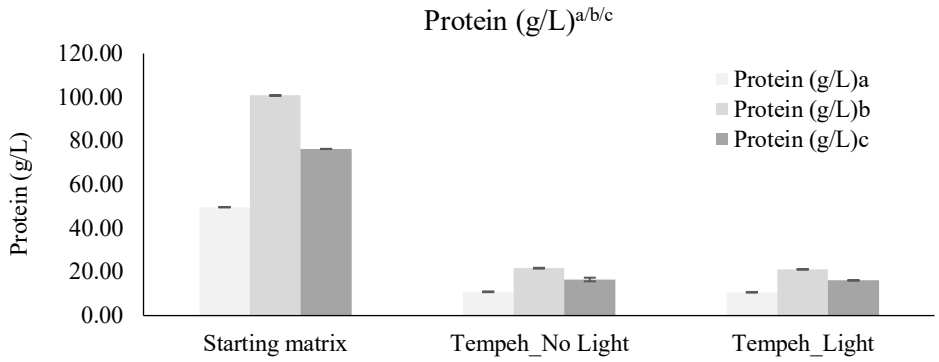

Fig. 65 Comparison of protein quantity in systems of tempeh

([a] BSA calibration line; [b] vanilla whey protein (WPI) calibration line; [c] chocolate whey protein (WPI) calibration line).

4.3.3. Growth of *Lentinula edodes* in race – tubes (dark/light conditions) Error! Bookmark not defined.

The race – tubes of *Lentinula edodes* were left to grow for 50 days. After this considerable time the systems showed complete colonization by mycelium of the exposed surface (on top), but only a hint of colonization of the underlying matrix (Fig. 66). Given the absence of colonization of the entire matrix by the mycelium, the test was interrupted despite the long incubation period, avoiding the heat shock that might have accelerated the fructification process and subsequent characterization experiments.

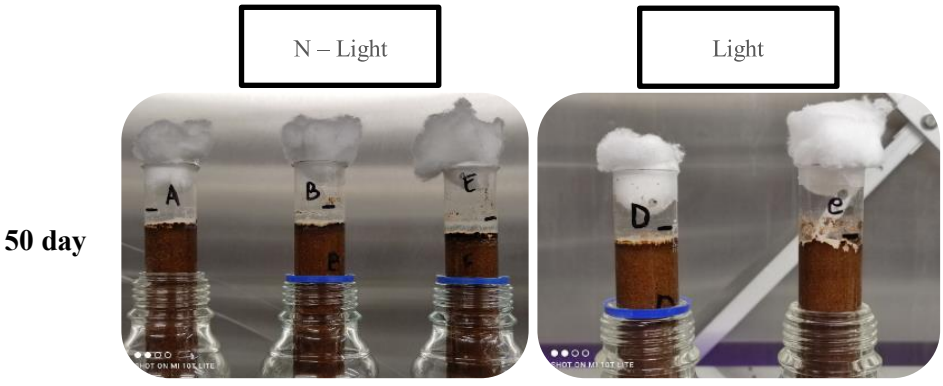

Fig. 66 *Lentinula edodes* fructified in race – tubes.

5. Conclusions and Future Outlook

The objective of this study was to valorise, from a sustainability perspective and following biotechnological approaches, two important by-products of the food industry, BSG and AP, using biosafety level 1 -BSL1- microorganisms (i.e. *Rhizopous oligosporus*, *Chlorella vulgaris* and *Lentinula edodes*). The experimental part started with the preparation and the maintenance of these (micro)organisms on synthetic medium and natural substrates of common or innovative use, and ended with the verification of the growth of the microorganisms on the complex matrices to be exploited.

By adopting microcosm setups in which the chickpea matrix inoculated with *R. oligosporus* is free, rather than entrapped inside plastic bags, it is more favourable for mycelial growth and maintenance of this microorganism. In the first case, it is possible to develop and maintain the growth of this fungus for about 45 days, unlike the second case, where the arrest of metabolic activity assessed through respirometric measurements was evinced in 10 days. The adoption of a free matrix as opposed to an entrapped one promotes better aeration within the system. A hermetically sealed system is characterized by poor air circulation, resulting in limited oxygenation. However, it is important to point out that considering a short time interval, ten days, both configurations allow for obtaining the same yields of carbon dioxide per g of total chickpea solids, leading to the conclusion that in this time interval the two systems can be adopted indiscriminately to grow *Rhizopus oligosporus*.

In co-cultures of free *R. oligosporus* and entrapped *C. vulgaris* in alginate beads, no significant synergy was found between the two microorganisms. The yields of carbon dioxide per g of total chickpea solids used were comparable to those obtained in the absence of the microalgae. One possible explanation for this phenomenon could be the difficulties of mass transfer between the interior and exterior due to the limited interface surrounding the microalgae beads and also the decreased metabolic activity typical of entrapped microorganism (Ruggeri et al., 1991). To better understand this aspect and the possibility of improving such microbeads further investigation is needed. Future developments could be to set up co-culture microcosms of *R. oligosporus* and *C. vulgaris* both as free microorganisms and with different inoculum ratio, or to tune the alginate beads preparation modifying certain parameters related to microalgae entrapment (e.g. alginate/microalgae volume ratio and the concentration of Ca^{2+} ions), or the photoperiod adopted (Lam & Lee, 2012). Ascertaining whether an optimal condition of *C. vulgaris*, allows significant synergy with *R. oligosporus* would be of great help in developing innovative applications based on such synergy. For instance, one could try to produce an innovative tempeh, the production improvement of which finds its basis in this synergy and the nutritional improvement of which finds its basis in the adoption of another nutritionally important microorganism, such as *C. vulgaris*.

The presence of a light source (red and blue light, 100 lx) seemed to have limited effects on the growth and mycelial growth rate of the fungus *R. oligosporus*. In the future, an alternative illumination source and a different intensity could be evaluated. Not only to achieve an optimal growth condition but also to improve certain nutritional characteristics of the fungal organism, such as the amount of vitamin D_2 obtainable.

In the development and maintenance of *L. edodes* in Petri dishes under both dark and illuminated conditions (red and blue light, 100 lx), the MFC substrate has proven to be a viable alternative to the classical MEA, in terms of mycelium extension, the rate of extension and the lag phase following the adopted modeling approach. The possibility of using MFC is particularly relevant given its low cost (Schwarze et al., 2022).

An important aspect emerged from the submerged cultures of *R. oligosporus* and *L. edodes*. In this type of fermentation, both microorganisms grew on the AP matrix, despite its marked acidity. This result lays the foundation for possible experiments for future applications, such as the fructification of *L. edodes*, optionally in co-culture with *R. oligosporus*, on an AP matrix. This would probably improve not only the growth and nutritional characteristics of *L. edodes*, but also the added value of the substrate, which could also be a source of nutrients at the end of the fermentation period and, as such, could be reused.

Another important aspect that emerged from the submerged cultures of *R. oligosporus* and *L. edodes* was the amount of protein detected in the slurries. An increase in the amount of protein was observed in all submerged configurations, compared to the initial readings. This could be explained by the fact that a higher fermentative activity (with the production of organic acids) is favoured in this type of culture conditions and that the native high molecular weight complex proteins in these matrices can be hydrolysed to generate a higher availability of shorter peptides in the liquid phase. Furthermore, under these conditions, a higher amount of protein was detected for both microorganisms in the presence of BSG than in AP. A possible explanation may result from the different compositions of the matrices. The amount of protein is higher in BSG, where it varies from 20% to 30% on a dry basis (Lynch et al., 2016) than in AP, where it varies approximately from 2% to 7% on a dry basis (Antonic et al., 2020).

With regard to the protein content found in tempeh, under both dark and illuminated conditions, there was a decrease in the final measured concentration compared to that detected in the starting matrix. A possible explanation for this decrease lies in the fact that, in contrast to submerged configurations, where a greater fermentative activity (with production of organic acids) is preferred, in solid-state cultures there is more preference towards biomass growth, with consumption of organic acids and utilisation of the proteins of the starting matrix (Ahnan-Winarno et al., 2021). A possible future development could be to search for the right percentages of BSG and AP with which to formulate the complex matrix to be fermented, to evaluate possible optimal conditions for obtaining a higher biomass yield in a shorter fermentation time.

In addition to the possible future developments already reported in the previous paragraphs, further prospects lie in improving the fermentation conditions the fruiting system of *L. edodes* in racing tubes.

In summary, it can be stated that the following conclusions emerged from the exploratory tests conducted:

- In long-term microcosms, 45 days, the growth of *R. oligosporus* is favoured on a free chickpea matrix rather than entrapped one. Whereas, with a short period, 10 days, both conditions are equally valid for the growth of this type of fungal culture.
- Limited synergy was observed between *R. oligosporus* and *C. vulgaris* (under the tested experimental conditions).

- Non-significant effects of the light source (red and blue light, 100 lx) on the growth of *R. oligosporus* were ascertained.
- The suitability of the MFC substrate for the development and maintenance of *L. edodes* in Petri dishes, both in dark and light conditions (red and blue light, 100 lx) was confirmed.
- The AP matrix constitutes a suitable substrate for the growth of *R. oligosporus* and *L. edodes*, despite its low starting pH value.
- In submerged cultures of *R. oligosporus* and *L. edodes*, a higher amount (g/L) of hydrolysed protein is measured in the liquid phase in the presence of BSG rather than AP (with the current method).
- Decreased protein content in tempeh using BSG/AP substrate compared with BSG substrate alone.

In conclusion, it can be stated that the BSG and AP matrices are compatible for the cultivation of the investigated fungal species (acting as complex media), but further testing is required. In order to assess the safety of the products elaborated here and to analyse their properties in more detail, so as to understand and evaluate their real applicability as food/integrators in human and animal nutrition. All this bearing in mind not only the added nutritional value, but also the environmental footprint and costs associated with this type of production process when moving to more advanced stages of development of the enhancement strategy.

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