Methods for Filamentous Fungi Biomass Production as Protein-Rich Foods

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***Abstract*: Fungi in various types and forms has been a part of human diet for thousands of years. Today, filamentous fungi are widely used as cell factories in the pharmaceutical, food and beverage industries. The fungal biomass itself is also a valuable product for food as a rich protein source for humans. This review outlines the microfungi used and their respective fermentation production methods. The review focuses on small-scale microfungi production studies using a range of medium and carbon sources.**

***Keywords*: fungal biomass, mycoprotein, microfungi, filamentous fungi, food security, media optimization.**

INTRODUCTION

Currently, the environmental, health and ethical impact of conventional animal industry is substantial and thus needs to be addressed. For example, the global animal agriculture & livestock production sector generates more greenhouse gasses (GHGs) than the global transportation sector's vehicular exhaust emissions combined, thereby potentially contributing to climate change [1, 2].

There also appears to be some concern and scepticisms regarding the possible health risks of processed meat and high cholesterol that comes with diets heavily dominated with such animal products [3, 4, 5].

The potential applications of these fungi are diverse with uses in proteins, enzymes, metabolites, and organic acids. The fungal biomass itself is also a valuable product for food, textile, construction industries. Fungi may also be a source of food ingredients such as vitamins and certain polyunsaturated fatty acids [10, 11].

Based on the size and visibility of the fungi's fruiting body to the eye, they may be macroscopic (e.g., button mushroom), or microscopic fungi (e.g., Fusarium spp.). Some species of fungi, such as *Aspergillus spp.*, *Neurospora spp.*, *Rhizopus* spp., *Fusarium* spp., etc. are categorized as GRAS (generally regarded as safe) by the USFDA and have been used as food for human consumption. Filamentous fungi like *Aspergillus* spp. and *Neurospora* spp. have been used traditionally in some East Asian foods and drinks. *Fusarium venenatum* one such popular edible filamentous fungi mycoprotein marketed under the brand name Quorn.

Depending on the fungal species and cultivation conditions, a filamentous fungal biomass can contain about 50% protein (dry weight). This makes these fungi good candidates for protein production for human consumption [11, 12].

Single-cell protein (SCP) refers to protein derived from cells of microorganisms such as yeast, fungi, algae, and bacteria, which are grown on various carbon sources for synthesis. These microbial proteins are called single cell protein (SCP), although some of these microbes may be multicellular as is the case with filamentous fungi [13, 14]. Microorganisms used for SCP usually contain 40% or more of crude protein by dry weight [13]. The production of SCP takes place in a fermentation process.

This literature review thus presents an overview of the various microfungal production media, and the parameters to mass produce certain fungi that have commercial or medical significance, with an emphasis on edible filamentous fungi and mycoprotein production.

DIFFERENT MEDIA & SUBSTRATES FOR FUNGAL BIOMASS PRODUCTION

Fungal biomass and protein production are influenced by fermentation conditions, parameters, strain viability, inoculum size, duration, nutrient media, etc.

The ideal nutrient media and its composition varies for and may be specific to different fungal species. The commonly used media with broad microfungi fermentation support include, Potato Dextrose Broth, Vogel's, Sabouraud broth, and Czapek Dox broth.

For *Fusarium venenatum*, a study by Fatemeh et al. (2018) used Vogel medium was used for pre culture cultivation, while sugar from dates was used in production medium [16]. Submerged fermentation was carried out in flasks for the first phase, and a benchtop scale STR was used to conduct the submerged fermentation in the next phase. Plackett-Burman Design with factors such as date sugar concentration, peptone, chemical nutrients in the media, temperature, time, shake rate, inoculate size, pH in two levels and Response Surface methodology, etc. were used to determine the fermentation condition for highest biomass and productivity. The

authors reported that an appreciable amount of total protein in first phase and even higher protein yield in the second phase was observed.

The study by Nitayavardhana et al. (2013) investigated *Rhizopus oligosporus* production on vinasse, a residue from the sugarcane-based ethanol industry [20]. For the fungal cultivation 2.5 litre airlift bioreactor was used on 75% (v/v) vinasse at 37°C and pH 5.0 with varying aeration rates. The study found that the fungal biomass yield depended on the aeration rate, and the highest yield was obtained at around 8.04 (gram biomass increase/gram initial biomass) at 1.5 volume$_{air}$/volume$_{liquid}$/min (vvm) which resulted in approximately 50% crude protein in the biomass. Moreover, amino acids contents in the yield were comparable to commercial protein sources for aquatic feeds such as fishmeal.

A paper by Mahboubi et al. (2017) explored the utilisation of dairy wastes for producing edible biomass for humans or as animal feed [21]. *Aspergillus oryzae* and *Neurospora intermedia* were cultivated on various types of dairy waste regarding production of biomass and chemicals such as ethanol and glycerol. For the substrates, dairy products and by-products used were: cream with 48% (w/w) total solids, crème fraiche with 40% (w/w) total solids, commercially produced sour milk containing 10% (w/w) solids, mild yoghurt with 10% (w/w) total solids, cheese whey with 6% (w/v) total solids and sour milk with 11% (w/w) total solids used in both its sterile (autoclaved) and non-sterile (unautoclaved) form. The fungal strains *A. oryzae* and *N. intermedia* were cultivated in different dairy media as well as in semi-synthetic medium with lactose under semi-aerobic conditions. After cultivation, the biomass was harvested from the medium by pouring out the cultivation medium through a fine mesh. The biomass was dried to constant weight in an oven for 24 h at 70°C. Based on the results obtained, the paper presents four main scenarios for using dairy waste to produce fungal proteins. In the first scenario, the fungal process is installed near the facilities responsible for production of the different dairy products in order to handle their waste streams. These installed processes would be focused on conversion of dairy waste to fungal protein for animal/fish or human consumption. A similar situation is proposed in scenario 2, but the vessel is installed at the sources of waste that includes dairy waste from the market or households and that can be converted into fungal protein. In the third scenario, dairy waste from the market or households and waste streams from production facilities are combined in a common fungal process for production of fungal protein. The fourth scenario pertains to incorporation of dairy waste in ethanol plants. This scenario appears to be the most feasible for the production of both fungal protein and ethanol from dairy waste. The paper concludes by noting that up to 0.48 g of biomass and 0.06 g of ethanol per gram of waste were obtained during cultivation of *A. oryzae* and *N. intermedia* in dairy waste and that the integration of this process at ethanol plants might be the most economically advantageous strategy.

Jin et al. (1999) presents a low-cost for the treatment of starch processing wastewater (SPW) with the production of fungal protein as well as glucoamylase enzyme [22]. The fungus used was *Rhizopus oligosporus* which can covert more than 95% starch materials in SPW to produce 4.5–5.2 g of dry fungal biomass from a litre of SPW in 14 h cultivation at 35°C and initial pH 4.0. The fungal biomass production was done on a laboratory scale batch system using an air lift bioreactor with working volume of 3.5 litres. The SPW containing medium was inoculated with a 10% (v/v) preculture. Biomass yield was expressed in gram dry biomass/litre of culture medium. The supernatant was collected for glucoamylase assay, and COD, glucose, and starch tests. The resultant fungal biomass has about 46% protein and is thought to be safe for human consumption. Additionally, the process using an air lift bioreactor was also carried out in a batch system. They also note another environmental benefit of this process which is the removal of 95% COD and total suspended solids. Moreover, the authors mention that this process is possibly feasible for the treatment of any starch processing effluent with bioproduct recovery, and can therefore contribute to the global management of the environment and waste resources.

Another study by Jin et al. (1999) investigated fungi that can utilise starch processing wastewater (SPW) to produce useful products [23]. *Aspergillus, Rhizopus, Trichoderma, Fusarium, Paecilomyces, Geotrichum and Chaetomiumi* were selected to be grown in SPW. It was observed that SPW derived from wheat and corn starch processing contained a large amount of organic matter. The suspensions were prepared from cultures grown on potato dextrose agar (PDA). They were inoculated in 250 cm^3 flasks which were incubated on an orbital shaker with a shaking rate of 150 rpm at 30°C for 24 h. The biomass in the medium was harvested by filtration of cultivation medium through a stainless-steel mesh. A total of 15 species of microfungi and yeasts were cultivated on SPW medium with initial pH values ranging from 3.5 to 5.0 at 30C. *A oryzae, R oligosporus, R arrhizus and T viride* showed high microbial activities to hydrolyse starch and utilise total organic carbon for biomass protein. These microfungi also grew fast, formed pellets and clumpy or coalesced biomass which was easy to harvest, and thus were selected for further studies. In terms of strain selection, the five strains of *A. oryzae* grew very well at specific growth rates ranging from 0.08 to 0.12 h~1 and produced 4.9– 5.6 g dm~3 of dry biomass, with 85–92% of COD reduction. The results show that from the selected microfungi, *A oryzae, R oligosporus and R arrhizus* had a high microbial and enzymatic capacity to utilise SPW as sole carbon and energy source. The authors conclude that raw SPW can be used as the sole source of carbon and energy without any pre-treatment by hydrolysis and sterilisation, and nutrient supplementation. Moreover, maintenance of aseptic conditions during the cultivation may be unnecessary.

Nair & Taherzadeh, 2016 explored the possibility of using edible *ascomycetes* filamentous fungi in sugar- or molasses-to-ethanol processes, to cultivate on vinasse or stillage to produce ethanol and protein-rich fungal biomass [24]. Here, two food grade fungal strains, *Neurospora intermedia* and *Aspergillus oryzae* var. *oryzae* were studied. The fungal cultures were prepared on potato dextrose agar (PDA) plates containing (in g/L) potato extract 4; dextrose 20; agar 15 and for spore suspensions (3 mL inoculum), these fungal plates were flooded with 20 mL sterile distilled water and the spores were released by gently agitating the mycelium. In shake flasks and airlift-bioreactors, a reduction of vinasse COD by 34% and viscosity by 21% was noted. The usage of sugars and glycerol showed a yield of approximately 202 to 223 g of dry fungal biomass of *N. intermedia* or *A. oryzae* respectively per litre of vinasse. The authors note that the current process at an ethanol facility producing about 100,000 m^3 of ethanol anually may provide yields up to 250,000 tons of dry fungal biomass alongside 8800–12,600 m^3 extra ethanol. The inoculum was inoculated in 100 mL of YPD broth containing (in g/L) dextrose 20; peptone 20; yeast extract 10 and they were incubated for 48 h at 35°C and 125 rpm. After cultivation, the fungal biomass was harvested and its dry weight calculated by drying the biomass at 105°C overnight.

This biomass was also used as inoculum for vinasse media-based fungal cultivation. The initial fungal growth screening was made on the vinasse diluted to 5, 10, 20, 30, 40, 50 (% v/v). Further screening was made on the factors such as cultivation time, media pH, temperature, and media sterilization (with and without). Ethanol concentration and fungal biomass yield were used as indicators to determine the optimum production conditions. A vinasse-based media was custom made with the addition of ammonium sulfate, and (or) potassium dihydrogen phosphate as sources of nitrogen and phosphorus. The fungal cultivations were carried out aerobically in the liquid vinasse medium (with specific dilution), in 100 mL volume (in 250 ml flasks). Cultivations were carried out for 4 days in an orbital shaking water bath at 35 °C and 125 rpm, with samples taken every 24 h. The optimum culture conditions were validated in a bench scale airlift bioreactor with a working volume of 3.5 L. The cultivation was carried out at pH 5.5 ± 0.2 (for N. intermedia) and 6.5 ± 0.1 (for A. oryzae) at temperature 35 ± 2 °C for 3 days. The results show that batch cultivations using vinasse, without any additional nutrient supplementation have an efficient fungal growth within the range, (g dry fungal biomass per litre vinasse) 31.7 to 202.4 for N. intermedia and 37.9 to 222.8 for A. oryzae. However, ethanol production remained limited since nutrients in vinasse most favoured the fungal biomass production. The paper concludes that usage of vinasse from sugar-to-ethanol plants could be achieved using ascomycetes filamentous fungi.

Prakash & S Karthick (2013) who studied the biomass content, anti-oxidative, and anti-tumour activity of extracts collected from *Fusarium venenatum* biomass, activated lyophilized *Fusarium venenatum* culture in an oats meal medium [25]. To evaluate the different media (Potato dextrose broth, Sabouraud dextrose broth, Sabouraud maltose yeast extract broth, Czapek'Dox broth, yeast phosphate soluble starch, and Vogel's minerals medium) for suitability, 100 mL of each medium was poured in 250 ml capacity flasks and autoclaved. Five flasks of each medium were inoculated with 1 ml of spore suspension of *Fusarium venenatum* and incubated at 28°C for 7 days. After incubation, the broth was filtered and the filtrate was discarded. 100 g of the collected mycelial biomass was washed with Millipore water and the washed biomass transferred to pre dried Whatman No.1 filter paper and dried using an oven at 60°C to a constant weight. The RNA content of biomass was reduced by subjecting the biomass to heat shock. Then, RNA of the biomass was determined using Ahangi et al. (2008) protocol, while Kjeldahl technique was used for the crude protein determination. The highest biomass production was recorded in Vogel's mineral broth with 5.40 g/L mycelial dry weight followed by Sabouraud maltose yeast extract broth and yeast phosphate soluble broth which revealed 4.40 and 3.0 g/L respectively. Czapek dox broth showed the least biomass production at 1.14 g/L. With regards to protein content, highest content was recorded in biomass derived from Vogel's mineral broth (45.12%), Sabouraud maltose yeast extract broth (23.11%) and yeast phosphate soluble broth (15.0%).

The usage of fish processing by-products and wastewater streams to produce fungal biomass has been explored in several studies. In a study by Ferreira et al. (2020), *A. oryzae* was deployed to a media containing wastewater such as salt brine, sludge, wastewater, etc [26]. from fish production. Submerged fermentation of the fungi in shake flasks (50 mL media) for 72 hours gave varying yields depending on the source and type of wastewater stream and sludge. Using only sludge gave a biomass yield of 42 g/L. However, the study notes that different methods and intensities of biomass recovery (drying, sieving, washing) had a significant effect on yield and protein content. Fermentation in salt brine and end-of-pipe output, however, gave the highest biomass yield after 48 h with salt brine giving a yield of about 4 g/L which was slightly over two times the yield from end-of-pipe wastewater stream. The study also notes that these two biomasses were 'purer' and had higher protein content compared to those obtained from using sludge as the main fermentation media. They mention that the entanglement of fungal filaments with media components & suspended solids is the likely reason for inflated biomass weight. The most influential factors affecting protein yield apart from wastewaters were fermentation duration, pH, dilution of media, and biomass recovery techniques.

Another study that used vinasse for cultivation was by Karimi et al. (2019) where *A. oryzae, N. intermedia, R. oryzae, Monascus purpureus, and Fusarium venenatum* were explored for their biomass yield and nutritional composition albeit with primary focus of using the biomass as fish feed [28]. The five fungal species were initially prepared and preserved on PDA plates and spore solution for inoculating vinasse medium were taken from the mature culture plates. The chemical and nutritional composition of the concentrated vinasse used was also determined. 250 mL shake-flasks containing 100 mL of vinasse medium were used for the primary cultivation for 72 h at 35°C and 125 with routine samples collected every 24 hours to examine the fermentation conditions using liquid chromatography. After 72 h cultivation, the biomass was sieved out, washed, and dried at 70°C. 5% vinasse media showed the best growth for *A. oryzae, N. intermedia*, and *R. oryzae*. Notably, *M. purpureus and F. venenatum*, failed to grow in any vinasse conceentrations. The higheset biomass yield was approximately 103.0 [protein 44.7%], 78.6 [protein 57.6%], and 27.9 (g DM/L) [protein 50.9%] for A. *oryzae, N. intermedia,* and *R. oryzae*, respectively. The authors mention that the fungi species appreciable amounts of amino acid and other relevant nutrients that should make the biomass suitable as fishmeal.

RESULTS & CONCLUSION

This review provides a general overview of the widely used and studied microfungi for human consumption and their respective production systems and parameters influencing their yield. The studies covered are predominantly small-scale fermentation setups involving the usage of simple and readily accessible media for microfungal cultivation. A range of carbon sources and media can be used and optimized as needed for a specific microfungi as observed in this review.

While *fusarium* spp. are very widely used and well-known filamentous microfungi, the review highlights the potential that a range of other microfungi hold and are yet to be exploited as a protein-rich food source for human consumption.

Although SCP production is very flexible and potentially cost-effective, some safety concerns are present. The use of waste-derived proteins and biomass as food sources human nutrition requires very strong safety profiling and assessments.

Discussion

The direct consumption of genetically modified organisms (GMO) in food has not received widespread acceptance and approval both by the competent regulatory authorities as well as the public. However, this is likely to change as scientific data pertaining to their safety grows along with improving genetic engineering technologies and reaches a conclusion in the coming years.

Certain fungi may produce mycotoxins that need to be controlled or eliminated before consumption during SCP production. This can be partly addressed by genetically engineering microbes or by selecting the organisms with mycotoxins as a priority. The overall production conditions may also be designed and optimized to address this concern.

ABBREVIATIONS

SCP: Single Cell Protein
GHG: Greenhouse Gases
GRAS: Generally Regarded as Safe
PDB: Potato Dextrose Broth
STR: Stirred-tank Reactor
ED: Effluent of Decanter
SS: Solid Stream
GMO: Genetically Modified Organism
PPL: Potato Protein Liquor
SPW: Starch processing wastewater
COD: Chemical Oxygen Demand
PDA: Potato Dextrose Agar
YPD: Yeast Peptone Dextrose

COMPETING INTERESTS

The authors declare no conflicts of interest or competing interests associated with this work.

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