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Sustainability Spotlight Statement

Ensuring global food security for a growing population requires sustainable, health-promoting protein sources beyond resource-intensive conventional livestock farming. Mycoprotein produced via submerged fermentation of edible fungi offers a promising alternative, with low carbon-footprint. This study advances the sustainable production of *Pleurotus ostreatus* mycoprotein by identifying key fermentation parameters that enhance mycelium biomass titer and protein content while controlling mycelial pellet morphology suitable for food applications. A significant advancement is the demonstration of oleic acid as a novel carbon source, highlighting a path towards valorizing lipid-rich feedstocks and reducing competition with food crops. This work directly supports UN SDG 2 (Zero Hunger) by enhancing mycoprotein availability and SDG 12 (Responsible Consumption and Production) by establishing efficient bioprocesses to support circular bioeconomy.

Mushroom Mycelium as a Sustainable High-Protein Food Source: Effects of Submerged Fermentation Conditions on Mycoprotein Production and Mycelium Morphology

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Abstract

Harnessing edible fungal mycelium as a source of health-promoting food presents a transformative pathway to enhancing global food security, and addressing the demand by world growing population in the 21st century. The mycoprotein from fungal mycelium also serves as a sustainable alternative to resource-intensive animal-sourced protein. This study investigated how submerged fermentation influences mycelium morphology and mycoprotein production by edible fungus *Pleurotus ostreatus*. Key parameters, including inoculum fragmentation, types of carbon sources, carbon-to-nitrogen (C/N) and carbon-to-phosphorous (C/P) ratios, and agitation rates, were systematically evaluated. Principal Component Analysis (PCA) was employed to identify the most influential parameters and elucidate their correlations with production metrics. Controlled inoculum fragmentation, inoculum density and agitation were found to be crucial for achieving uniformity of pelletized mycelium and improving productivity. Protein content and production increased with a decreasing C/N ratio, achieving a maximum of 39.7% (of dry biomass) and 3.89 g L^{-1}, respectively, while not significantly influenced by the C/P ratio. Oleic acid, a plant-based fatty acid, was demonstrated for the first time as a sole non-sugar carbon source for *P. ostreatus* cultivation, achieving a biomass yield comparable to glucose. Maximum biomass production (12.9 g L^{-1}) and productivity (1.61 g L^{-1} d^{-1}) were attained through desirable inoculum fragmentation, an inoculum density of 40 mg L^{-1}, an agitation rate of 150 rpm, a carbon loading of 3.6 g L^{-1}, a C/N ratio of 2.6 and a C/P ratio of 52.9. These findings provide valuable insights into establishing an efficient and sustainable biorefinery for mycelium-based foods.

Keywords: Fungal Mycelium; Mycoprotein; Submerged Fermentation; Pellet Morphology; Sustainable Biorefinery

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1. Introduction

Securing a sustainable protein supply for a growing global population represents one of the most critical food security challenges.[1] A strategic transformation framework is needed towards building a more resilient and sustainable food supply to support global food security, as resource-intensive livestock farming cannot meet the accelerating demand alone.[2] Mycoprotein, referring to the whole-cell, protein-rich biomass derived from fungi, stands at the forefront of this transition.[3] Cultivated from edible mycelium, it offers not only a scalable, high-quality vegan protein, containing all essential amino acids, but also provides balanced nutrition and health-promoting benefits, such as a high content of β-glucan fiber known to improve gut health and help regulate blood cholesterol levels, along with other key metabolites like choline, an essential nutrient that supports liver function and brain health.[3, 4] The edible fungus *Pleurotus ostreatus* is an especially promising microorganism for this purpose. Its fruiting body is well known as oyster mushroom, and considered nutritious owing to the significant content of carbohydrates (43.42 - 66.54 % biomass, dry basis (d.b.)), proteins (17.06 - 55.4 % biomass, d.b.), and dietary fibers (7.43 - 23.63 % biomass, d.b.).[5-7] *P. ostreatus* is also a good source of vitamins (*e.g.*, A, C, E and B2), minerals (*e.g.*, sodium, potassium, magnesium and calcium), and essential amino acids like lysine, phenylalanine, methionine, threonine, leucine and valine.[5, 8, 9] The species has been explored for the production of various bioactive compounds such as enzymes (*e.g.*, laccase and cellulase)[10, 11] and exopolysaccharides (*e.g.*, β-glucans)[7], which find diverse applications in the field of environment and agriculture, and food, beverage, health, pulp, textile and other industries[12, 13].

Cultivation of fungal mycelium is a sustainable approach, offering profound environmental advantages over conventional livestock farming, including substantially lower land use, water consumption, and carbon emissions.[14] By growing mycelium in efficient, controlled fermentation systems, this technology can directly meet the growing market demand for vegan proteins and novel food ingredients, decoupling food production from large-scale agriculture. Moreover, fungi can be successfully cultivated on waste substrates, such as pomelo peel powder, solid digestate from anaerobic digester, wheat straw, tree leaves, apple peels and banana peels.[15-17] Solid-state fermentation has been widely prevalent for fungal cultivation mainly attributed to the lower input of energy and water and cost-effectiveness in terms of potential to propagate on cheaper substrates and valorize agro-industrial wastes.[18] However, the cultivation method is constrained by the requirement of large footprint, inconvenient separation of mycelium biomass that is adhered to the fermented solid substrate, and challenges to maintain homogeneity of pH, temperature and moisture throughout fermentation, which may potentially lead to batch-to-batch variability in the characteristics of the biomass produced.[19] Submerged fermentation on the contrary is advantageous in terms of much smaller footprint, shorter cultivation period, capability of regulating and maintaining uniform culture conditions throughout fermentation, and easiness of scaling-up.[20] Furthermore, many fungi species, including *P. ostreatus*, form spherical mycelium pellets in submerged fermentation, which facilitates efficient harvesting of

the mycelial biomass from the culture medium for subsequent downstream applications.[21, 22] While solid-state fermentation of *P. ostreatus* has been extensively investigated for the traditional cultivation of its fruiting bodies, there are comparatively few studies on its submerged cultivation.[19, 23] Submerged fermentation of *P. ostreatus* is advantageous for large-scale mycoprotein production owing to its significantly shorter production cycles (days vs. weeks), higher volumetric productivity, and the ability to maintain precise control over mycelium morphology and biomass composition suitable for developing food ingredients. Moreover, submerged fermentation has been an emerging area for developing mycelium-based foods, such as cultured caviar and mycoboba from *Aspergillus awamori* (manufactured by Optimized Foods), meat analogue for Harbin red sausage by *Fusarium venenatum* [24], oncom from *Neurospora intermedia* [25], and dairy analogue for yogurt from *Fusarium* strain *flavolapis* (manufactured by Nature's Fynd), suggesting the need of more comprehensive research.

The type and composition of the substrate play a crucial role in determining the production of biomass as well as metabolites thereof. For instance, biomass production in submerged fermentation of *Monascus purpureus* was reported to be significantly influenced by the choice of carbon (*e.g.*, glucose, sucrose, xylose and citric acid) and nitrogen (*e.g.*, yeast leavening, NH_4Cl, peptone and soybean meal) sources.[21] Similarly, the availability of glucose and yeast extract, and supplementation of olive oil was observed to have critical impact on protein production in *A. oryzae* and *N. intermedia*.[26] Investigating such non-sugar carbon sources (*e.g.*, fats/oil) is critical for developing more sustainable and cost-effective bioprocesses, as lipids and fatty acids can often be derived from low-cost agri-food industry byproducts, thereby avoiding competition with food crops. However, these lipids are typically used only as supplements rather than as the primary substrate; to the best of our knowledge, no studies have yet investigated the use of a long-chain fatty acid as the *sole* carbon source for submerged fungal cultivation. Furthermore, like nitrogen forms a key part of proteins, phosphorus forms a critical portion of phosphorylated biomolecules, *e.g.*, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), and the reductant nicotinamide adenine dinucleotide phosphate (NADPH), which are essential molecules involved in energy transfer in the cells.[27] Only a few studies have explored the effect of phosphorus on production of mycelium biomass and protein, with existing research being limited to other products like manganese-dependent peroxidase, glucosamine and lipid in fungi species, *e.g.*, *Phanerochaete chrysosporium*, *Mucor indicus* and *Umbelopsis vinacea*.[27-29] Beyond specific nutrients, the stoichiometric relationship between carbon and nitrogen (C/N ratio) and carbon and phosphorus (C/P ratio) are critical determinants with potential influence on cell growth and metabolic pathways. Precise regulation of these ratios is therefore indispensable for targeted modulation of metabolic fluxes, enabling the preferential allocation of cellular resources towards biomass accumulation or protein biosynthesis.[30] However, in contrast to well-studied platforms like bacteria and microalgae, these stoichiometric relationships remain critically under-investigated in fungal submerged cultivation. Specifically, while the C/N ratio has received limited attention, the effect of the C/P ratio

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on biomass and protein synthesis remains entirely unexplored. Moreover, other parameters, including inoculum properties (*e.g.*, inoculum density) and agitation speed have been identified as influential process conditions for mycelial growth, in addition to pellet morphology in submerged fermentation of certain species like *Ganoderma curtisii* and *Aspergillus awamori*.[31, 32] Based on the current state-of-the-art for submerged fungal fermentation, a comprehensive investigation is still required to evaluate the influence of inoculum properties, different sugar and non-sugar carbon sources, and key stoichiometric ratios (C/N and C/P) on the interplay between mycelial morphology, biomass accumulation, and protein synthesis to ultimately develop an economically viable and environmentally sustainable process for global food security.

Therefore, to address the research gap, this study aimed at developing a robust submerged fermentation process for *Pleurotus ostreatus* to maximize the co-production of mycelial biomass and protein. The central hypothesis was that the systematic modulation of physical factors to control mycelial morphology, combined with the regulation of chemical factors to tailor nutrient availability and metabolic flux, would substantially enhance both biomass titer and protein content. The objectives of this research were to: (1) evaluate the effects of key inoculum and fermentation parameters, including inoculum fragmentation level, inoculum density, and agitation rate, on mycelial pellet morphology; (2) systematically investigate the influence of media formulation factors, including both sugar and non-sugar (such as long-chain fatty acid) as sole carbon sources and the stoichiometric C/N and C/P ratios, on the production of mycelium biomass and protein; and (3) to elucidate the complex correlations between various submerged fermentation parameters and the resulting production metrics, thereby identifying the most favorable conditions for sustainable mycoprotein production.

2. Materials and methods

2.1. Organism and culture conditions

2.1.1. Reagents and chemicals

All the chemicals were of analytical grade. Glucose and fructose were procured from Ward's Science (Rochester, NY, USA). Lactose, xylose and lauric acid were procured from Thermo Fisher Scientific (Waltham, MA, USA). Magnesium sulfate and monopotassium phosphate were purchased from Ambeed Inc. (Buffalo Grove, IL, USA). Yeast extract and potato dextrose agar were purchased from Hardy Diagnostics (Santa Maria, CA, USA). Oleic acid was purchased from TCI (Portland, OR, USA). 3,5-Dinitrosalicylic acid was procured from Alfa Aesar (Ward Hill, MA, USA). Chloramphenicol was purchased from G Biosciences (Saint Louis, MO, USA). All the reagents were prepared in de-ionized water.

2.1.2. Genome sequencing and species identification

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As the source of fungal mycelium, the Oyster mushroom fruiting bodies were sourced from a local store (Ithaca, NY, USA). A 2 mm x 2mm piece of tissue was aseptically removed from the mushroom flesh in the middle of the stem butt-end and then cultured on potato dextrose agar (PDA) for approximately 10 days.[33] The Genomic DNA of the strain was extracted following a previous method [34] with minor modification: a small fragment of mycelium was collected from a mature plate and placed into a Polymerase Chain Reaction (PCR) tube with 50 µL of nuclease-free water. The mixture was briefly vortexed to ensure homogeneity. The tube was then incubated in a thermocycler (BIO-RAD, Model T100 Thermal Cycler, Hercules, CA, USA) for 15 minutes at 95 °C to facilitate cell lysis and DNA release through heat shock.[34] Following incubation, the sample was immediately cooled in an ice-water bath for 1 minute to stabilize the released genomic DNA. The resulting extract was used directly as a template for PCR amplification without further mechanical or chemical treatment. PCR was carried out using ITS 5 (Forward, GGA AGT AAA AGT CGT AAC AAGG, position 1737 - 1758) and ITS 4 (Reverse, TCC TCC GCT TAT TGA TAT GC, position 2390 - 2409) primers (Supplementary information, Fig. S1). The amplification was performed in a 25 µL total reaction volume containing 12.5 µL of 2X Master Mix, 0.5 µL of Forward and Reverse Primer each, 0.15 µL GoTaqFlexi Taq Polymerase, 9.85 µL of nuclease-free water and 1.5 µL of the extracted DNA. The PCR tubes were then placed in a thermocycler and run using the appropriate conditions: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 minute, annealing at 56°C for 1 minute and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. A 3 µL aliquot of the resulted sample was then electrophoresed on 1.5% (w/v) agarose gel. The gel was loaded with a 100bp DNA ladder (Promega Bench Top DNA Ladder) as molecular weight markers for comparison. The gel box (Instaview Blue LED Transilluminator, Model, E1201-BLT) (Accuris Instruments, Edison, NJ, USA) was run at 120 Volts for 30 minutes until the ladder bands were appropriately separated. The gel was visualized using the gel box orange filter. Based on the primers used, we observed the sample bands appearing on the agarose gel as expected (Supplementary information, Fig. S2). The PCR sample was then cleaned using ExoSAP and subjected to sanger sequencing by Cornell Biotechnology Resource Center (BRC) (Ithaca, NY, USA). The ITS sequences were then compared with other ITS sequences in the GenBank database using the native nucleotide Basic Local Alignment Tool (BLAST) function accessible through the National Center for Biotechnology Information (NCBI) (Supplementary information, Fig. S3 and S4), based on which the species was identified as *Pleurotus ostreatus*.

2.1.3. Media, inoculum preparation and culture conditions

The fungal mycelia were cultivated on agar plates in basal media containing (g L^{-1}): glucose (30.0), yeast extract (5.0), KH_2PO_4 (1.0), $MgSO_4$ (0.244) and agar (20.0), as previously described by Shen et al. [35] with minor modifications. The agar plates were incubated at 30 °C under static conditions for 6–7 days until the mycelia colonized approximately 90% of the agar surface area, as determined by the radial expansion of the mycelial front from the center of the plate. To ensure the collection of

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actively growing biomass, plates were harvested before the mycelium reached the edges of the petri dish.[35] The plates were maintained at 4 °C for further use. Inoculum preparation was performed in 300-mL shake flasks containing 150 mL of liquid basal media (without agar). The liquid media was inoculated with 3-4 units of 8-10 mm mycelial discs cut out from the pre-grown agar plates using sterile forceps, and the seed flasks were incubated at 30 °C and 150 rpm for 6 days in a shaker incubator (MaxQ™ 6000, Thermo Scientific, Waltham, MA, USA). The pH of the media was adjusted to 6.0 by adding 2 M HCl or 2 M NaOH. The medium was sterilized by autoclaving at 121 °C for 15 min. The basal medium was selected based on preliminary trials (data not shown), where it supported faster mycelial growth compared to potato dextrose agar/broth to reach comparable biomass levels for inoculation.

For inoculation of the experimental flasks, 80 mL of the seed culture was transferred into a sterile 100-mL beaker containing majority of smaller pellets of comparable sizes. The pellets were fragmented through homogenization by a mechanical homogenizer (T25 digital Ultra Turrax, IKA, Staufen, Germany) at 5000 rpm for 40 seconds. This specific fragmentation level was selected based on the study described later in the Section 2.2. The resulting inoculum granulometry exhibited a mean fragment size of 924.5 µm. For all subsequent experiments, 1 mL of this homogenized pellet suspension was used as inoculum, corresponding to an inoculum density of ~20 mg L^{-1} biomass (dry basis (d.b.)) in 150 mL of liquid media. The experimental flasks comprised of the same basal media as was used for that of inoculum generation (unless otherwise specified), except that it was supplemented with 0.1 g L^{-1} chloramphenicol to prevent microbial contamination in the submerged fermentation of fungi.[35]

2.2. Evaluation of the effect of inoculum fragmentation through homogenization

Pleurotus ostreatus was investigated for the production of biomass and morphology of pellets under varying levels of inoculum fragmentation comprising of different homogenization speeds and durations. Pre-grown seed culture was subjected to four types of fragmentation treatments following a method previously reported by Shen et al.[35], with modifications based on our preliminary experimental trials (data not shown) on *P. ostreatus*: F1 (pellets homogenized at 3000 rpm for 5 seconds), F2 (homogenized at 5000 rpm for 10 seconds), F3 (homogenized at 5000 rpm for 40 seconds), and control (no homogenization). The pellet fragmentation was achieved using a mechanical homogenizer (T25 digital Ultra Turrax, IKA, Staufen, Germany). Inoculation was performed using the method as mentioned earlier in Section 2.1. The cultures were incubated for 10 days under continuous shaking of 150 rpm at 30 °C.

2.3. Submerged fermentation with variable media composition

2.3.1. Evaluation of the effect of different carbon sources and carbon loading

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Five carbon sources including glucose, lactose, fructose, xylose and oleic acid were studied as the sole carbon substrate for submerged fermentation of *P. ostreatus*. Three carbon loadings to cultivation media were investigated including 0.88, 3.60 and 6.33 g L^{-1}, corresponding to glucose concentrations of 2.19, 9.0 and 15.82 g L^{-1}, lactose concentrations of 2.08, 8.55 and 15.03 g L^{-1}, fructose concentrations of 2.19, 9.0 and 15.82 g L^{-1}, xylose concentrations of 2.19, 9.0 and 15.82 g L^{-1}, and oleic acid concentrations of 1.14, 4.71 and 8.28 g L^{-1}, respectively. Yeast extract was used as nitrogen source for all assays at a fixed concentration (5 g L^{-1}). The cultures with different carbon loadings (0.88, 3.60 and 6.33 g L^{-1}) were incubated under constant agitation of 150 rpm at 30 °C until the exhaustion of carbon source (*e.g.*, total sugars or fatty acid) in the respective media, *i.e.*, on Day 5, Day 8 and Day 12.

2.3.2. Evaluation of the effect of different C/N and C/P ratios

P. ostreatus was cultured in media containing different concentrations of nitrogen, including 0.06, 0.39, 0.72, 1.05 and 1.38 g L^{-1}, which were prepared by adding yeast extract at equivalent concentrations (0.5, 3.5, 6.5, 9.5 and 12.5 g L^{-1}, respectively). The experiment was performed using the basal liquid media containing 9 g L^{-1} glucose (and 1 g L^{-1} KH_2PO_4), which corresponded to equivalent C/N ratios of 65.5, 9.4, 5.0, 3.4 and 2.6, respectively.

Further, the organism was cultured under varying concentrations of phosphorus (0, 0.07, 0.52, 0.98 and 1.43 g L^{-1}) in the media, which correspond to KH_2PO_4 concentrations of 0, 0.3, 2.3, 4.3 and 6.3 g L^{-1}. The study was carried out in media containing 9 g L^{-1} glucose (and 5 g L^{-1} yeast extract), which corresponded to equivalent C/P ratios of 52.9, 6.9, 3.7 and 2.5, respectively.

The cultures were incubated for 8 days under constant agitation of 150 rpm at 30 °C.

2.4. Evaluation of the effect of different inoculum densities and agitation rates

The organism was investigated in submerged cultivation at varying inoculum densities of 20 and 40 mg L^{-1} biomass (d.b.), under different agitation rates of 75, 150 and 255 rpm at 30 °C. The cultures were grown for 8 days in media supplemented with glucose at a carbon loading of 3.6 g L^{-1}, yeast extract at a nitrogen loading of 1.38 g L^{-1} and KH_2PO_4 at a phosphorous loading of 0.07 g L^{-1}, which were selected based on previous studies carried out in Section 2.3.

2.5. Analytical methods

2.5.1. Determination of the size distribution of inoculum fragments and pellets

The size distribution of inoculum fragments was determined using Mastersizer particle size analyzer (MS-2000, Malvern Instruments Limited, Westborough, MA, USA) by gradually adding 30 mL sample after different fragmentation treatments. The instrument mathematically interprets light scattering pattern by the fragments and fits the measured data to estimate a volume-based (%, v/v) particle size distribution in the range of 10 – 3000 µm, which is obtained as a bell-shaped plot.

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For determination of pellet sizes after submerged fermentation, 10 mL of homogenously mixed culture was poured into a petri dish, and mycelial pellets were spread out across the dish to remove any clumps and aggregates, and visualize discrete units of pellets. Images of the petri dish were captured inside a photo light box and the sizes of individual pellets were estimated using the software, ImageJ, developed by the National Institutes of Health (Bethesda, MD, USA).

2.5.2. Estimation of biomass, residual sugar and fatty acid concentrations

For sampling of biomass, the experimental flask was mixed thoroughly by gentle shaking to avoid sedimentation of mycelium pellets, and 10 mL of the culture was collected by pouring into a sterile measuring cylinder. The mycelium biomass was separated from the liquid medium using vacuum filtration through a pre-weighed Whatman filter paper (United Scientific, Libertyville, IL, USA), assisted by a vacuum pump (Rocker 400, Rocker Scientific, Kaohsiung City, Taiwan). The biomass residue was washed thoroughly using de-ionized water (150 mL) for two cycles and subjected again to vacuum filtration to remove residual water and any surface-adhered medium components. The filtered and washed samples were dried at 50 °C in a convective oven (VWR Oven F Air 3.65CF, VWR International, Radnor, PA, USA) until it attained a constant weight with change of no more than ±0.5%.[21] Moisture content of mycelium from different assays was observed to be within a range of 90.0 to 96.3 % (Supplementary information, Table S5). The concentration of biomass in the fermentation media was expressed as dry weight (DW) of biomass per liter of media (g L^{-1}).

Biomass productivity was calculated as per Eq. (1):

$$Biomass\ productivity\ \left(g\ L^{-1}d^{-1}\right) = \frac{Biomass\ concentration\ (g\ L^{-1})}{Fermentation\ period\ (d)} \quad (1)$$

The initial media and cell-free spent media were collected and analyzed for the content of residual reducing sugar through DNS (3,5-dinitrosalicylic acid) assay as described earlier by Miller [36], and measuring the absorbance using UV-Vis Spectrophotometer (UV5Bio, Mettler Toledo, Columbus, OH, USA) at 540 nm. Similarly, the cell-free supernatant was analyzed for residual concentration of fatty acid spectrophotometrically using commercial free fatty acid assay kit (Sigma-Aldrich, St. Louis, MO, USA) and determining the absorbance at 570 nm.

Biomass yield with respect to substrate ($Y_{x/s}$) was calculated as the ratio of biomass concentration (g L^{-1}) at a given time point and the corresponding concentration of carbon source consumed, as per Eq. (2):

$$Biomass\ yield\ (g\ g^{-1}) = \frac{Biomass\ concentration\ (g\ L^{-1})}{Concentration\ of\ carbon\ source\ consumed\ (g\ L^{-1})} \quad (2)$$

2.5.3. Estimation of intracellular proteins

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Harvested mycelium biomass initially separated from liquid media through vacuum filtration was subjected to lyophilization using a freeze-dryer with cycles of 12 h freezing and 12 h drying (EW-03336-44, Harvest Right, Salt Lake City, UT, USA). To evaluate the nutritional quality of the harvested mycelium biomass, the intracellular protein content was analyzed as previously described by Bakratsas et al. [23] with minor modifications. 50 mg of freeze-dried biomass was resuspended in 5 mL of phosphate buffer (pH 7.0) and subjected to ultrasonication (Q125, QSonica, Newtown, CT, USA) at 8 kHz (40% intensity) in a pulse mode (15 s ON, 3 s OFF) for 6 min. The sample was placed in ice water bath during ultrasonication to eliminate the risk of protein degradation owing to excess heat generating from the sonication process. Intracellular contents were obtained in the supernatant by centrifuging (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) the sample at 5000 rpm for 15 min. Protein content was estimated through Bradford Assay (Pierce Bradford Protein Assay Kit, Thermo Scientific, Waltham, MA, USA), using bovine serum albumin (BSA) as standard. In brief, 1.5 mL Bradford reagent was mixed with 30 µL of cell-free supernatant and the mixture was incubated for 10 minutes, followed by the measurement of absorbance at 595 nm using a spectrophotometer. The absorbance value was converted into mass of proteins using the correlation based on calibration curve ($y = 1.0404\,x$; $R^2 = 0.9923$):

$$Concentration\ of\ protein\ in\ the\ sample\ (mg\ mL^{-1}) = \frac{Absorbance\ (595\ nm)}{1.0404} \quad (3)$$

Protein content in the biomass (d.b.), protein concentration in the fermentation media, productivity and protein yield were calculated as per Eq. (4), (5), (6) and (7):

$$Protein\ content\ (\%) = \frac{Mass\ of\ protein\ (g)}{Mass\ of biomass\ (d.b.)\ (g)} \times 100\% \quad (4)$$

$$Protein\ concentration\ \left(g\ L^{-1}\right) = Protein\ content\ (\%) \times Biomass\ concentration\ (g\ L^{-1}) \quad (5)$$

$$Protein\ productivity\ \left(g\ L^{-1}d^{-1}\right) = \frac{Protein\ concentration\ (g\ L^{-1})}{Fermentation\ period\ (d)} \quad (6)$$

$$Protein\ yield\ \left(g\ g^{-1}\right) = \frac{Protein\ concentration\ (g\ L^{-1})}{Concentration\ of\ carbon\ source\ consumed\ (g\ L^{-1})} \quad (7)$$

2.6. Statistical analysis

Experiments evaluating carbon source types and carbon loading for glucose and lactose were performed in triplicate; all other experiments were conducted in duplicate (Supplementary information, Table S3). Results were expressed as the mean ± standard deviation of the replicates. Statistical significance of the difference between the means was estimated through one-way or two-way analysis of variance (ANOVA) with a post-hoc Tukey test using the Origin Pro software (OriginLab, Northampton, MA, USA) at a significance level (α) of 0.05. Principal Component Analysis and Pearson Correlation were performed using Origin Pro software.

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3. Results and discussion

3.1. Effect of inoculum fragmentation

The size of mycelium fragments in the inoculum critically influences the development of new pellets and biomass accumulation.[31] To this end, inoculum was subjected to varying levels of fragmentation to obtain mycelium fragments of various sizes, *viz.*, control: no homogenization; F1: 3000 rpm, 5 seconds; F2: 5000 rpm, 10 seconds; and F3: 5000 rpm, 40 seconds. The mean sizes of mycelium fragments resulting from different treatments including control, F1, F2 and F3 were 1110.25, 1089.13, 1036.37 and 924.5 µm, respectively. Fig. 1A shows the size distribution of mycelium fragments in the inoculum. After fragmentation, the fraction of mycelium fragments (%, v/v of the inoculum) having sizes in the range of 1000 – 2500 µm were 53.9, 51.9 and 46.3, respectively, in control, F1 and F2 treatments. Whereas, F3, which was subjected to the highest degree of fragmentation resulted in a major fraction (64.5%, v/v) of the fragments in a size a range less than 1000 µm, while only 35.5% exhibited sizes more than 1000 µm. This fragmentation had a direct effect on the development of new pellets, which were harvested after 10 days. The pellets sizes were observed to decrease with increasing degree of fragmentation, resulting in mean diameters of 10.9, 6.41, 3.95 and 2.26 mm, respectively, from control, F1, F2 and F3 treatments (Fig. 1B). Furthermore, the pellet size distribution became less scattered and more even with increasing fragmentation level. The control and lower fragmentation levels (F1 and F2) resulted in lower pellet count, which did not differ significantly among the treatments ($p > 0.05$); whereas, F3 exhibited substantially higher pellet count (count: 2400) relative to other treatments ($p < 0.05$) (Fig. 1C). As observed from Fig. 5A, while control, F1 and F2 resulted in larger-sized mycelium pellets with spiky and rougher surfaces, F3 generated more compact pellets with relatively smoother surfaces and more homogenous size distribution. The underdeveloped pellets were observed as numerous fine and short thread-like structures dispersed all over the medium, and were excluded from size and count estimations. To the best knowledge of the authors, this is the first study demonstrating the effect of inoculum fragmentation level on resulting pellet sizes and number in submerged cultivation of *P. ostreatus*.

Moreover, effect of inoculum fragmentation levels was investigated on different growth parameters, including biomass production, productivity and yield. Lower biomass titer and productivity of 1.8 g L^{-1} and 0.18 g $L^{-1}d^{-1}$, respectively, were observed for control, whereas, relatively higher titers (6.65 – 12.8 g L^{-1}) and productivities (0.66 – 1.28 g $L^{-1}d^{-1}$) were observed for F1, F2 and F3 (Fig, 1D), although the values did not vary significantly among the different fragmentation treatments ($p > 0.05$). Biomass yields across different treatments varied in the range of 0.66 – 1.57 g g^{-1} (Fig. 1E). These observations are in close alignment with the study reported for *Inonotus hispidus* culture, where, the biomass production was observed to increase with increment in homogenization rate and time from 6000 to 26000 rpm and 1 to 3 min, respectively, resulting in maximum biomass of 10.29 g L^{-1}.[35] Moreover, Shen et al. [35] reported that lower shear rate and shorter shear time yielded loose mycelial

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clumps and heterogeneous pellets, similar to our control; whereas, high shear rate and longer shear time produced well-dispersed evenly distributed mycelia, analogous to our F3 treatment. The dispersed morphology seems essential for creating higher surface area per unit volume of fragmented mycelium which can potentially enhance the uptake of nutrients and oxygen from the liquid medium, consequently leading to improved growth. Furthermore, the enhanced biomass titer and productivity under different fragmentation treatments (F1, F2 and F3) may also be attributed to availability of a greater number of densely-branched hyphal tips per unit length of an inoculum fragment, promoting higher germination capacity.[35] Another study also reported a similar pattern for biomass production in *Aspergillus niger* involving dispersion of inoculum.[37] Implementation of pellet recycling strategy revealed that fragmentation of mycelium pellets before cultivation could enhance biomass production from 27.0 g L^{-1} (1st batch) to 32.1 g L^{-1} (8th batch); whereas, direct inoculation with pellets (without fragmentation) resulted in decline in biomass production from 28.2 g L^{-1} (1st batch) to 9.2 g L^{-1} (6th batch), thus, demonstrating the significance of inoculum fragmentation.[37]

In the present study, since, no significant differences were observed between the treatments F1, F2 and F3 in terms of biomass titer, productivity and yield (Fig. 1D and E), therefore F3 was selected as the inoculum fragmentation level for further experiments, owing to significantly higher biomass production and productivity (compared to control), as well as compact and relatively uniform pellet sizes (Fig. 5A).

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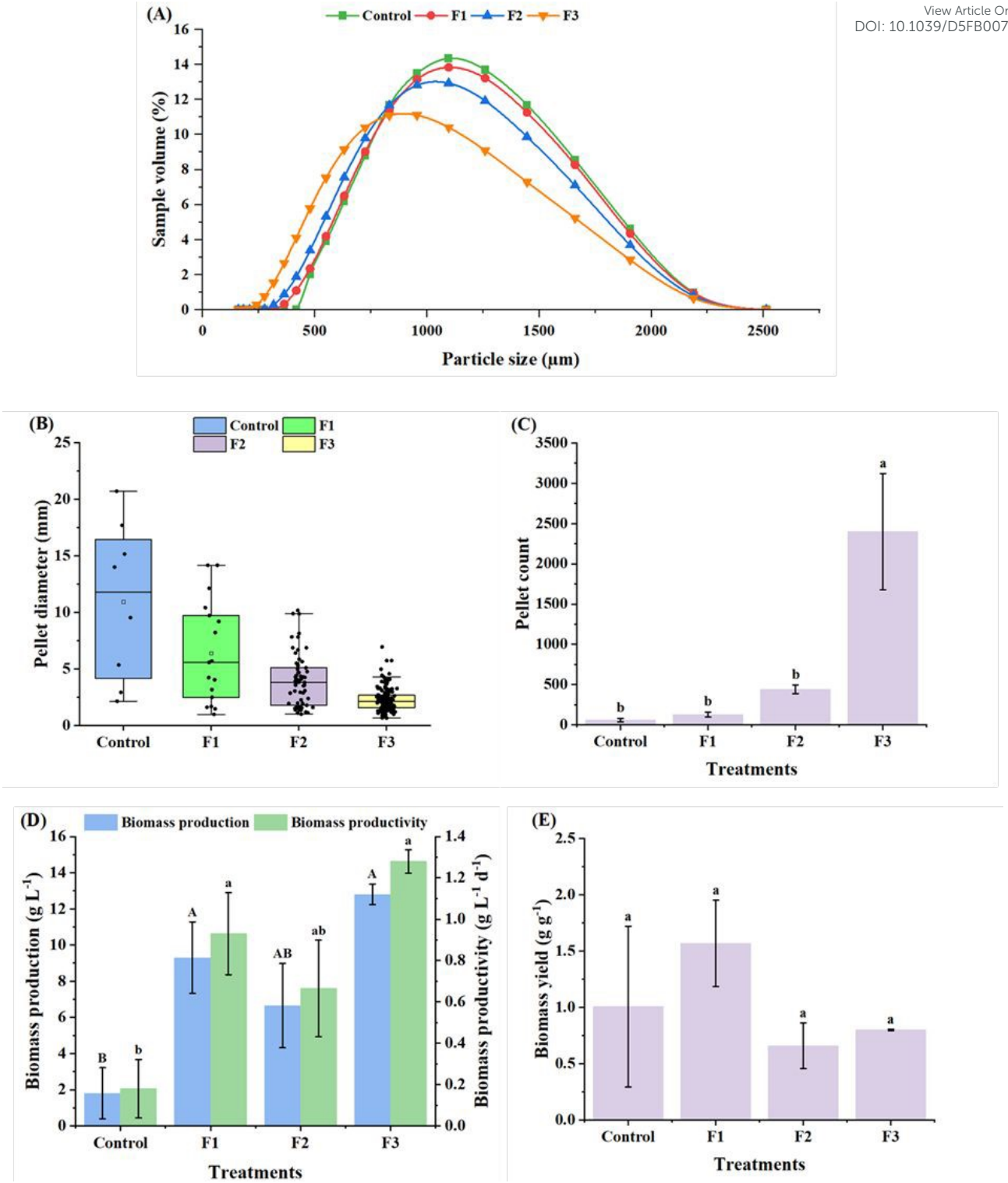

392

393 **Fig. 1.** Effect of different fragmentation levels on (A) size distribution of mycelium fragments in
394 inoculum, (B) diameter of mycelium pellets in the culture on Day-10 (Quartile summary of the Box
395 and Whisker plot is provided in Supplementary information, Table S1), (C) number of pellets in the
396 culture, (D) biomass production and productivity, and (E) biomass yield. Control: no homogenization;

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F1: 3000 rpm, 5 s; F2: 5000 rpm, 10 s; and F3: 5000 rpm, 40 s. * Groups that share same letters are considered statistically insignificant.

3.2. Effect of media composition on submerged fermentation of *P. ostreatus*

3.2.1. Effect of carbon source and loading on mycelium biomass

P. ostreatus was cultivated with different sugar and non-sugar carbon sources including monosaccharides (glucose, fructose and xylose), disaccharide (lactose), and long-chain fatty acid (oleic acid) at varying carbon loadings of 0.88, 3.60 and 6.33 g L^{-1}, which were harvested on Day 5, Day 8 and Day 12, respectively. Among all sole carbon sources, the highest mycelium biomass production and productivity were obtained using glucose. Biomass production was observed to improve with increment in carbon loading of glucose from 0.88 – 6.33 g L^{-1} (equivalent glucose concentrations: 2.19 – 15.82 g L^{-1}), resulting in a maximum titer of 11.2 g L^{-1} (Fig. 2A). However, highest biomass productivity (1.09 g $L^{-1}d^{-1}$) was obtained at carbon loading of 3.6 g L^{-1} (Fig. 2B). Bakratsas et al. [23] reported similar patterns in *P. ostreatus* biomass production which was observed to increase from 6 to approx. 14 g L^{-1} with the increase in glucose concentration from 5 to 20 g L^{-1}. It was interesting to observe that oleic acid, as a non-sugar sole carbon source, achieved a higher biomass yield than majority of sugar-based carbon sources in this study. A biomass production of 8.33 g L^{-1} was obtained from oleic acid at a carbon loading of 6.33 g L^{-1}; and its biomass productivity was 0.69 g L^{-1} d^{-1} at the same carbon loading which was not significantly different from the productivity attained at 3.6 g L^{-1} carbon loading. Furthermore, oleic acid exhibited higher biomass yield in the range of 1.0 – 1.27 g g^{-1} at different carbon loadings, and was observed to be comparable with glucose at higher loadings (3.6 – 6.33 g L^{-1}) (Fig. 2C).

Filamentous fungi are known to assimilate fatty acids from fats and oils within endogenous organelles like peroxisome mediated by β-oxidation and glyoxylate pathways resulting in the production of carbohydrates, energy and acetyl-CoA.[38] Oleic acid is an 18-carbon unsaturated fatty acid with 76.5% (w/w) carbon content in a molecule; hence, this higher energy density possibly accounts for the higher biomass production owing to higher energy accumulation in *P. ostreatus* in the present study. Oleic acid is the major fatty acid comprising of 55–83% of olive oil.[39] Nazir et al. [26] demonstrated the use of olive oil (40 g L^{-1}) as sole carbon source to grow *Aspergillus oryzae* in presence of yeast extract, attaining a biomass titer of 13.21 g L^{-1}, which was relatively higher than the biomass production (5.63 g L^{-1}) obtained using 20 g L^{-1} glucose as sole carbon source. Moreover, researchers have demonstrated the use of olive oil as a medium supplement to enhance biomass production. For instance, Rosales-López et al. [31] reported that supplementation of 1% v/v olive oil to the culture medium could increase biomass production by 32% and 240% relative to optimized basal medium (containing glucose) and commercially available potato dextrose broth, respectively, in submerged fermentation of *Ganoderma curtisii*. However, to the best knowledge of the authors, there are no other studies demonstrating the

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cultivation of filamentous fungi utilizing a long-chain fatty acid as the sole carbon source. This finding is particularly significant from a sustainability standpoint, as it demonstrates the potential to valorize plant-based or lipid-rich waste streams, such as used cooking oils or agricultural byproducts, which do not compete with the arable land and food crops required for sugar-based feedstocks like glucose and fructose. In the present study, an attempt was also made to utilize lauric acid as carbon source under different carbon loadings (0.88 – 6.33 g L^{-1}). However, no growth was observed, primarily owing to the higher melting point (~43 °C), leading to a clear phase separation, and flaking out of the added lauric acid as a solid layer on top of the aqueous media under specified culture conditions (30 °C and 150 rpm). Whereas, oleic acid, could form an oil-in-water emulsion [40] under the given conditions. Although, initial addition of oleic acid led to formation of a distinct layer on the surface of the aqueous medium; however, this phase separation gradually diminished during the cultivation period at 150 rpm. While no external emulsifying agents were utilized, the dispersion of the fatty acid was likely facilitated by the continuous mechanical agitation in conjunction with the biological activity of the fungus. *P. ostreatus* is known to secrete extracellular lipases [41] and polysaccharides with surfactant-like properties (*e.g.*, β-glucan) [42], which can reduce interfacial tension and promote the emulsification of lipids in submerged culture. This biological stabilization of the oleic acid likely enhanced its bioavailability to the inoculum fragments for uptake and growth.

The biomass production, productivity and yield were observed to be comparable between other sugars like lactose, fructose and xylose at the highest carbon loading of 6.33 g L^{-1}, with values significantly lower ($p < 0.05$) than that obtained with glucose and oleic acid (Fig. 2A, 2B and 2C). However, substantially low biomass production and productivity were observed with xylose at 3.6 g L^{-1} carbon loading. These results are in alignment with other studies which have identified glucose as a superior carbon source that provided higher cell biomass and product titers, among other sugars. For instance, higher production of biomass and exopolysaccharides (EPS) have been reported in *P. ostreatus* and other filamentous fungi, *e.g.*, *Inonotus hispidus*, using glucose as carbon source, in contrast to other substrates, including lactose and fructose, at the same concentration.[19, 35] Li et al. [43] justified the slower growth rate of *Neurospora crassa* on xylose through the gene expression profile of sugar transporters; deciphering that out of 39 putative sugar transporter genes in the genome of *N. crassa*, 15 and 6 genes were expressed respectively when D-xylose and D-glucose were used as carbon sources. This suggests that glucose, being the preferred and most efficiently metabolized sugar, is handled by a small number of specialized and effective transporters. However, xylose triggers a broader, less-specialized response, upregulating a larger suite of transporters as part of a wider metabolic adjustment to a less-desirable carbon source.

3.2.2. Effect of carbon source and loading on mycelium protein

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The protein content (% biomass, d.b.) and protein production from submerged fermentation of *P. ostreatus* were observed to increase with higher carbon loadings from 0.88 to 6.33 g L^{-1}, and attained respective maximum values with glucose (31.1% and 3.47 g L^{-1}), followed by oleic acid (22% and 1.83 g L^{-1}) (Fig. 2D and 2E). However, protein content and production were found to be relatively lower with other carbon sources like lactose, fructose and xylose, which could be attributed to corresponding lower biomass yield and production from these sugars, as compared to glucose and oleic acid. Hamza et al. [19] reported similar relationship between biomass and protein production in *Pleurotus ostreatus*, where protein production exhibited exponential increase from Day 6 to Day 10, with exponential growth of biomass following Day 5 of cultivation. Furthermore, Bakratsas et al. [23] demonstrated significantly higher protein content and production in *P. ostreatus* biomass cultivated using glucose (29.0 ± 2.0% and 3.32 ± 0.20 g L^{-1}) as compared to lactose, fructose and xylose on Day 8 of cultivation, which is well in accordance with the findings of the present study. The protein content and production from oleic acid were 7.99 – 21.98% and 0.12 – 1.83 g L^{-1}, respectively, under carbon loadings from 0.88 to 6.33 g L^{-1}, which were significantly higher than majority of sugar substrates, but lower than glucose. This could be attributed to possible metabolic switch in the cells, resulting in the diversion of energy from fatty acid towards accumulation of fat in the cells, as a priority over synthesis of proteins. This is corroborated by Nazir et al. [26], wherein, *A. oryzae* biomass accumulated 14% protein and 34% fat from 40 g L^{-1} olive oil, while it produced 48% protein and 3% fat from 20 g L^{-1} glucose. Similarly, protein productivity and protein yield from glucose were observed to attain maximum values of 0.29 g L^{-1} d^{-1} and 0.30 g g^{-1}, respectively, which were significantly higher than that from oleic acid (0.15 g L^{-1} d^{-1} and 0.22 g g^{-1}) (Fig. 2F and 2G) and other sugars.

Overall, the superior performance of protein production by *P. ostreatus* in presence of glucose, followed by oleic acid, and relatively lower performance in presence of other sources, *e.g.*, lactose, fructose and xylose, could be attributed poor efficiency of assimilation of these sugars into the cells as compared to glucose and oleic acid. As the maximum biomass productivity and protein yield were observed with glucose at carbon loading of 3.6 g L^{-1} (culture period: 8 days), these set of conditions were selected for performing further experiments, keeping in view the industrial viability of the process. Fig. 5B shows the morphology of the pellets grown using different carbon sources at loading of 3.6 g L^{-1}. Glucose and lactose were observed to produce several round pellets with relatively uniform size distribution, as compared to fructose and xylose which yielded majority of pellets with rough and spiky surfaces and a non-uniform size distribution. Pellets generated from oleic acid-containing medium had interesting morphological features which were prominently distinguishable from the ones yielded from sugar substrates. For instance, oleic acid yielded a greater number of compact pellets with even smoother surfaces and a more homogenous size distribution as compared to the rest of the carbon sources, including glucose and lactose. The enhanced pellet morphology (compact, smooth, uniform) observed with oleic acid suggests a potential role of lipids in modulating mycelial aggregation and

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morphology. This aligns with Cheng et al. [44], who improved *Pleurotus* sp. pellet compaction and circularity using olive oil (containing oleic acid) combined with the surfactant Tween 80 (a synthetic source of oleic acid). Cheng et al. [44] speculated that Tween 80 might assist in olive oil emulsification, implying the lipid phase influences pellet formation. Observations from our study indicate oleic acid alone may promote similar characteristics, possibly by altering hyphal surface interactions due to its hydrophobicity, thus mimicking surfactant effects. Alternatively, this could be attributed to the potential of *P. ostreatus* to secrete extracellular lipases [41] and polysaccharides with surfactant-like properties (*e.g.*, β-glucan) [42], facilitating the emulsification of oleic acid. Although, the exact mechanism warrants further investigation, these findings underscore the significant impact of lipid-based carbon sources on fungal pellet morphology.

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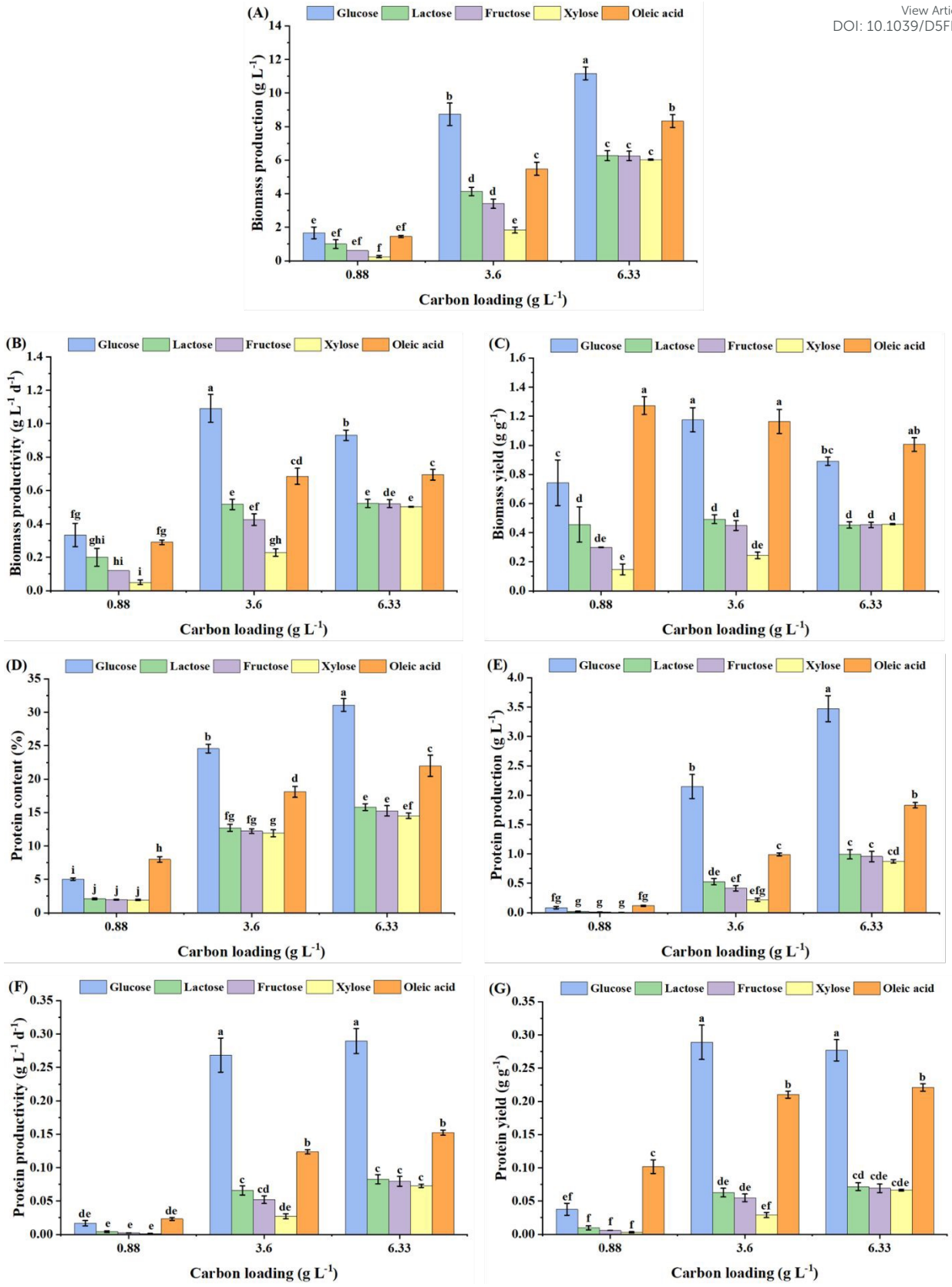

513

514 **Fig. 2.** Effect of different carbon loadings and carbon types on (A) biomass production, (B) biomass
515 productivity, (C) biomass yield, (D) protein content, (E) protein production, (F) protein productivity

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and (G) protein yield in *Pleurotus ostreatus*. * Groups that share same letters are considered statistically insignificant.

3.2.3. Effect of C/N and C/P ratios on mycelium biomass

P. ostreatus was cultivated under different C/N ratios of 65.5, 9.4, 5.0, 3.4 and 2.6, which were achieved by varying the nitrogen loading in the media to 0.06, 0.39, 0.72, 1.05 and 1.38 g L^{-1}, respectively, at a fixed carbon loading. Biomass production and productivity were observed to increase significantly with increment in nitrogen loading and decrement in C/N ratio (Fig. 3A), attaining highest values of 9.8 g L^{-1} and 1.23 g L^{-1} d^{-1}, respectively, at a N-loading of 1.38 g L^{-1} (C/N=2.6). These trends are in alignment with the findings of Bakratsas et al. [23], where increasing production of *P. ostreatus* biomass was achieved with increase in the concentration of yeast extract from 5 to 15 g L^{-1}, which can be indirectly attributed to increase in nitrogen loading. Biomass yield was remarkably low (0.4 g g^{-1}) at the lowest nitrogen loading (highest C/N ratio, *i.e.*, 65.5), however, comparable yields in the range of 0.88 – 1.09 g g^{-1} were obtained with further increment in nitrogen loading from 0.39 to 1.38 g L^{-1} (Fig. 3B). Cao et al. [32] reported similar observations in *Aspergillus awamori*, wherein, a high C/N ratio of 153 (control) resulted in the lowest production (~5 g L^{-1}) and yield (~0.5 g g^{-1}) of biomass, however, a reduction in C/N ratio to 15 led to substantial increment in the values (14.00 g L^{-1} and 0.98 g g^{-1}), relative to the control.

The improved growth metrics achieved at lower C/N ratios could be attributed to higher nitrogen concentration in the media, which is critical for the endogenous production of nucleotides, amino acids and other supporting cofactors. Increased availability of easily assimilated organic nitrogen sources (like those in yeast extract) directly fuels pathways like the glutamine synthetase cycle, boosting the production of amino acids needed for protein synthesis and overall cell structure.[45] Simultaneously, ample nitrogen ensures the production of purine and pyrimidine bases for nucleotide synthesis, supporting DNA replication and RNA production required for cell division and growth.[30] Furthermore, many essential enzymatic cofactors (*e.g.*, nicotinamide adenine dinucleotide and flavin adenine dinucleotide) also contain nitrogen atoms. With a preferable carbon substrate such as glucose providing energy and carbon skeletons, abundant nitrogen allows these precursors to be readily assimilated into biomass, resulting in higher biomass titers. Conversely, a high C/N ratio leads to faster nitrogen depletion, and subsequent nitrogen starvation causing earlier transition of the fungi into stationary phase, and consequent reduction in biomass growth and yield.[32]

In the next step, *P. ostreatus* was evaluated under varying C/P ratios of 52.9, 6.9, 3.7 and 2.5, which were obtained by supplementing different loadings of phosphorus to the media, *viz.*, 0.07, 0.52, 0.98 and 1.43 g L^{-1} (corresponding to KH_2PO_4 concentrations of 0.3, 2.3, 4.3 and 6.3 g L^{-1}), in addition to the control unit without phosphorus (0 g L^{-1}). The highest biomass production (4 g L^{-1}) and productivity (0.5 g L^{-1} d^{-1}) were achieved at the highest C/P ratio of 52.9 (corresponding to a

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phosphorous loading of 0.07 g L^{-1}), which were significantly higher than the control ($p < 0.05$), but were comparable with the performance at lower C/P ratios or higher phosphorous loadings (Fig. 3D). Biomass yield was obtained in the range of 0.9 – 1.04 g L^{-1} d^{-1}, clearly demonstrating that variation of phosphorous loading did not impose any impact on the yield (Fig. 3E). Liang et al. [28] reported a similar pattern in *Phanerochaete chrysosporium* involving increase in biomass titer from 0.002 to 2.006 g L^{-1} with initial increase in KH_2PO_4 concentration from 0 to 0.05 g L^{-1}, however, further increment in KH_2PO_4 loading (0.5 – 4.0 g L^{-1}), led to reduced growth rate producing only 2.397 – 2.678 g L^{-1} of biomass, which was not significantly different from the biomass titer attained at lower KH_2PO_4 loading of 0.05 g L^{-1}. Hence, it can be inferred that although availability of phosphorus is critical for boosting the growth of fungi, excess availability of phosphorus beyond a certain threshold does not impose substantial impact on biomass production.

3.2.4. Effect of C/N and C/P ratios on mycelium protein

Similarly, the increase in nitrogen loading and corresponding reduction in C/N ratio (from 65.5 to 2.6) led to significant increase in protein content from 1.84% to 39.7%, and equivalent protein production from 0.01 to 3.89 g L^{-1} (Fig. 3C). There is lack of studies delving into the effect of C/N ratio on protein production in submerged fermentation of filamentous fungi. However, the effect is well-studied in other proteinaceous cell factories. For instance, microalgae *Chlorella vulgaris* exhibited similar pattern in enhancement of protein content from 27.61% to 61.56% with the decrease in C/N ratio from 32:1 to 12:1, which has been primarily attributed to higher availability of nitrogen to the intracellular protein synthesis machinery at lower C/N ratio.[46] Likewise, as the C/N ratio declined from 50 to 24, single-cell protein production was observed to increase from 3.7 to 8.4 g L^{-1} in red yeast *Sporidiobolus pararoseus*.[47] A lower C/N ratio, associated with a higher nitrogen availability stimulates the cells to build more proteins primarily to support cell growth [47], which possibly explains the reason for higher protein content associated with higher biomass production in the present study. Moreover, the highest protein content attained in present study (39.7%) aligns well with the range (42.8 – 44.0%) reported for *Pleurotus ostreatus* cultivated using synthetic media as well waste substrates, *e.g.*, wine lees and fibre sludge-derived cellulosic hydrolysate.[10, 23]

The maximum protein content (24.0%) and protein production (0.96 g L^{-1}), achieved at a phosphorous loading of 0.07 g L^{-1} (equivalent C/P ratio of 52.9), exhibited increments of 10.2% and 52.1%, respectively, relative to the control without phosphorus (Fig. 3F). However, further increase in phosphorous loading did not cause any significant changes in the protein content and production. Similar observations were reported in *Mucor indicus*, where variation in KH_2PO_4 concentration between 0 and 7.5 g L^{-1} did not cause any significant changes in the protein content which was secreted within a range of 505 to 520 g kg^{-1} biomass under different conditions.[29] In the present study, the plateauing of biomass production at higher phosphorous loadings beyond 0.07 g L^{-1}, could be attributed to feedback

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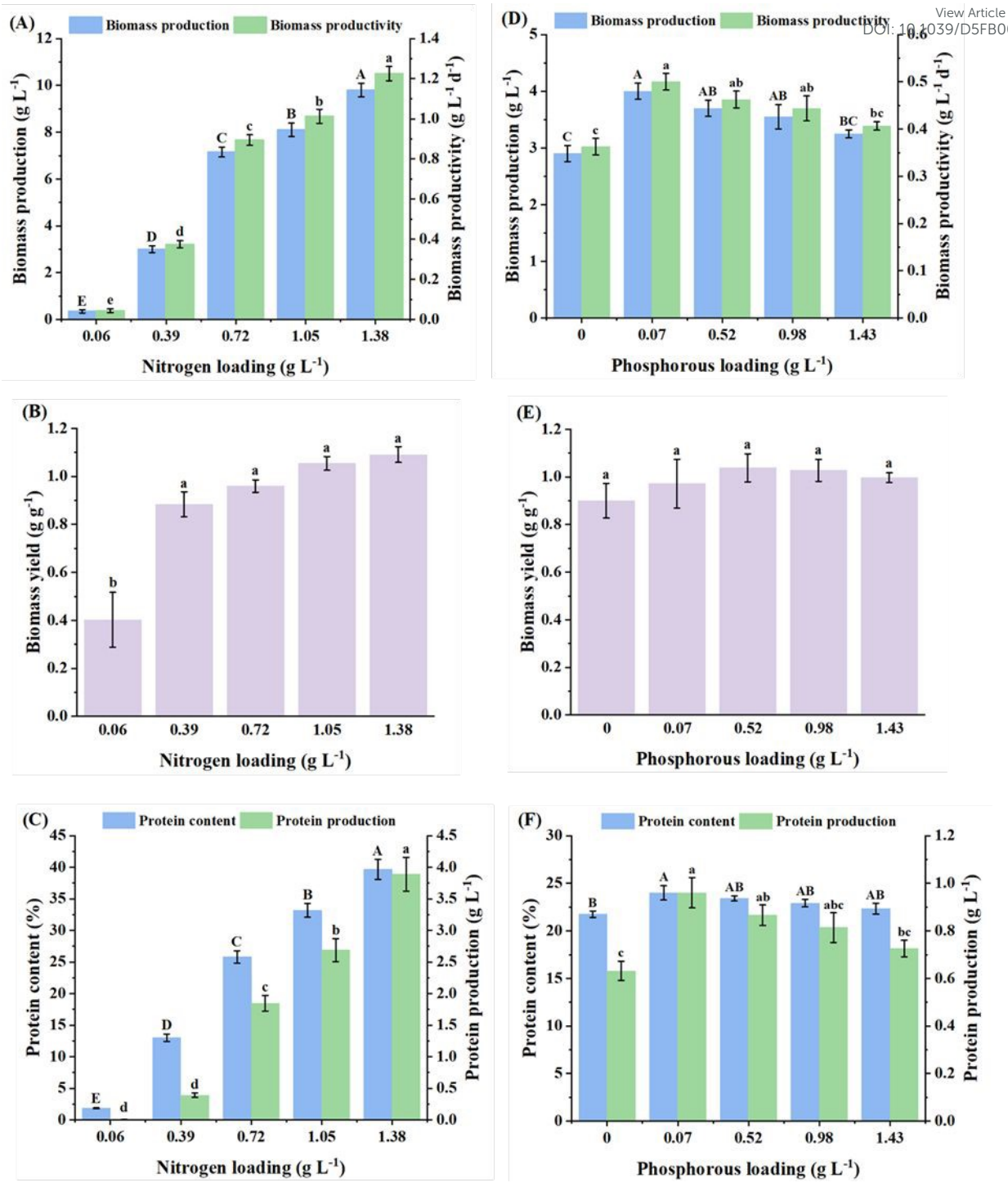
inhibition, implying that at 0.07 g L^{-1}, the fungal cells were able to fill their internal phosphorus reservoirs (*e.g.*, ATP, polyphosphate granules), thereby, signaling the transporters to slow down or stop importing more phosphorus.[48] Beyond this concentration, even with abundant external phosphorus, the cell's regulatory systems might stop taking it up, thus plateauing the growth. Furthermore, since the accumulation of protein is associated with growth as previously reported for other species, *e.g.*, *Chlorella pyrenoidosa* [49], likewise in the present study, saturation in protein content and production were observed with plateauing of biomass production beyond 0.07 g L^{-1} phosphorous loading.

To the best knowledge of the authors, this is the first study demonstrating the effect of C/P ratio on the production of biomass and protein in submerged fermentation of filamentous fungi. However, the effects of C/P ratio observed in the present study are comparable with the outcomes of other studies concerning non-fungi-based cell systems. For instance, variation of C/P ratio between 282 to 474 did not cause a significant change in biomass production, showing saturation within a range of 48.1 – 48.9 g L^{-1} in the yeast *Sporidiobolus pararoseus*.[47] Based on the present findings, nitrogen loading of 1.38 g L^{-1} and phosphorous loading of 0.07 g L^{-1}, corresponding to C/N and C/P ratios of 2.6 and 52.9, respectively, were selected for further experiments. Variation of C/N and C/P ratio did not impose a significant impact on the morphology of the mycelium pellets in terms of size distribution pattern and external appearance of the surface (data not shown). In general, these pellets exhibited the same morphological attributes as those cultured using glucose at a carbon loading of 3.6 g L^{-1} (Fig. 5B (i)). This implies that pellet morphology remains consistent under fixed sources of carbon and nitrogen, unless other fermentation parameters are altered (*e.g.*, inoculum fragmentation, inoculum density and agitation).



608

609 **Fig. 3.** Effect of varying nitrogen loadings on (A) biomass production and productivity, (B) biomass
610 yield, and (C) protein content and protein production in *Pleurotus ostreatus*. Different nitrogen
611 loadings (0.06 – 1.38 g L^{-1}) correspond to equivalent C/N ratios of 65.5, 9.4, 5.0, 3.4 and 2.6,
612 respectively. Effect of varying phosphorous loadings on (D) biomass production and productivity, (E)
613 biomass yield, and (F) protein content and protein production in *Pleurotus ostreatus*. Different
614 phosphorous loadings (0.07 – 1.43 g L^{-1}) correspond to equivalent C/P ratios of 52.9, 6.9, 3.7 and 2.5,
615 respectively. * Groups that share same letters are considered statistically insignificant.

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3.3. Effect of inoculum density and agitation rate

Inoculum density and agitation rate were observed to have significant effects on growth as well as the morphology of pellets in terms of size and number. Biomass production, productivity and yield were observed to increase with increase in inoculum density from 20 to 40 mg L^{-1} under different agitation rates (Fig. 4A, 4B and 4C). Although these growth parameters exhibited significant improvement with the increase in agitation rate from 75 to 150 rpm, further increment in agitation to 225 rpm, resulted in substantial decline in biomass growth in terms of titer, productivity and yield (Fig. 4A, 4B and 4C). Cao et al. [32] reported similar observations in *Aspergillus awamori* culture where maximum biomass concentration was attained at 150 rpm, however, the production was observed to decline under both lower (50 rpm) as well as higher (250 rpm) agitation rates. The decline in biomass growth at higher agitation rate has been attributed to the fragmentation of pellets induced by high hydrodynamic shear. The broken pellets subsequently attempt to restructure into new pellets; the underlying process consumes energy, thereby diverting it away from active biomass formation and consequent decline in growth.[32] In the present study, highest biomass production (12.9 g L^{-1}), productivity (1.61 g L^{-1} d^{-1}) and yield (1.43 g g^{-1}) were attained using an inoculum density of 40 mg L^{-1} at 150 rpm; which were 23.4, 23.4 and 23.1% higher than the respective values at lower inoculum density (20 mg L^{-1}) under the same agitation rate. These observations are in alignment with the study reported by Shen et al. [35], where biomass production was observed to increase with the increment in inoculum density (from 8 to 40 mg L^{-1}), resulting in a maximum titer of 10.22 g L^{-1} at 40 mg L^{-1} inoculum concentration in *Inonotus hispidus* fermentation.

The lowest agitation rate of 75 rpm resulted in single, large-sized mycelium pellets with average sizes of 63.8 mm and 72.7 mm at inoculum densities of 20 and 40 mg L^{-1}, respectively (Fig. 5C). Fig. 5C shows the morphology of the pellets harvested on Day 8. The pellet sizes reduced and the pellet count increased significantly with the increase in agitation to 150 rpm. Whereas, further increment in agitation rate (from 150 to 225 rpm) did not impose a significant difference on the size nor the number of the pellets under specific inoculum densities (Fig. 4D, 4E and 5C). The average pellet sizes obtained at 150 rpm were 2.02 mm (20 mg L^{-1}), 1.85 mm (40 mg L^{-1}), and at 225 rpm were 1.83 mm (20 mg L^{-1}) and 1.66 mm (40 mg L^{-1}), as shown in the Box and Whisker plot in Fig. 4D. A few other studies have reported similar observations in terms of reduction in pellet size with increase in agitation rate. For instance, average pellet diameter of *Neurospora intermedia* was observed to reduce from 6.54 ± 0.62 mm to 1.92 ± 0.33 mm, with the increase in agitation from 100 to 150 rpm.[22] Although inoculum density did not have a remarkable effect on the size of the pellets at higher agitation rates (150 – 225 rpm), the pellet number was observed to increase significantly ($p < 0.05$) with the increase in the inoculum density at the highest agitation rate of 225 rpm, *i.e.*, 705 pellets at 20 mg L^{-1} to 1154 pellets at 40 mg L^{-1} (Fig. 4D and 4E).

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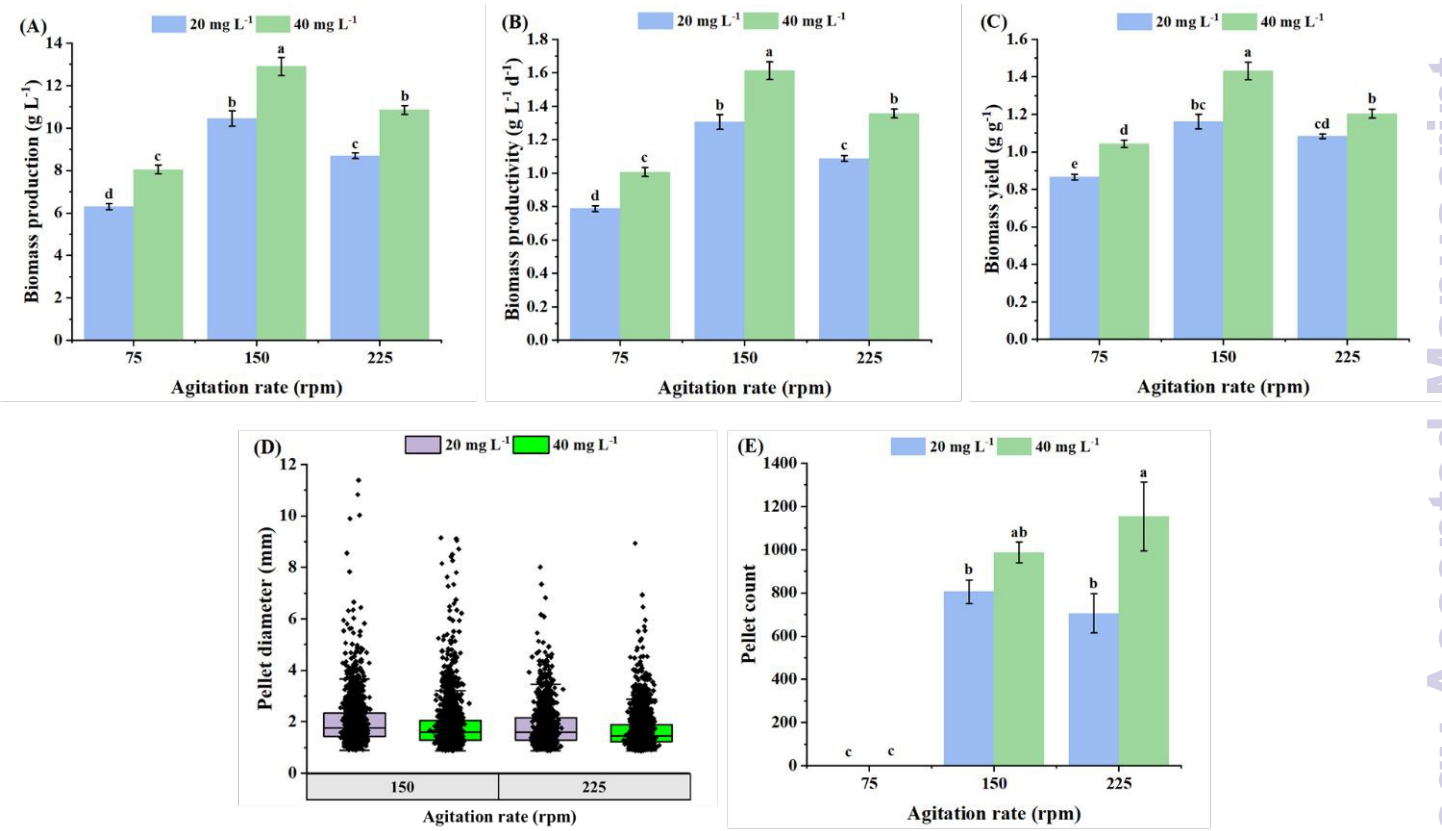

Fig. 4. Effect of different agitation rates and inoculum densities on (A) biomass production, (B) biomass productivity, (C) biomass yield, and (D) diameter of mycelium pellets on Day 8 (Quartile summary of the Box and Whisker plot is provided in Supplementary information, Table S2). Only a single pellet was observed at 75 rpm with a mean diameter of 63.8 mm and 72.7 mm at inoculum densities of 20 and 40 mg L^{-1}, respectively, and therefore, has not been included in the Box and Whisker plot, and (E) number of pellets in 10 mL culture. Inoculum densities are on dry weight basis. * Groups that share same letters are considered statistically insignificant.

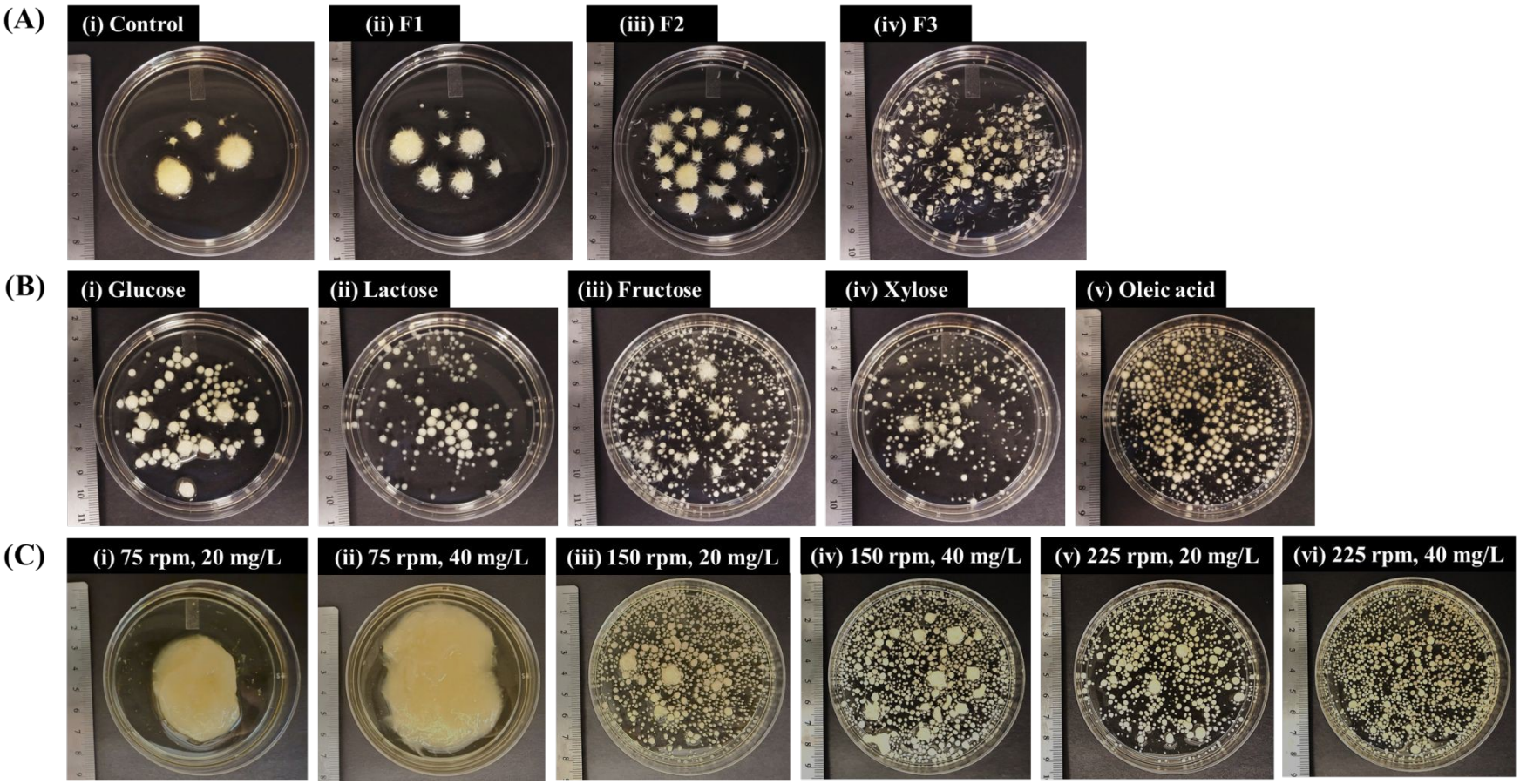

Fig. 5. Morphology of mycelium pellets under different (A) fragmentation levels. Control: no homogenization; F1: 3000 rpm, 5 s; F2: 5000 rpm, 10 s; and F3: 5000 rpm, 40 s; (B) carbon sources at 3.6 g L^{-1} carbon loading harvested on Day 8; and (C) inoculum densities and agitation rates.

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From a technological perspective, controlling *P. ostreatus* pellet morphology via inoculum fragmentation, inoculum density, agitation, and media formulation can profoundly impact industrial downstream processing. In the present study, compact, uniform pellets, achieved using fragmentation level F3, inoculum density of 20 mg L^{-1}, and agitation rate of 150 rpm, are significantly more advantageous for industrial-scale operations than large, loose, and heterogeneous mycelial clumps. The production of compact, uniform pellets is further supported by media components like glucose and oleic acid, which facilitate rounder pellet formation. In terms of primary recovery, such uniform pellet characteristics can facilitate more predictable filtration and dewatering rates. The compact nature of these pellets, combined with their smoother surface topography, reduces the entrapment of interstitial water within the fungal matrix, which can enhance drying efficiency and reduce the energy footprint of moisture removal. Beyond harvesting, compact pellets provide a higher biomass density (as observed in Sections 3.1 and 3.3), that can improve the efficiency of mechanical cell disruption, ensuring a more consistent release of intracellular proteins. Furthermore, a standardized size distribution is critical for texturization processes like extrusion, where uniform raw material ensures consistent rheological behavior and heat transfer.[50] This morphological control, governed by both physical and chemical parameters, is therefore essential for ensuring consistent quality and streamlining the transition from fungal biomass to a structured food ingredient.

3.4. Principal Component Analysis

Principal Component Analysis (PCA) was conducted to study how submerged fermentation parameters influence biomass and protein production, and substrate consumption. Essentially it reduces the dimension of variables from the primary data into principal components and visualizes how much variance of each experimental factor was weighted. It was found that two major principal components, PC1 and PC2, describe 77.92% of the total variation; and a third principal component, PC3, provides an additional 12.7% of the variation (as shown in Supplementary information, Fig. S5). The loading plot (Fig. 6A) demonstrates the relative weight of each variable in PC1 and PC2, where all factors except for phosphorous loading contribute positively to PC1, and carbon loading and substrate consumption contribute negatively to PC2. The dots in score plot represent the coordinates of individual data points in the new principal component space, with different carbon sources being distinguished by color. The results suggest that the majority of observations with glucose as carbon source contribute positively to PC2, while observations with other carbon sources including lactose, fructose, xylose and oleic acid contribute negatively to PC2. Pearson Correlation Analysis (Fig. 6B) was performed to understand the correlations among all variables. The analysis suggests that among three key medium constituents including carbon, nitrogen and phosphorous loadings, carbon loading had a significantly positive correlation with production of both biomass and protein, and substrate consumption. Meanwhile, nitrogen loading only had a strong positive correlation with protein production, rather than

biomass. Phosphorous loading did not have any significant correlation with production or substrate consumption. These results are in good agreement with our previous findings.

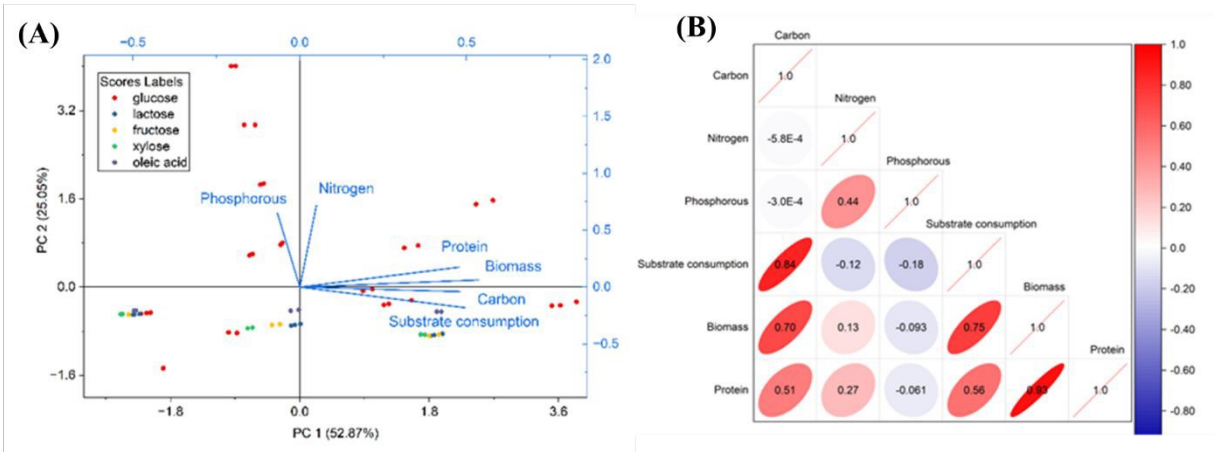

Fig. 6. (A) Biplot of Principal Component Analysis; and (B) Pearson Correlation Matrix on the medium composition, carbon substrate consumption, biomass and protein production from submerged fermentation of *P. ostreatus*

4. Conclusions

The study herein developed a robust framework for enhancing mycelium-based protein production from *Pleurotus ostreatus* in submerged fermentation. The results highlight that precise control of mycelial morphology can be achieved under specific inoculum fragmentation, agitation and inoculation density. The media composition influences protein production, where a low C/N ratio (2.6) and a high C/P ratio (52.9) provided the highest protein content of mycelium (39.7% of biomass, d.b.). By integrating these findings with appropriate agitation and inoculum density, the process achieved a maximum biomass titer of 12.9 g L^{-1}, with a productivity of 1.61 g L^{-1} d^{-1}. Furthermore, statistical analysis via PCA confirmed that carbon loading strongly influenced both biomass and protein production, whereas nitrogen loading was a significant driver primarily for protein synthesis, and phosphorous loading showed no significant correlation with either metric. This outlines a potential strategy for leveraging carbon and nitrogen-rich waste streams to optimize sustainable co-production of mycelium biomass and protein at industrial-scale. Moreover, this work establishes oleic acid as a viable non-sugar carbon source, demonstrating the potential to improve the economic feasibility of large-scale production by valorizing lipid-rich waste streams (*e.g.*, used cooking oils or agricultural byproducts), thus avoiding competition with arable land and food crops required for conventional sugar feedstocks. This research advances the development of mycelium as a sustainable, food-grade vegan

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protein, offering a promising alternative to conventional animal-sourced protein. However, industrial-scale submerged fungal cultivation could be constrained by high broth viscosity, limited oxygen transfer, and pellet clumping that can obstruct/adhere to bioreactor components like baffles, leading to process inconsistencies. Future research must address these scale-up dynamics by managing rheological behavior and maintaining morphological stability in large-scale bioreactors. Concurrently, further exploration can be directed towards deciphering the molecular-level mechanisms governing the variable performance of the species in response to media formulation.

Author contributions

Krishna Kalyani Sahoo: Investigation, Visualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Bruno Meireles Xavier:** Resources, Supervision. **Mohammad Afiq Hafiy Mohammad Taufiq:** Investigation, Writing - Original Draft. **Ke Wang:** Conceptualization, Supervision, Funding acquisition, Formal analysis, Writing - Original Draft, Writing - Review & Editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the Supplementary Information.

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Data availability

The data supporting this article have been included as part of the Supplementary Information.