MICROBIAL PROTEIN FROM RECOVERED NITROGEN: NUTRITIONAL QUALITY, SAFETY, AND FEASIBILITY ASSESSMENT

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ABSTRACT:

In contrast to traditional agriculture, microbial protein (MP) production is highly efficient in nitrogen (N) usage and can be employed to valorize a variety of recovered resources, thereby increasing the overall sustainability of food production. The present study aimed to establish the potential of seven recovered N sources originating from different waste streams for MP production using ethanol and acetate as growth substrates. The evaluation was based on specific growth rate, biomass yield, nutritional quality (*i.e.* macromolecular composition, amino acid (AA) and lipid profile) and food safety (*i.e.* concentration of heavy metals, polyaromatic hydrocarbons (PAH), pesticides and antibiotics) of the MP. The majority of the recovered N sources did not affect the kinetics and had a minor impact on the biomass yield, compared to their commercial equivalents. The nutritional content of the biomass was similar to soy flour and did not show major variations in AA and lipid profile for the different recovered N sources. Considering the heavy metal content, an average-weighing adult should not consume more than 53 – 213 g of the microbial biomass produced on recovered N per day due to its high copper content. A substantial amount of PAH were also found in the biomass. A daily consumption of 20 g/person/day would impose 2.0 – 2.8 times higher dietary exposure than the mean PAH exposure through nutrition in the EU, indicating a potential concern for human health. On the other hand, the biomass was free of antibiotics, and the traces of pesticides found did not raise any major concern for food applications. Based on the results of this work, no evidence was found to restrict the application of microbial biomass produced on recovered nitrogen as food.

Keywords: microbial protein, recovered nitrogen, nutritional quality, food safety

1 INTRODUCTION

About 80% of the nitrogen (N) supplied as fertilizer to produce edible crops is lost in the environment before it ends up on our plate. In the case of animal products, due to higher inherent losses within the feed and food chain, the N losses reach values up to 90% (Pikaar et al., 2017). The majority of this N ends up in our waterways and soil, causing eutrophication, hypoxia, soil degradation, and biodiversity loss (Sutton et al., 2013). Microbial protein (MP), the biomass of bacteria, yeasts, filamentous fungi, and microalgae that can be used for food applications, is a protein production strategy characterized by high N efficiencies (Goldberg, 1985). Thanks to use of bioreactor-based processes, MP offers the significant advantage over traditional agriculture to be produced in a closed system with control over incoming and outcoming fluxes (Pikaar et al., 2017). This approach yields a highly nutrient-efficient process where unused N streams can be recycled internally or treated before being released into the environment (Pikaar et al., 2018). MP has been presented by a multitude of studies as an attractive solution to tackle: *i)* the N inefficiency of animal and plant-based food production, *ii)* the excessive release of organic N in the environment, and *iii)* the potential to use recovered N from waste. In addition, the versatility of microbes enables the use of many different recovered N sources (*e.g.* urea, ammonium salts, ammonia water or gas and nitrate salts) (Goldberg, 1985), making MP highly promising in view of circular N management.

In the context of MP production from waste-derived N, one can distinguish two main approaches: *i)* MP cultivation directly in the N-rich liquid waste stream (with existing proofs of concepts using human urine and different types of wastewater originating primarily from the food processing industry) and *ii)* MP production with N recovered from waste via different technologies. The latter offers the potential to separate N from other components and contaminants present in the waste stream and/or concentrate N (thereby leading to higher biomass concentrations), facilitating the use of a much wider variety of waste streams than the first, direct approach. Examples of some of these N recovery technologies are struvite precipitation (Mehta et al., 2015; Zarebska et al., 2015), air or steam stripping and acid scrubbing (Bonmatí

and Flotats, 2003; Collivignarelli et al., 1998; Ippersiel et al., 2012), vacuum or vacuum membrane stripping (Ippersiel et al., 2012; Ukwuani and Tao, 2016), (bio) electrochemical cells (Rodríguez Arredondo et al., 2015), reverse (Gong et al., 2013; Mondor et al., 2008) or forward osmosis (Holloway et al., 2007) and electrodialysis (Pronk et al., 2006; van Linden et al., 2019). From these recovery technologies, struvite precipitation, air stripping and acid scrubbing are among the most mature technologies (Chang et al., 2022).

Among all the experimental studies investigating MP production from recovered N, only two considered the use of heterotrophic yeasts and bacteria cultivated with water-soluble substrates (*i.e.* acetate and cheese whey) (Guida et al., 2022; Matassa et al., 2022). The rest of the available scientific studies used inorganic gaseous substrates such as CO_2 and H_2 or organic gaseous CH_4 and biogas to produce their microbial feed/food product (Allegue et al., 2020; Khoshnevisan et al., 2020, 2019; Oesterholt et al., 2019; Pan et al., 2021; Pihlajaniemi et al., 2020; Tsapekos et al., 2020; Yang et al., 2022, 2021; Zha et al., 2021). However, using these gaseous substrates for MP production can give rise to high land area requirements, for instance in the case of CO_2 for microalgae cultivation or dedicated crops for anaerobic digestion to produce CH_4 and CO_2 (Ciliberti et al., 2016; Majid et al., 2014; Ritala et al., 2017), high capital investments, safety concerns (*i.e.* explosivity risks) and low volumetric productivities compared to heterotrophic MP production from liquid substrates (Senior and Windass, 1980; Vasey and Powell, 1984). On the other hand, heterotrophic MP production, for instance using ethanol and acetate, could yield a potentially carbon-neutral process when using substrates produced by combining CO_2 and H_2 through carbon capture and utilization technologies (Van Peteghem et al., 2022).

A limited number of studies have investigated the impact of using waste-derived N on microbial growth and productivity in comparison to their commercial equivalent (Christiaens et al., 2017; Guida et al., 2022; Khoshnevisan et al., 2020). Moreover, clear proof of the impact of using recovered N sources on both process performance parameters and the nutritional characteristics of the final protein product is lacking.

Given that N recovery can be capital- and energy-intensive (van Eekert et al., 2012), such considerations are essential to safeguard the economic competitiveness of these concepts. Furthermore, the degree to which organic and inorganic contaminants, potentially present in the waste-derived N, can accumulate in these novel food/feed ingredients has never been studied so far. However, such validation is crucial to comply with human food regulations (Turck et al., 2016).

In view of the above, the present study aimed to compare the MP production potential of seven selected recovered N sources originating from different waste streams (*i.e.* coke oven gas (COG), pig manure, municipal organic waste, (agro-)industrial waste), using ethanol and acetate as substrates (Figure 1). The selected N sources were recovered with three different technologies, namely air stripping combined with acid absorption, steam stripping combined with condensation and condensation of COG followed by neutralization with sulphuric acid. The suitability of each recovered N source for MP production was evaluated based on the specific growth rate, lag phase, biomass and protein yield and compared with their commercial equivalent through a series of 96-well plate screening tests. Two model microorganisms were used for MP production: the yeast *Metschnikowia pulcherrima* and the bacterium *Corynebacterium glutamicum*. Finally, a fed-batch experiment was performed at bench scale to produce yeast biomass and evaluate its nutritional quality and safety based on *i)* its macromolecular composition (*i.e.* proteins, lipids, carbohydrates, ash, and moisture) and the comparison with existing vegetable and MP alternatives such as brewer's yeast, spirulina and soy flour and *ii)* the level of food/feed contaminants that could arise from the waste-derived N matrix (*i.e.* heavy metals, poly aromatic hydrocarbons (PAH), antibiotics and pesticides).

2 MATERIALS AND METHODS:

2.1 MICROORGANISMS

The yeast *M. pulcherrima* MUCL 46194 was obtained from the Mycothèque de l'Université catholique de Louvain (BCCM™/MUCL collection, Belgium). The bacterium *C. glutamicum* DSM 20300 originated from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). These species were selected based on their *i)* ability to assimilate ethanol and acetate, *ii)* potential use as an MP product, and *iii)* best protein yield in comparison to three other MP-suited microorganisms grown on ethanol according to Van Peteghem et al. (2022). Both species are reported as food ingredients in the generally regarded as safe list (GRAS) published by the U.S. Food and Drug Administration (FDA) (FDA, 2022). Furthermore, *C. glutamicum* is recognized by the European food safety agency (EFSA) as a feed additive and novel food (Koutsoumanis et al., 2021) and is widely employed for industrial amino acid (AA) production (Kinoshita et al., 1957). *M. pulcherrima* has been used to modulate the sensory profile of wine (Morata et al., 2019) and is thus already used in the food and beverage industry.

2.2 SOURCES OF RECOVERED NITROGEN

The recovered N sources used in this study were obtained from different full-scale N recovery facilities in Belgium, the Netherlands, and Germany (Table 1). In most waste treatment facilities considered in this study, the waste was treated through anaerobic digestion (AD) to recover energy in the form of biogas. During this process, digestate is produced and separated through centrifugation into a solid and a liquid fraction (*i.e.* centrate). Depending on the targeted end product, N is recovered from the liquid fraction through stripping and acid absorption (*i.e.* ammonium sulfate – $(NH_4)_2SO_4$ and ammonium nitrate – NH_4NO_3) or via steam stripping and condensation (*i.e.* ammonia water – NH_4OH). In the case of N recovery from COG, the COG derived from the coking process is directed into a spray-type saturator. Here, ammonium sulfate is produced through the neutralization of ammonia with sulphuric acid. Downstream,

the ammonium sulfate lye is centrifuged and dried into salt crystals. Upon collection, the recovered N-sources were characterized and stored in sealed recipients at room temperature (Table S1, Figure S1).

2.3 EXPERIMENTAL PROCEDURES

The impact of the selected recovered N sources on the specific growth rate, lag phase, biomass and protein yield, as well as on the protein content of the MP, was determined via 96-well plate experiments. Among all the different N sources, those with the highest N content were selected as more attractive in view of future process upscaling, given that these sources imply lower transportation costs (from the N recovery facility to the MP production site) and do not limit volumetric productivity due to the excessive dilution that would be needed to supply sufficient N. Therefore, only $(NH_4)_2SO_4$ from COG, NH_4NO_3 from pig manure and municipal organic waste (PMOW), and NH_4OH from municipal organic waste (MOW) were used in fed-batch bioreactor experiments to characterize the biomass quality and identify potential contaminations from the recovered N source (Figure 1).

2.3.1 Inoculum preparation

Prior to the experiments, cultures were pre-grown at 28°C on LB Agar (Lennox, Sigma Aldrich, Germany). Subsequently, one colony was picked and transferred to LB broth (Lennox, Sigma Aldrich, Germany). The inoculum was prepared by: *i)* centrifuging at 6,603 g for 5 min (Centrifuge 5424 R G, Eppendorf, Belgium), *ii)* washing with 0.01 M phosphate-buffered saline (PBS, Sigma-Aldrich, Germany), *iii)* resuspending the washed pellet in 0.01 M PBS and, *iv)* correcting the cell concentration to a final optical density (OD) of 2.1 measured at 600 nm using a spectrophotometer (Infinite M200 Pro, Tecan, Switzerland). Finally, the washed pellet was resuspended in 0.01 M PBS. Each experiment was inoculated at 10% (v/v). This inoculum preparation procedure was followed in all experiments, while more specific information for each experiment is given in sections 2.3.2 and 2.3.3.

2.3.2 High throughput experiments

The impact of the different N sources on the kinetics and yield of MP production was assessed through 96-well plate tests performed at pH 6.8 and 28°C by using a plate reader (Infinite M200 Pro, Tecan, Switzerland), with OD measurements every 15 minutes and orbital shaking (180 rpm). The working volume of each well was 0.2 mL, and every condition was tested in ten replicates, by inoculating ten wells of a 96-well plate with the same inoculum. Adapted ammonium mineral salts (AMS) medium (excl. N source) was used as a cultivation medium with acetate (750 mg acetate/L) and vitamins (Table S2). Acetate was considered more suited for well plate experiments due to its lower volatility (boiling point at atmospheric pressure: 118°C) in comparison to ethanol (boiling point at atmospheric pressure: 78°C) (Green and Southard, 2019). The recovered and commercial N sources (Table 1) were filter-sterilized (0.2 µm PVDF filters, Chromafil®) and dissolved in the medium. At the end of each experiment, a homogenized sample was taken from the ten replicates to determine the acetate and ammonia concentrations. The same sample was then centrifuged at 20,807 g for 5 min. The supernatant was collected and stored at -20°C before analysis.

2.3.3 Fed-batch bioreactor experiments

The evaluation of the impact of selected N sources on the quality of the MP biomass was performed by cultivating *M. pulcherrima* in a 5L bioreactor (Applikon Biobundle, Getinge, Sweden) equipped with Ez-control (temperature, stirring, oxygen concentration, pH and level regulation), acquisition software BioXpert V and a peristaltic feeding pump WM 530SN/R2 (Watson-Marlow, England). The temperature was kept at 28°C using a heating blanket and cooling finger. Fed-batch operation was selected where each experiment was carried out in biological duplicates with a starting volume of 2 L and a final volume of 2.5L. Feeding was initiated after a batch phase of approximately 18 hours, at a rate of 200 mL/day, based on preliminary experiments. The pH was kept at 6.0 to operate within the preferred pH range of *M. pulcherrima*, using 0.5 M NaOH and HCl. Oleic acid was used as an antifoam and manually added during

the daytime or dosed every four hours via a peristaltic pump overnight (ISMATEC ISM834C, France). The dissolved oxygen (DO) level was maintained at a minimum of 30% of saturation by adjusting the aeration (1 – 2 volume of air per working volume per minute (vvm)) and agitation levels (400 – 600 rpm). In contrast to the well plate experiments, after the culture was pre-grown on LB broth, centrifuge, and washed, it was transferred to an adapted AMS medium (Table S2) with 2 g ethanol/L and 1.1 g NH_4Cl/L.

The cultivation medium inside the bioreactor was adapted AMS medium (Table S2), ethanol (2 g/L), supplemented with a recovered N source to reach a C/N ratio of 20 according to the findings of Van Peteghem et al. (2022). To achieve a high biomass concentration (*e.g.* 10 – 30 g CDW/L), a concentrated AMS solution (C/N 20, Table S3) with 40 g ethanol/L and the respective recovered N source was used as feed. The feeding solution was adapted to pH 6.0 using 2M HCl.

Sampling (ca. 25 mL) was performed three times per day for four consecutive days. Upon sampling, a volume of 8 mL of each sample was centrifuged at 20,817 g for 5 min. Subsequently, the supernatant was filtered (0.2 µm PVDF filters, Chromafil®) and stored (-20°C) for the determination of anions (*i.e.* NO_2^-, NO_3^-, Cl^-, SO_4^{2-}), cations (*i.e.* NH_4^+, Na^+), acetate and ethanol. The remaining sample was stored at -20°C for protein and total suspended solids (TSS) analyses. At the end of each experiment, the biomass was harvested through centrifugation at 7,500 g for 45 min (Avanti JXN-30 series, rotor: JLA-8.1000, Beckman coulter, Belgium), washed with 0.1 M PBS and again centrifuged (7,500 g, 45min). The resulting biomass paste was frozen at -20°C and was then lyophilized for seven days (Lyovapor L-200, Buchi). Finally, the biomass was ground using a mortar and pestle and stored at 4°C until subsequent analysis (section 2.5.2).

2.4 Analytical techniques

The biomass concentration, expressed as g cell dry weight (CDW) per L, was quantified as TSS according to standard methods 2540 D for the fed-batch experiments. In the case of the 96-well plate experiments, a calibration curve was used to convert OD into CDW for each organism. An Ion Chromatographer (930 Compact IC Flex; Metrohm®, Switzerland), equipped with a Metrosep C6-250/4.0 column and conductivity

detector, was used to determine the cations. The moisture content and ash fraction of the lyophilized biomass were determined gravimetrically by drying at 105°C (moisture content) overnight and incineration at 550°C for 1.5 hours (ash fraction). Anions were analyzed with an Ion Chromatographer (930 Compact IC Flex) with Metrosep A supp 5-150/4.0 column and conductivity detector (Metrohm, Herisau, Switzerland). The concentration of ethanol and acetate were determined using an HPLC (Prominence-i LC 2030 Plus, Shimadzu) equipped with a refractive index detector (RID-20A, Shimadzu) and an Aminex HPX-87H column with 9 µm particle size (7.8 x 300 mm, Bio-Rad), at an eluent (5 mM H_2SO_4) flow rate of 0.60 mL/min at 41°C.

The subsequent analysis were carried out for the biological duplicates of each condition tested in fed-batch, except for the analysis for antibiotics where only one replicate was used.

The analysis of cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), zinc (Zn), and arsenic (As) was preceded by digestion of the lyophilized biomass suspensions with HNO_3 (65 %, VWR) in a closed vessel (MARS 6 I-wave system, CEM, Belgium) and mercury (Hg) analysis was preceded by digestion with Au in a closed vessel. All metal concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer, NexION 300, United States of America).

Pesticides (Table S9) were extracted from lyophilized microbial biomass (5 g) by shaking the samples for 3 h at 300 rpm with acetonitrile (ACN). Samples were centrifuged for 5 min at 12,857 g, the supernatant was collected, evaporated with a rotary evaporator and dissolved in a mixture of ultra-pure water and ACN (9:1 v/v). The final detection and quantification were done through a multi-residue method (Claus and Spanoghe, 2020) using a LC–MS/MS (Waters ACQUITY UPLC, Xevo TQDmass spectrometer). Recoveries, limits of detection (LOD) and limits of quantification (LOQ) were generated through the spike-placebo method. Recovery values for the microbial biomass matrix for azoxystrobin and imazalil were respectively 23 ± 6% and 10 ± 0.36%.

For antibiotics analysis (Table S4), a sample of 1 g was suspended in 4 mL water, homogenized with antibiotics internal standards and left to equilibrate for 10 min. Subsequently, 6 mL of ACN-ULC grade was added, samples were vortexed for at least 30 s and centrifuged for 10 min at 1,900 g and 18°C. The supernatant was collected and evaporated at 60°C. The residue was redissolved in 1 mL ammonium acetate (0.2 mol/L), vortexed for 2 min, centrifuged for 5 min at 1,900 g and 18°C, and filtered (PTFE, 0.2 µm) before analysis. The analysis was performed using LC-MS/MS (Waters, Acquity, UPLC Premier HSS T3 with VanGuard FIT, 1.8 µm, 2,1 x 100 mm).

The PAH were extracted from lyophilized biomass by pressurized liquid extraction (PLE) using an ASE350 (Dionex). Extraction cells were filled with diatomaceous earth (Celite 545, Sigma-Aldrich), ca. 1.5 g of sample and 20 µL solution with recovery standards. Extraction was performed with hexane:acetone 3:1 v/v and the specific PLE settings were adopted from De Witte et al. (2019). Subsequently, the supernatant was evaporated to 1 mL with a Turbovap II evaporator and applied on a glass column (10 nm internal diameter) filled with 2.0 g of aluminum oxide (Aluminum oxide 90 active basic, Merck) and deactivated with 10% of deionized type 1 water. The extract was eluted with 15 mL hexane and again evaporated to 1 mL. Next, the extract was applied on a second glass column (10 nm internal diameter), filled with 1.0 g of silica (Silica gel 60, Merck) and conditioned with 10 mL hexane, and eluted with 28 mL of hexane. The extract was again evaporated and reconstituted in 0.5 mL isooctane, after which it was analyzed using GC-MS (Agilent 7890A GC) equipped with a PAH column (Agilent, 30 m, 0.25 mm, 0.25 µm) and MS detector (Agilent 5975C) (De Witte et al., 2019).

The total (crude) protein concentration was determined through the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, Belgium) with bovine serum albumin as a standard. The total carbohydrate content was analyzed with the colorimetric method of Josefsson (1983), measuring the colored derivative generated after L-tryptophan, sulfuric, and boric acid addition using a spectrophotometer (Spectronic 22, Thermo Scientific, Belgium) at 520 nm and D-glucose (≥99.5%, Carl Roth) as a standard. The determination

of the AA profile of the lyophilized biomass was done by HPLC with UV detection according to the AOAC method 2018.06, preceded by acid hydrolysis (6M HCl) for 24 hours and derivatization using 6-amino quinolyl-N-hydroxysuccinimidyl carbamate. Tryptophan content was determined after alkali hydrolysis followed by HPLC with fluorescence detection (Reference ISO 13904:2016). The fatty acid profile was established according to the ISO 12966-1:2014 reference method. Fatty acids were extracted from the lyophilized biomass by the Soxhlet method, hydrolyzed, converted into their methyl esters, and measured using a GC equipped with a flame ionization detector.

2.5 CALCULATIONS

2.5.1 Specific growth rate and lag time estimation

The OD corrected with the un-inoculated control of each sample was log-transformed and used to fit the Richards equation (Equation 1) using the nls.lm optimization algorithm from the minpack.lm package in R (Begot et al., 1996). The specific growth rate for each condition could be calculated from this fit, as described in Candry et al. (2018).

$$ln\left(\frac{\Delta OD}{\Delta OD_{min}}\right) = A * \left(1 + v * e^{1+v} * e^{\frac{\mu}{A} * (1+v)^{1+\frac{1}{v}} * (\lambda - t)}\right)^{-\frac{1}{v}} \quad \text{Equation 1}$$

The Richards equation estimates: carrying capacity (A), specific growth rate (μ), lag time (λ), and a shape factor with no biological meaning (v).

2.5.2 Biomass and protein yields

Biomass ($Y_{X/S}$) and protein ($Y_{P/S}$) yields for the well plate experiments were calculated according to equations 2 and 3. Biomass, substrate, protein, and acetate are indicated by X, S, p, and Ac, respectively. The initial and final amounts of biomass and acetate are expressed as CDW_i, Ac_i, and CDW_f, Ac_f, respectively.

$$Y_{X/S}\left[\frac{g\ CDW}{g\ Ac_{consumed}}\right] = \frac{(CDW_f[g\ TSS] - CDW_i[g\ TSS])}{(Ac_f[g] - Ac_i[g])} \quad \text{Equation 2}$$

269 $$Y_{p/S}\left[\frac{g\ protein}{g\ Ac_{consumed}}\right] = Y_{X/S}\left[\frac{g\ CDW}{g\ Ac_{consumed}}\right] * p\ \left[\frac{g\ protein}{g\ CDW}\right]$$ Equation 3

270 ### 2.5.3 Biomass nutritional quality and safety assessment

271 The potential of microbial biomass, produced with recovered N sources, as a human food source was
272 evaluated based on the nutritional quality and the presence of contaminants. About 30 g of *M. pulcherrima*
273 biomass was produced in a fed-batch bioreactor and subsequently lyophilized into dry microbial biomass.
274 The nutritional quality of the produced microbial biomass was compared to brewer's yeast, spirulina, and
275 soy flour. These reference products cover the same applications as the ones envisioned for the biomass
276 produced in this study (*e.g.* dietary supplements, a substrate for food extrusion of textured meat
277 alternatives, and additives in baked products). For the analyzed metals a distinction was made between
278 the different metals as follows: Cr and Ni are referred to as ultra-trace elements, since they are suggested
279 to have an essential biochemical function in humans but not clearly identified (Berdanier et al., 2007), Zn
280 and Cu are referred to as inorganic elements present in food essential to the development and functioning
281 of the human body (Solan, 2021) and all other measured metals (*i.e.* As, Pb, Cd, Hg) are considered as
282 elements in food that have a relatively high density and are toxic or poisonous at low concentrations
283 (Schrenk and Cartus, 2017) and are therefore referred to as heavy metals. As a reference level for
284 contamination of food by heavy metals and PAH, the highest and lowest limits defined by the European
285 Commission for various foodstuff were used (European Commission, 2011; European Parliament and the
286 Council of the EU, 2006). For the safety assessment, a recommended intake of 20 g/person/day was used
287 to calucalte related risks (Reed et al., 1990; Schulz and Oslage, 1976). This intake corresponds to the
288 recommende intake for brewer's yeast, which had the highest intake from the three chosen reference
289 products and therefore is used for the estimation of the highest risk.

290 ### 2.5.4 The amino acid score

291 The nutritional quality of the biomass was assessed via the AA score (Oser, 1959), which determines the
292 effectiveness of meeting the essential AA at a recommended level of protein intake (equation 4). A score

equal to or higher than 1 indicates a specific AA fulfills or exceeds the nutritional requirement per amount of provided protein.

$$AA\ score = \frac{mg\ AA/g\ protein}{mgAA/g\ protein\ in\ required\ pattern} \quad \text{Equation 4}$$

2.6 Statistical analysis

Prior to any statistical test, the normality of the distributions of lag time, growth rate, biomass, and protein yield was verified using QQ-plots and the Shapiro-Wilk test. An independent sample t-test was performed for the normally distributed data to identify a significant difference between average growth rates, lag times, and yields. The homogeneity of the variances was verified with Levene's test. The Wilcoxon signed-rank test was applied for all conditions with a non-normal distribution. P-values below 0.05 were considered statistically significant. All analyses were conducted in R (v3.6.1).

2.7 Data availability

The majority of the raw data is available in supplemental file. Other relevant data can be provided upon request.

3 Results

3.1 High throughput screening of the impact of the recovered N sources on growth kinetics and yields

The impact of different recovered N sources on the kinetics and yields of *M. pulcherrima* and *C. glutamicum*, was evaluated through 96-well plate experiments. The specific growth rates achieved using recovered N in the form of $(NH_4)_2SO_4$ presented insignificant differences (Table S5) from those achieved when the commercial equivalent was supplied as N source to *C. glutamicum* and *M. pulcherrima* (Figure 2; Table S5). The specific growth rates when the microorganisms were cultivated using NH_4NO_3 showed a similar trend. The specific growth rate of *C. glutamicum* and *M. pulcherrima* when using commercial

315 NH_4NO_3 was not significantly different (p = 0.44 – 0.76) from the recovered N source (Table S5). In the case
316 of *C. glutamicum* grown on recovered NH_4OH from PMOW and MOW, the average specific growth rate
317 was between 1.6 and 1.9 times lower compared to commercial NH_4OH. The growth rate of *M. pulcherrima*
318 grown with NH_4OH recovered from MOW was also 1.3 times lower than commercial NH_4OH. The other
319 two recovered NH_4OH sources (from PMOW and industrial and agro-industrial organic waste (IWAW))
320 showed an average specific growth rate of 0.29 h^{-1} that was not significantly different (p = 0.78 – 0.96)
321 from the commercial N source (0.29 ± 0.05 h^{-1}).
322 The biomass yield on acetate when using recovered N was equal to or lower (1.0 – 1.3 times) compared to
323 commercial N (Figure 3). A similar trend was observed for the protein yields, which were approximately
324 equal (0.91 – 1.0 times) and up to 2.2 times lower for the recovered N sources. Overall, biomass yields for
325 *C. glutamicum* were 1.3 – 2.8 times higher compared to *M. pulcherrima*, while protein yields were
326 comparable for both the bacterium (0.048 – 0.11 g protein/g $Ac_{consumed}$) and the yeast (0.06 – 0.10 g
327 protein/g $Ac_{consumed}$).
328 In summary, *M. pulcherrima* showed the smallest differences between specific growth rates, lag time
329 (Figure S2), and protein yields on commercial and their respective recovered N sources compared to *C.*
330 *glutamicum*. Typically, protein yields were lower for the recovered N sources in comparison to their
331 commercial equivalent. Only *M. pulcherrima* grown on $(NH_4)_2SO_4$ recovered from COG showed a protein
332 yield 1.1 times higher than that obtained with the commercial and recovered resources (Figure 3). In
333 addition, NH_4OH recovered from IWAW was the only recovered NH_4OH that yielded a significantly similar
334 specific growth rate for both *M. pulcherrima* and *C. glutamicum* grown on commercial NH_4OH. Similarly,
335 the biomass yield on NH_4OH recovered from IWAW was comparable to that obtained with commercial
336 NH_4OH for both *M. pulcherrima* and *C. glutamicum*. Considering these, $(NH_4)_2SO_4$ recovered from COG,
337 NH_4NO_3 recovered from PMOW, and NH_4OH recovered from IWAW were chosen for the subsequent fed-
338 batch experiments.

3.2 CONTAMINANTS PROFILE OF THE PRODUCED MICROBIAL BIOMASS

3.2.1 Heavy metals

The heavy metal content in the microbial biomass ranged from: 3.3 – 13 µg/kg biomass for mercury (Hg), 6.1 – 545 µg/kg biomass for cadmium (Cd), <8.3 – 29 µg/kg biomass for lead (Pb) and <17 – 20 µg/kg biomass for arsenic (As) (Figure 4A). In the samples from recovered NH_4OH and NH_4NO_3, respectively, Pb and As were not detected. Overall biomass from recovered NH_4OH had the lowest content in all heavy metals except for Cd compared to biomass produced with the other two recovered N sources.

3.2.2 Polycyclic aromatic hydrocarbons

All the PAH that were analyzed in this study were detected in the microbial biomass except for indo(123cd)pyrene (Figure 4B). The microbial biomass produced with recovered $(NH_4)_2SO_4$ from COG and NH_4OH from IWAW contained a similar amount of each one of the quantified PAH. Biomass produced with recovered NH_4NO_3 from PMOW had a PAH profile similar to the other recovered N sources, but a consistently lower content (1.1 – 26 times lower). The three types of microbial biomass contained high amounts of chrysene (38 – 93 µg/kg biomass), pyrene (21 – 65 µg/kg biomass), fluorathene (13 – 43 µg/kg biomass), benzo(a)anthracene (11 – 38 µg/kg biomass) and fenanthrene (8 – 23 µg/kg biomass). The content of the other PAH found in the microbial biomass varied between 0.8 and 9.6 µg/kg biomass.

3.2.3 Antibiotics and pesticides

The microbial biomass was analyzed for 85 different types of antibiotics (*e.g.* veterinary drugs) (Table S4). None of the analyzed veterinary drugs were detected in any of the biomass products. A list of all the analyzed pesticides can be found in Table S6. From the 110 analyzed pesticides two could be detected and quantified in at least one of the *M. pulcherrima* samples. Specifically, azoxystrobine was present in one of the two microbial biomass samples produced with recovered $(NH_4)_2SO_4$ from COG (24 µg/kg biomass), in

363 the other duplicate azoxystrobine was not detected. For biomass produced with recovered NH_4OH from
364 IWAW, azoxystrobine had an average content of 18 ± 3 µg/kg biomass. Imazalil was found in one of the
365 two *M. pulcherrima* samples produced with recovered NH_4OH, at a content of 7 µg/kg biomass.

366 ## 3.3 NUTRITIONAL QUALITY OF THE MICROBIAL BIOMASS

367 ### 3.3.1 Composition of the biomass product

368 All products, the reference and microbial biomass of *M. pulcherrima* produced here, are powdery food
369 products with a similar moisture content (≈ 5%) (Figure 5). The protein content in the microbial biomass
370 varied between 26 – 31% using the three different recovered N sources (Figure 5). *M. pulcherrima* grown
371 with recovered NH_4NO_3 from PMOW displayed the highest protein content, while the lowest content was
372 found in the biomass produced with $(NH_4)_2SO_4$ from COG. Similar to the protein, the carbohydrate content
373 was comparable for the three tested recovered N sources and varied between 27 – 33%. The lipid content
374 presented a higher variability between the different conditions (25 – 36%). Specifically, microbial biomass
375 produced with recovered NH_4NO_3 contained the lowest amount of lipids, and microbial biomass produced
376 with recovered $(NH_4)_2SO_4$ the highest. A slightly higher variability in the ash content over the different
377 samples of microbial biomass (3.8 – 6.7%) was noted.
378 Overall, the protein content of the microbial biomass (26 – 31%) produced in this work is 1.2 up to 2.2
379 times lower compared to the reference products (38 – 58%). On the other hand, the carbohydrate content
380 of *M. pulcherrima* (27 – 33%) is similar to spirulina and soy flour (24 – 32%), but brewer's yeast has 1.3 –
381 1.5 times more carbohydrates than *M. pulcherrima*. From all the major defined organic constituents, the
382 lipid content of the microbial biomass differs the most from the reference products (1.2 – 4.7 times higher
383 in the microbial biomass). The moisture and ash content covered a similar range for the reference products
384 (moisture: 4.7 – 5.2%; ash 4.5 – 6.2%) and microbial biomass (moisture: 5.0 – 7.0%; ash: 3.8 – 6.7%).
385 Amongst the products compared, *M. pulcherrima* biomass produced with recovered NH_4NO_3 and soy flour
386 show the best conformity in terms of macromolecular composition.

3.3.2 Amino acid profile

The amino acid profile of the three samples of *M. pulcherrima* biomass is highly similar for both essential and non-essential AA for human nutrition (Figure 6A). Here, all biomass samples from *M. pulcherrima* had an average AA score between 1.1 and 1.3 (Figure S4), where a value of 1.0 represents a perfect match with human requirements. Considering the individual AA, the three microbial biomass samples were mainly short in histidine, isoleucine, leucine, methionine and valine. For these five essential AA, the AA score ranged from 0.33 – 0.94. In the case of biomass production from recovered $(NH_4)_2SO_4$ and NH_4OH, lysine also did not reach an AA score of 1.0.

Overall, the reference products contained equal or higher amounts of each AA in their protein (g AA/g protein) than the microbial biomass from this study. The only two exceptions were the conditionally essential AA cysteine and the non-essential AA serine. All proposed reference products have an AA score above 1.0 for all the essential AA (Figure S4).

3.3.3 Fatty acid content

Small variations were noted in the fatty acid profile of the biomass produced with the three selected recovered N sources. The microbial biomass produced from NH_4NO_3 had the overall lowest lipid content (25 ± 1 %) (Figure 5). Specifically, the biomass contained less palmitoleic acid (10 – 17 times), oleic acid (1.2 – 1.3 times) and linoleic acid (1.4 – 2.3 times) compared to the other biomass produced in this study (Figure 6B). The microbial biomass was generally rich in unsaturated fatty acids, which comprised 24 – 35% of the total biomass, with the majority of it (22 – 30% of the total biomass) being oleic acid. Linoleic acid was the second most abundant fatty acid (1.9 – 4.4 % of the total biomass).

The fatty acid content of the selected reference products was substantially different than the microbial biomass produced in this work (Figure 6B). For instance, the fatty acids lignoceric acid (24:0) and eicosenoic acid (20:1), present in all microbial biomass, were not detected in any of the reference products. In

addition, brewer's yeast did not contain any essential fatty acids linoleic and α-linoleic acid. In the case of the produced microbial biomass, the former was the second most prevalent fatty acid in *M. pulcherrima* biomass. Oleic acid could be found in the three reference products but was 4.8 – 87 times more concentrated in the microbial biomass. In the case of spirulina and soy flour 27 and 58% of the total lipid content, respectively are essential fatty acids. For the microbial biomass, this ranged from 7.7 to 12%. All reference products contained more saturated fatty acids, 12 – 34% of the total fatty acids, compared to the microbial biomass (3.0 – 5.5% of the total fatty acids). Considering the complete product, soy flour and spirulina contain the highest amount of saturated fatty acids (2.7 – 3.0% of the biomass), while brewer's yeast (1.0% of the product) and microbial biomass (1.1 – 1.4% of the product) had similar content.

3.3.4 Minerals and ultra-trace elements

The measured ultra-trace elements of the microbial biomass produced from the three selected recovered N sources showed little variability, with values of 0.08 – 0.20 mg/100 g biomass for chromium (Cr) and 0.10 – 0.24 mg/100 g biomass for nickel (Ni). The mineral zinc (Zn) also displayed a small variability: 0.56 – 0.81 mg/100 g biomass (Figure 7). The copper (Cu) content of *M. pulcherrima* biomass produced from recovered NH_4NO_3 was the highest at 8.5 mg/100 g biomass. In contrast, the Cu content for biomass produced on recovered $(NH_4)_2SO_4$ and NH_4OH amounted to 3.6 and 2.0 mg/100 g biomass, respectively. The Cr content in the microbial biomass product generated in this study (0.08 – 0.20 mg/100 g) was lower or similar to the reference products (0.03 – 0.10 mg/100 g). The second quantified ultra-trace element, Ni, was 2.7 – 6.4 more concentrated in soy flour than in the microbial biomass. The mineral Cu was 1.2 – 39 times more concentrated in the microbial biomass in comparison to the reference products. Finally, Zn was 3.3 – 24 times more concentrated in the reference products as to the microbial biomass.

4 DISCUSSION

4.1 POTENTIAL OF RECOVERED N SOURCES FOR MICROBIAL PROTEIN PRODUCTION

From all the tested recovered N sources, NH_4OH recovered from MOW was the only N source that significantly impacted the specific growth rate of *M. pulcherrima* and *C. glutamicum* (Figure 2). The recovered NH_4OH from PMOW also negatively impacted the specific growth rate of *C. glutamicum*. Overall recovered N sources had little to no effect on the specific growth rates of *M. pulcherrima* and *C. glutamicum.* Guida et al. (2022) reached a similar conclusion for *C. glutamicum*, *Cupriavidus necator*, *Yarrowia lypolitica*, *Wickerhamomyces anomalus* and *Komagataella phaffii* grown with recovered NH_4OH from brines used in municipal wastewater treatment. Also, Khoshnevisan et al. (2020) observed no substantially different growth rates for methanotrophic bacteria produced with electrochemically extracted N from digestate (derived from MOW) and commercial NH_4Cl. In contrast, a series of studies did observe significant differences in growth rate when recovered and commercial N sources were used (De Paepe et al., 2020; Kurcz et al., 2018; Tsapekos et al., 2019; Zeng et al., 2023; Zha et al., 2021). However, in these studies, the difference in growth rate was presumably linked to the lack of other nutrients in the medium or different substrate concentrations. For example, Tsapekos et al. (2020) saw a higher growth rate with methanotrophic bacteria grown with commercial NH_4Cl compared to digestate because the latter was short in iron and copper. In summary, in this study and in results reported in literature, recovered N sources rarely impacted the specific growth rate of microorganisms used to generate MP products.

Next to the specific growth rate, the overall production potential of MP is also determined by the biomass yield. In accordance with the specific growth rate, the biomass yield of NH_4OH recovered from MOW grown with *M. pulcherrima* and *C. glutamicum,* as well as the biomass yield from recovered NH_4OH from PMOW grown with *C. glutamicum* was substantially lower (1.24 – 1.31 times) in comparison to their

commercial equivalent (Figure 3). Therefore, these specific recovered N sources are less suited for MP production. The biomass yields for *M. pulcherrima* and *C. glutamicum* were for all recovered $(NH_4)_2SO_4$, about 20% lower compared to commercial $(NH_4)_2SO_4$, even though their specific growth rates were not significantly different. Guida et al. (2022) also found more variation in the biomass yields for the same recovered N source grown with different heterotrophic yeasts and bacteria, while specific growth rates were not significantly different. The study of Tsapekos et al. (2020) revealed that semi-continuously fed methanotrophs (dilution rate: 0.008 h^{-1}) with pure methane and electrochemically recovered NH_4^+ from digestate had a slightly higher biomass production (2.01 ± 0.09 g CDW/L) in comparison to commercial NH_4Cl (1.81 ± 0.12 g CDW/L). On the other hand, Yang et al. (2021) did not observe a significant difference in biomass yield for commercial NH_4Cl and electrochemically recovered NH_4^+ from reject water and digestate with methanotrophic bacteria. In a similar set-up with NH_4^+ electrochemically recovered from MOW, biomass yields on recovered N were around 20% lower than growth with commercial N (Khoshnevisan et al., 2020). Based on the above, one can conclude that with careful selection of the recovered N sources it is possible to reach similar productivities as their commercial equivalents, thereby enabling N-neutral food production. Obviously, much depends on the selected recovered N source and microorganism(s).

4.2 Microbial biomass produced with recovered nitrogen could be safe for human consumption

Even though there is an increased interest in N recovery technologies, information on the complete composition of the generated recovered N product is lacking (Bloem et al., 2017; Deng et al., 2021). Moreover, these products are mainly used as a fertilizer for plant production. Introducing the recovered N in our food system via the more resource-efficient MP production route could give rise to safety concerns. However, the *M. pulcherrima* biomass from this study, produced for human consumption with recovered N, posed a minimal safety risk for most contaminants if a daily recommended intake of 20 g of

biomass was respected and special attention is given to the PAH content. Overall, the detailed safety assessment performed here could aid future policy strategies aimed at the introduction of recovered N in various biotechnological applications.

4.2.1 (Heavy) metals

Yeasts and bacteria can bioaccumulate metals (Eltarahony et al., 2021), *i.e.* if heavy metals are present in the recovered N, they could accumulate in the biomass. Metals like As, Cd, Hg, Pb, and Cr can only originate from the recovered N sources, while Zn, Ni, and Cu are also added via the trace element solution in the cultivation medium to support microbial growth (Table S3). Metals can be present in a wide variety of nutritious foods, some of these metals are considered essential for human nutrition (Cu, Zn, Cr), and some are toxic (*i.e.* As, Cd, Hg, Pb, Ni). However, given that all metals are toxic when a certain intake is exceeded, the provisional intake from the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the EFSA was considered to determine the tolerable daily intake (EFSA-CONTAM, 2014, 2010, 2009; EFSA, 2020; JEFCA, 1995). Based on the recommended intake of brewer's yeast (20 g/person /day)[1] and provided one consumes no other products that contain heavy metals (EC, 1997), the estimated intake of all metals ranged from 0.07 to 1,719 µg metal/day for the different MP from recovered N (Table S7). This corresponded to a maximal of 38% of the tolerable daily intake for an adult with an average body weight of 62 kg (Walpole et al., 2012). Surprisingly, not the heavy metals As, Cd, Hg or Pb (JEFCA, 1995), but the mineral, copper restricted the tolerable daily consumption of the different *M. pulcherrima* biomass samples. In other words, an average-weighing adult should not consume more than 53 – 213 g per day of MP produced on recovered N due to its copper content (Table S7). Theoretically, all copper accumulated in the biomass could originate from the growth medium. Possibly, the copper content could be reduced by reducing the copper concentration of the medium. The total amount of zinc added through the defined

[1] From the three refence products considered in this study, brewer's yeast had the highest recommended intake and therefore is used for the estimation of the highest risk.

503 medium is lower than the zinc content measured in the biomass, which indicates that at least a part of the
504 zinc in the biomass could originate from the recovered N sources. In summary, the microbial biomass
505 produced with recovered N from this study can be safely consumed by an average-weighing adult if the
506 recommended daily intake of 20 g is respected.

507 ### 4.2.2 Polycyclic aromatic hydrocarbons

508 PAH naturally occur in oil deposits and coal (Diggs et al., 2011) and due to the hydrophobic and lipophilic
509 nature of PAH, they can easily accumulate in the food chain (Pensado et al., 2005). The PAH can
510 contaminate food products through environmental (*e.g.* deposition on crops, oil spills in the sea) or
511 anthropogenic (*e.g.* cooking, industrial food processing) pathways (Bansal and Kim, 2015). No provisional
512 tolerable weekly intake or equivalent recommendation for PAH in food was found, possibly due to the fact
513 that less information is available about the toxicity of PAH in comparison to heavy meals for instance.
514 Instead, the risk of consuming 20 g of *M. pulcherrima* biomass daily by an average weighing adult of 62 kg
515 is assessed (section 4.1.1). The dietary exposure of the marker benzo[a]pyrene (BaP) for carcinogenic PAH
516 in food is calculated to range from 24 – 52 ng/person/day for *M. pulcherrima* biomass from recovered N
517 (Table S8). In contrast, the mean dietary exposure in the EU of BaP is estimated at 242 ng/person/day
518 (EFSA, 2008). In other words, the BaP intake via *M. pulcherrima* biomass from recovered N could be
519 considered acceptable. However, if chrysene, benzo[b]fluoranthene, and benz[a]anthracene (PAH4), are
520 also considered, the dietary exposure to BaP and chrysene and PAH4 from *M. pulcherrima* biomass from
521 recovered N is close to (0.85 – 1.1 times) or 2.0 – 2.8 times higher than the mean dietary exposure in the
522 EU. To effectively assess the risk related to this intake, the 95% lower confidence limit of the benchmark
523 dose for a 10% increase in tumor-bearing animals ($BMDL_{10}$), was chosen as a reference point to calculate
524 the margin of exposure (MOE) (EFSA, 2008; Larsen, 2006) (Table S8). Specifically, the MOE for *M.*
525 *pulcherrima* biomass from recovered NH_4NO_3 (ranging from 13,868 – 20,466 for the measured PAH)
526 indicated a low concern for consumer health, as this exposure is also in line with the MOE median in Europe

(9,346 – 17,949) (EFSA, 2008). However, the MOE for *M. pulcherrima* biomass from recovered $(NH_4)_2SO_4$ and NH_4OH varied between 5,594 and 8,524 for BaP and chrysene, and PAH4, which the EFSA Scientific Committee (EFSA, 2005) indicates as a potential concern for consumer health. This analysis reveals that, to use recovered N sources for MP production, special attention should be paid to their PAH content as well as the PAH accumulation in the biomass.

4.2.3 Other contaminants

Considering the waste streams ammonia water and salts are typically recovered from *e.g.* animal manure or sludge from agro-industrial wastewater treatment, food contaminants such as veterinary drugs and pesticides may also occur in the recovery product (Vaneeckhaute et al., 2017). In the *M. pulcherrima* biomass from recovered NH_4NO_3, none of the 85 tested antibiotics (Table S4) and 110 pesticides (Table S6) were detected. In the case of *M. pulcherrima* biomass from recovered $(NH_4)_2SO_4$ and NH_4OH, no antibiotics were found. However, the fungicides imazalil and azoxystrobin were both detected in biomass from recovered NH_4OH. The latter was also detected in *M. pulcherrima* biomass from recovered $(NH_4)_2SO_4$. This was unexpected given that the recovered $(NH_4)_2SO_4$ originates from COG, and azoxystrobin is neither used nor expected to appear at any point in the N recovery process from COG during storage or transportation. Nevertheless, it cannot be excluded that both fungicides entered the *M. pulcherrima* biomass during the harvesting and drying of the biomass, given that these processing steps were executed in a non-food grade lab environment, where these fungicides could have been present. Regardless of the origin of these fungicides, the low amount present in the *M. pulcherrima* biomass poses minimal risk. An average-weighing adult would have to consume 425,000 up to 1,016,000 g biomass/day to exceed the acceptable daily consumption of any detected pesticides (Table S9). In comparison, to meet the reference intake of protein about 161 up to 198 g biomass/day should be consumed (Table S10).

4.3 NUTRITIONAL QUALITY OF *M. PULCHERRIMA* BIOMASS FROM ETHANOL AND RECOVERED N

The macromolecular composition of the *M. pulcherrima* biomass generated in this work was closest to the composition of soy flour. Typically, the biomass contained similar or lower amounts of essential and conditionally essential AA than the three reference products, except for cysteine. The two essential fatty acids, linoleic and α-linoleic acid were both present in the produced microbial biomass, while these are absent in brewer's yeast.

4.3.1 Protein content and amino acid profile

In accordance with the results from the well plate experiments (Figure S3), the protein content (26 – 31%) showed little variation for the chosen recovered N sources (Figure 5). Given that ammonium was added in equimolar concentrations and *M. pulcherrima* cannot utilize nitrate (Senses-Ergul et al., 2006), it was evident that the different recovered N sources did not induce significant variations in the protein content. The protein content of the biomass in this study was also in line with the protein content of *M. pulcherrima* grown on ethanol and commercial NH_4Cl found in literature (31 – 33%) (Van Peteghem et al., 2022), but lower in comparison to the chosen reference products (Figure 5). Accordingly, the intake of 100 g of *M. pulcherrima* biomass covers 53 – 62% of the dietary reference intake for protein (EFSA Panel on NDA, 2012) (Table S10). Intake of 100 g of the reference products represents 77 to 115% of the dietary reference (Table S11).

In addition to the bulk protein content, the AA profile is another principal marker used to evaluate the nutritional quality of food. As for the protein content, the AA profile was almost identical for the three recovered N sources (Figure 5). This suggests the possibility that the type of recovered ammonium salt has little to no effect on the protein composition of microbial cells. All AAs essential for human diet were present in the *M. pulcherrima* biomass, but their overall content was lower than those of the reference products. While none of the reference products had an AA score lower than one for any essential AA, the *M. pulcherrima* biomass was deficient in histidine, isoleucine, leucine, methionine, and valine. It was also

found that *M. pulcherrima* biomass grown with ethanol and NH_4Cl had a deficiency for isoleucine, leucine, histidine, and methionine (Van Peteghem et al., 2022). Surprisingly, the *M. pulcherrima* biomass had a 1.5 up to 2.8 higher content of the sulfur-containing cysteine in comparison to the reference products. Cysteine is a conditionally essential AA and is important for infants and adults with a poor vitamin B status or that consume alcohol (WHO and UNU, 2007).

4.3.2 Lipid content and profile

M. pulcherrima is known for its antimicrobial, wine aroma-improving and lipid-accumulating properties (Morata et al., 2019). The *M. pulcherrima* biomass produced in this study had a total lipid content of 25 – 36%. Typically, lipids are accumulated in yeast biomass under N-limiting conditions (Certik and Shimizu, 1999). In none of the experiments, N was fully consumed, but concentrations were typically low at the end of the experiments (0.02 – 0.51 g ammonium/L). The final ammonium concentration was the lowest for *M. pulcherrima* grown with recovered NH_4OH (0.02 – 0.04 g ammonium/L) and the highest for recovered NH_4NO_3 (0.24 – 0.51 g ammonium/L), which corresponded to respectively the highest (36%) and lowest (25%) lipid content in the biomass. It is possible that N-based protein synthesis was jeopardized in favor of lipid synthesis (Abeln, 2020). The lipid content was also higher than all reference products (7.7 – 21%). Evidently, the intake of 100 g *M. pulcherrima* biomass covers a more significant part of the dietary lipid reference intake (43 – 51%) than for the reference products (11 – 30%) (Table S10 and S11). However, the majority of the lipids in the *M. pulcherrima* biomass were unsaturated fatty acids (95 – 97% of the total lipid content), while for the reference products, 66 – 87% of the total lipids were unsaturated. As a result, the consumption of 100 g of the reference products covers equal or higher portions (5 – 15%) of the saturated lipid dietary reference intake in comparison to the *M. pulcherrima* biomass (5 – 7%) (Table S10 and S11). In general, nutritional guidelines recommend reducing the intake of saturated fatty acids to minimize cholesterol levels and thus the risk of cardiovascular disease (EFSA Panel on NDA, 2010).

As for AA, some fatty acids are essential in a human diet since the human body cannot produce them. Both the essential fatty acids, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), were present in the *M. pulcherrima* biomass (Figure 5). Moreover, the linoleic acid content in the *M. pulcherrima* biomass was higher than the reference products brewer's yeast and Spirulina. The overall fatty acid composition of the *M. pulcherrima* biomass was in line with the composition from *M. pulcherrima* grown on sugars and was similar to olive oil (Abeln, 2020; USDA Agricultural Research Service, 2019). The most dominant fatty acid present in the biomass was oleic acid (84 – 86% of the total lipids). It cannot be excluded that some of the oleic acid in the biomass originated from its addition as antifoam. However, regardless of its origin, this fatty acid composition reflects that of the *M. pulcherrima* biomass that would be produced in an industrial context, where the addition of a food-grade antifoam such as oleic acid, would be required. Oleic acid is one of the most common monounsaturated fatty acids in human nutrition (Krishnan and Cooper, 2014) and diets rich in this fatty acid are suggested to be beneficial for regulating body weight and preventing obesity (Liu et al., 2016; Tutunchi et al., 2020).

4.3.3 Other macromolecular elements in biomass

Next to lipids and protein, the *M. pulcherrima* biomass was also rich in carbohydrates (27 – 33%) (Figure 5). Typically, the main carbohydrate storage compounds in yeasts are glycogen (*i.e.* polysaccharide) and trehalose (*i.e.* disaccharide) (Sols et al., 1971). For human nutrition, more complex carbohydrates are preferred over sugars (Who et al., 2003). Overall, the dietary reference intake for carbohydrates covers a similar range for the *M. pulcherrima* biomass (11 – 13%) and the reference products (9 – 16%) (Table S10 and S11). Principally, the moisture or water content plays an important role in the preservation of the product (Vickie et al., 2014). By making water unavailable for pathogenic or spoilage causing bacteria, the shelf life of this product can be extended (Vaclavik et al., 2014). The *M. pulcherrima* biomass was freeze-dried to reduce the moisture content to 5 – 8% to ensure long term preservation (Dupont et al., 2014). Similarly, the chosen reference products were also dried products. The final measured component of the

biomass was ash. The ash fraction typically represents the inorganic elements in the biomass, like essential minerals such as Ca^{2+}, K^{+}, Mg^{2+}, but also toxic heavy metals like As, Cd, Hg, Pb, Ni (Campanella et al., 1999; Sarwar et al., 1985). The total content of all metals measured in this study represents only 0.08 – 0.15% of the ash fraction. This indicates the predominance of non-toxic minerals.

4.4 Biomass from recovered N could cover more than half of the recommended daily protein intake, provided that nucleic acid, PAH and Cu content is reduced

In addition to the potential contamination arising from the utilization of recovered N sources, the safety of consuming dried yeast biomass is also determined by the nucleic acid (NA) content. Typically, yeast biomass has a nucleic acid content of about 10%(Ravindra, 2000; Reed et al., 1990) and given that the safe level for NA intake for humans is 4 g/day (Hall et al., 1967), the daily recommended intake for brewer's yeast is set at 20 g/person/day (Reed et al., 1990; Schulz and Oslage, 1976). However, commercial MP products such as Quorn™ or Torula yeast can be produced by including a NA reduction step, which reduces the final NA content to below 2% (Akin and Chao, 1974; Ritala et al., 2017; Trinci, 1994; Viikari and Linko, 1977). Hence, the suggested maximum daily consumption limit for Quorn™ is more than 100 g (Trinci, 1992). With an average dietary reference intake proposed by EFSA of 50 g protein/day (European Parliament and the Council of the EU, 2011), about half of the daily protein demand could be covered by *M. pulcherrima* biomass. However, in this case, the Cu content for *M. pulcherrima* biomass from recovered NH_4NO_3 would need to be reduced by 50% and the PAH content should be decreased by a factor 10 for all recovered N sources to be safe for human consumption.

4.5 IS THE CONSUMPTION OF MICROBIAL BIOMASS WITH RECOVERED N REALISTIC TODAY?

The type of process described in this paper can contribute to a more circular food production system, and the uncontrollable release of ammonia or other reactive nitrogen species can be avoided (Matassa et al., 2015; Pikaar et al., 2017). However, even though the biomass composition indicates that the use of recovered N does not result in any major concerns towards safe consumption (section 4.2), the commercial exploitation of such a process may present some challenges. The concept presented here is built on two main principles: *i)* the direct use of microbial biomass as food (ingredient) and *ii)* the use of recovered nutrients from various types of waste for food production. To bring microbial biomass onto the market, it must comply with food regulations and standards like any other type of food. In the case of microbial biomass, the product likely falls under the "novel food" regulations, where "novel food" is generally defined as food produced by a method that has not previously been used or that does not have a significant history of consumption (Good Food Institute, 2022). In other words, regulatory frameworks do exist, but in most cases, the product would have to be approved by governmental institutions like the Food and Drug Administration (FDA, United States of America), EFSA (European union) and National Health Commission (NHC) of the People's Republic of China before it can be commercialized. Yet, international legislation regarding the use of recovered N for food production is less straightforward. Specifically, no specific restrictions are in place in terms of quality of the substrates used for novel food production (Vapnek et al., 2020). In other words, recovered nutrients could in theory be used as long as they do not jeopardize the quality of the final product. However, strict regulations do exist when recovered nutrients are used as fertilizers for food or feed production, including limits on the levels of heavy metals and other toxic compounds potentially present in the fertilizer product (European Commission, 2019; US Law, 1976).

Moreover, insects (*i.e.* novel food) may be produced exclusively with feed grade materials[2] in the EU (IPIFF, 2021). It therefore remains unclear if the authorities would accept the concept proposed in this work.

One of the basic principles for bringing (novel) food onto the market is its compliance to legislation on contaminants in food. The presence of contaminants in food is often unavoidable, due to their ubiquitous presence or natural origin. To safeguard public health governments have defined maximum levels for certain contaminants in various foodstuff. In the case of the *M. pulcherrima* biomass produced from recovered N, none of the heavy metal concentrations exceeded the highest limit set for various foodstuff by the EU Commission (Table S12; Figure 4A). Moreover, none of the biomass samples from the selected recovered N sources had As and Hg concentrations above the lowest limit (European Parliament and the Council of the EU, 2006). In the case of As and Hg, maximum limits were respectively set for different types of rice products and fishery products. The Cd content in the biomass exceeded the limits set for some infant and child formulae, fruits, vegetables and tree nuts. The content in all samples was below any limit defined for different types of food supplements. Similarly, the biomass would not be suited as an infant and child formula based on the Pb content. While the Pb content remained well below maximum levels set for fruits, vegetables, meat and cereals. The FDA has established similar maximum levels for heavy metals in comparable food items as the EU regulations (European Parliament and the Council of the EU, 2006; FDA, 2023). The EFSA defined BaP and PAH4 as good indicators to assess health risk related to PAH present in food (EFSA, 2008). Accordingly the EU Commission defined maximum limits for BaP and PAH4 in food (European Commission, 2011). The lowest limits are set for infant formulae (1 µg BaP or PAH4 /kg) and all *M. pulcherrima* biomass produced with recovered N exceeded this limit (Figure 4B). Moreover all *M. pulcherrima* also exceeded the maximum levels of PAH4 set for all foodstuff including smoked fishery

[2] Products of non-animal origin or the following products of animal origin: fishmeal, blood products from non-ruminant, di and tricalcium phosphate of animal origin, hydrolyzed proteins from non-ruminant, hydrolyzed proteins from hides and skins of ruminant, gelatin and collagen from non-ruminants, eggs and egg products, milk, milk based-products, milk-derived products and colostrum, honey and rendered fat.

682 products, seafood, oils and fats (Table S13). In the United states however, no regulations regarding
683 maximal levels of PAH in food are in place (Sampaio et al., 2021). Regardless, PAH contamination should
684 be prevented or significantly reduced. It has been proven that PAH can be effectively removed from real
685 food matrices (*e.g.* oils, smoked meat or fish) through a physicochemical interaction with (low density)
686 polyethylene, which are plastics that are frequently used for food packaging (Šimko, 2018). This method
687 could potentially also be applied to remove PAH from the *M. pulcherrima* biomass, but this will have to be
688 verified first. A final consideration, apart from legislation, safety and productivity that could hamper the
689 implementation of recovered N in biotechnological processes that target food production (*e.g.* MP
690 production) is public acceptance. Several studies showed public resistance towards the use of urine
691 derived fertilizer for edible crops (Khalid, 2017; Segrè Cohen et al., 2020; Simha et al., 2021), implying that
692 the more direct nutrient recycle proposed in this work might also suffer from public resistance. Careful
693 product quality control, however, could favor the social acceptance of the MP produced with recovered
694 nutrients as food.

695 # 5 CONCLUSION

696 This work delivered a proof of concept that estimates the feasibility of using recovered N sources for MP
697 production. The study showed that selected recovered N sources had little to no impact on the overall
698 nutritional quality and specific growth rate of microbial biomass. *M. pulcherrima* biomass had a lower
699 protein content and lower amounts of essential AA in comparison to the reference products. However,
700 the choice of a different MO with higher protein content could increase the nutritional quality of the
701 product. Based on the analyzed contaminants, it was concluded that a daily consumption of 20 g of *M.*
702 *pulcherrima* would not pose adverse health effects, provided that the PAH content of the biomass is
703 reduced by about 15% for biomass produced with recovered $(NH_4)_2SO_4$ and by 22% for biomass produced
704 from recovered NH_4OH . The final implementation of this concept, however, depends on the acceptance

of governmental institutions that regulate the acceptance of novel food as well as the overall social acceptance.

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Figure captions

Figure 1: Experimental methodology followed to assess the impact of selected recovered nitrogen sources on the kinetics, yield, quality and safety of microbial protein production

Figure 2: Specific growth rate for the commercial and recovered N sources in 96 well-plates experiments fed with acetate. The recovered N source from COG stands for cokes oven gas, and OW for organic waste. The median of the replicates is shown as a horizontal black bar inside the boxplot, and the outliers are shown with black dots outside the plots (n = 10). Here, COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, IOW: industrial organic waste, IWAW: industrial and agro-industrial organic waste and MOW: municipal organic waste.

Figure 3: The yield of protein and biomass (sum of protein and other non-measured components such as carbohydrates, lipids, and ash) based on acetate (Ac) consumption with different N recovery products. Average values ± standard deviation of replicates (n = 10) are presented. Here, COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, IOW: industrial organic waste, IWAW: industrial and agro industrial organic waste and MOW: municipal organic waste.

Figure 4: The average content of contaminants detected in the microbial biomass. A: heavy metals in the biomass product grown on selected recovered N sources and ethanol. Horizontal dotted lines represent the lowest and highest limits for heavy metals defined for various types of food sources according to the EU regulation (European Parliament and the Council of the EU, 2006). B: poly aromatic hydrocarbons (PAH) in the biomass produced from ethanol and selected recovered N sources. Horizontal dotted lines represent the lowest and highest limits for benzo(a)pyrene and ∑PAH4 (sum: benzo(a)pyrene, benzo(a)anthracene,

benzo(b)fluoranthene and chrysene) defined for various types of food sources according to the EU regulation (EU Commission, 2011). The error bars represent the standard deviation of the biological replicates (n = 2). For one of the duplicates from biomass produced with $(NH_4)_2SO_4$ determined values for fenanthrene, anthracene, fluoranthene, pyrene and benzo(a)anthracene did not pass the quality requirements, therefore the result of only one of the duplicates is depicted and no error bars are shown. *For one of the two replicates the concentration of benzo(a)pyrene was below the detection limit, the value that could be detected is shown. Here, COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, and IWAW: industrial and agro-industrial organic waste.

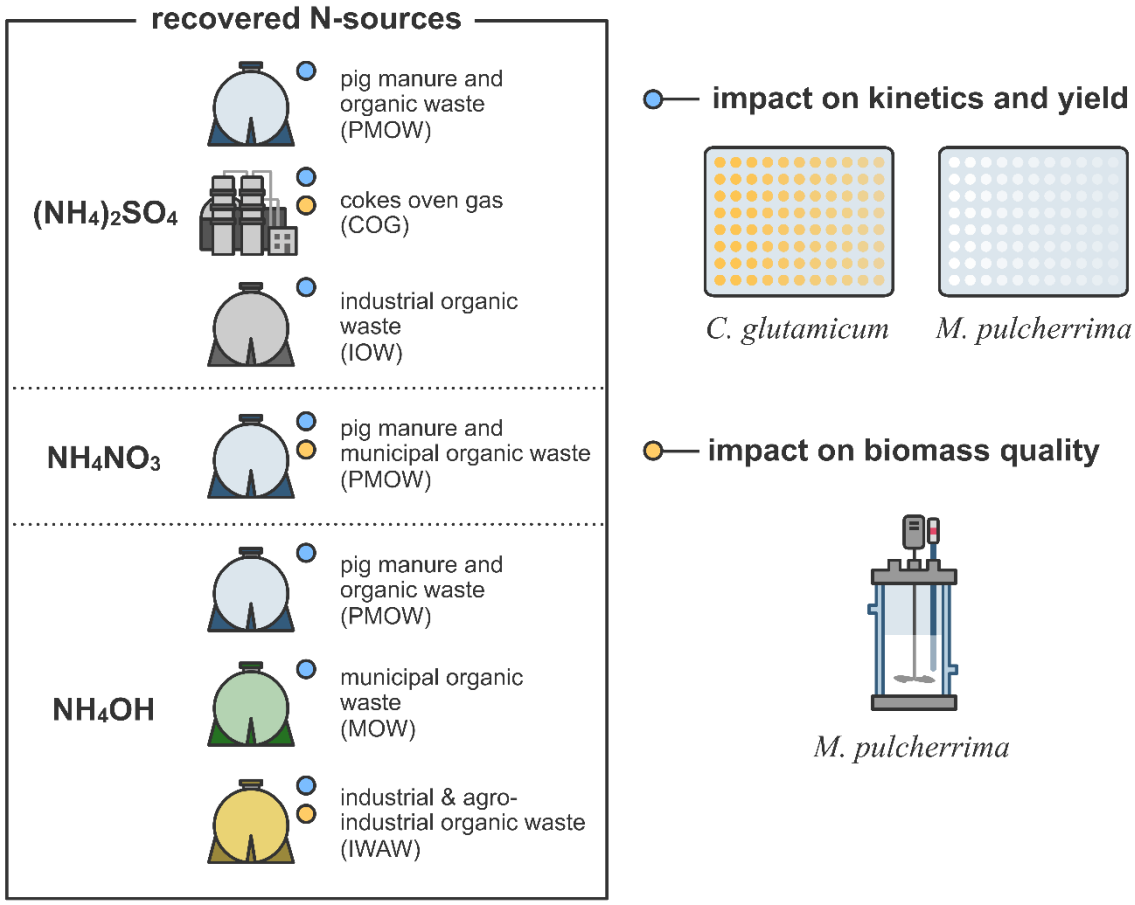
Figure 5: Composition of biomass from *M. pulcherrima* grown with the three selected recovered nitrogen sources and reference products. The reported composition is based on proteins, carbohydrates, lipids (fatty acids), moisture, and ash. Average values ± standard deviation from biological duplicates are presented. The reference product data were obtained from the FoodData central dataset provided by the U.S. department of agriculture (USDA Agricultural Research Service, 2019). Here, COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, and IWAW: industrial and agro-industrial organic waste.

Figure 6: A: Amino acid profile of the microbial biomass produced in this study and of the reference products brewer's yeast, spirulina and soy flour. The essential, conditionally essential and non-essential amino acids defined for human nutrition are shown. The black line indicates the essential AA requirements in a human diet (WHO and UNU, 2007). B: Overview of fatty acids found in the biomass of *M. pulcherrima* grown with three selected recovered nitrogen sources and ethanol. A square root scale was used for the y-axis to improve readability. Average values ± standard deviation from biological duplicates are presented

and the reference product data were obtained from the FoodData central dataset provided by the U.S. department of agriculture (USDA Agricultural Research Service, 2019). The microbial biomass of M. pulcherrima was produced with three selected recovered nitrogen sources and ethanol. Average values of the biomass products ± standard deviation from biological duplicates are presented. Here, COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, IWAW: industrial and agro-industrial organic waste. Arg: arginine, Cys: cysteine, Gly: glycine, Pro: proline, Tyr: tyrosine, His: histidine, Ile: isoleucine, Leu: leucine, Lys: lysine, Met: methionine, Phe: phenylalanine, Thr: threonine, Trp: tryptophan, Val: valine, Ala: alanine, Asp: aspartic acid, Glu: glutamic acid, Ser: serine.

Figure 7: Overview of the concentration of some minerals (*i.e.* Copper and Zinc) and ultra-trace elements (*i.e.* Chromium and Nickel) in the biomass product produced from ethanol and selected recovered N sources. The values on the y-axis are shown with a square root scale to enhance readability. The bars represent average values ± standard deviation (n=2) depicted as error bars for the data obtained in this study. For the nickel content in spirulina and the zinc content of brewer's yeast solely the positive error bar is shown given the negative one is below zero and therefore cannot be shown on a square root axis. In the case of the reference products, bars represent mean values ± standard deviation for brewer's yeast (n=18) (Rubio et al., 2018), Spirulina (n=11) (Muys et al., 2019), and soy flour (n≥8) (Porter and Jones, 2003). COG stands for cokes oven gas, PMOW: pig manure and municipal organic waste, and IWAW: industrial and agro-industrial organic waste.

1088 Figures



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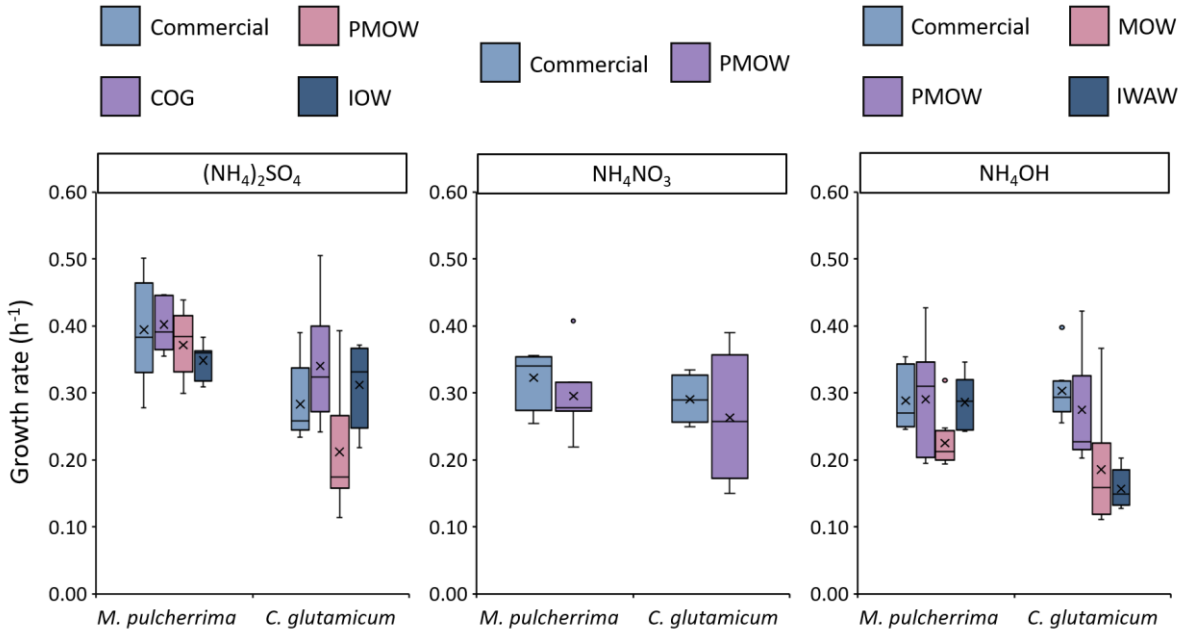

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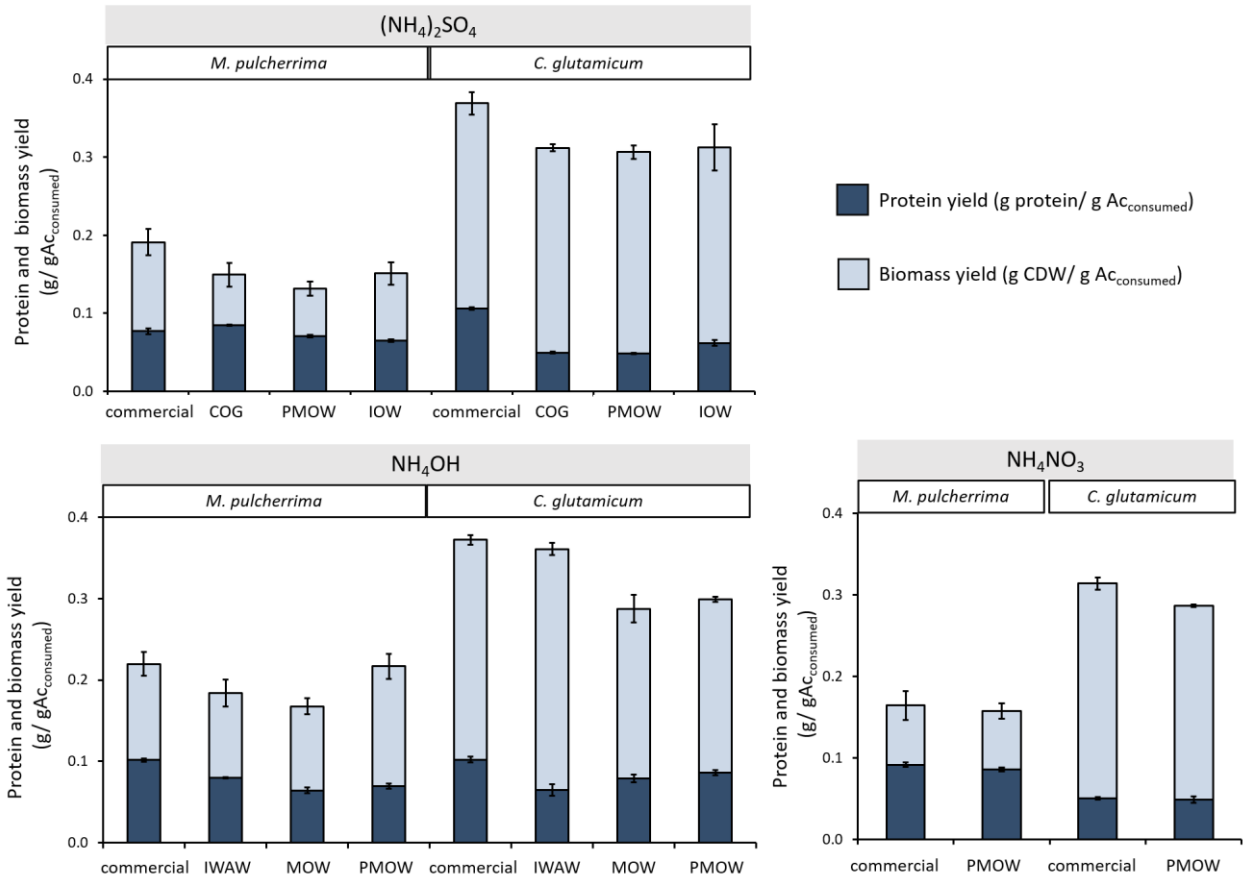

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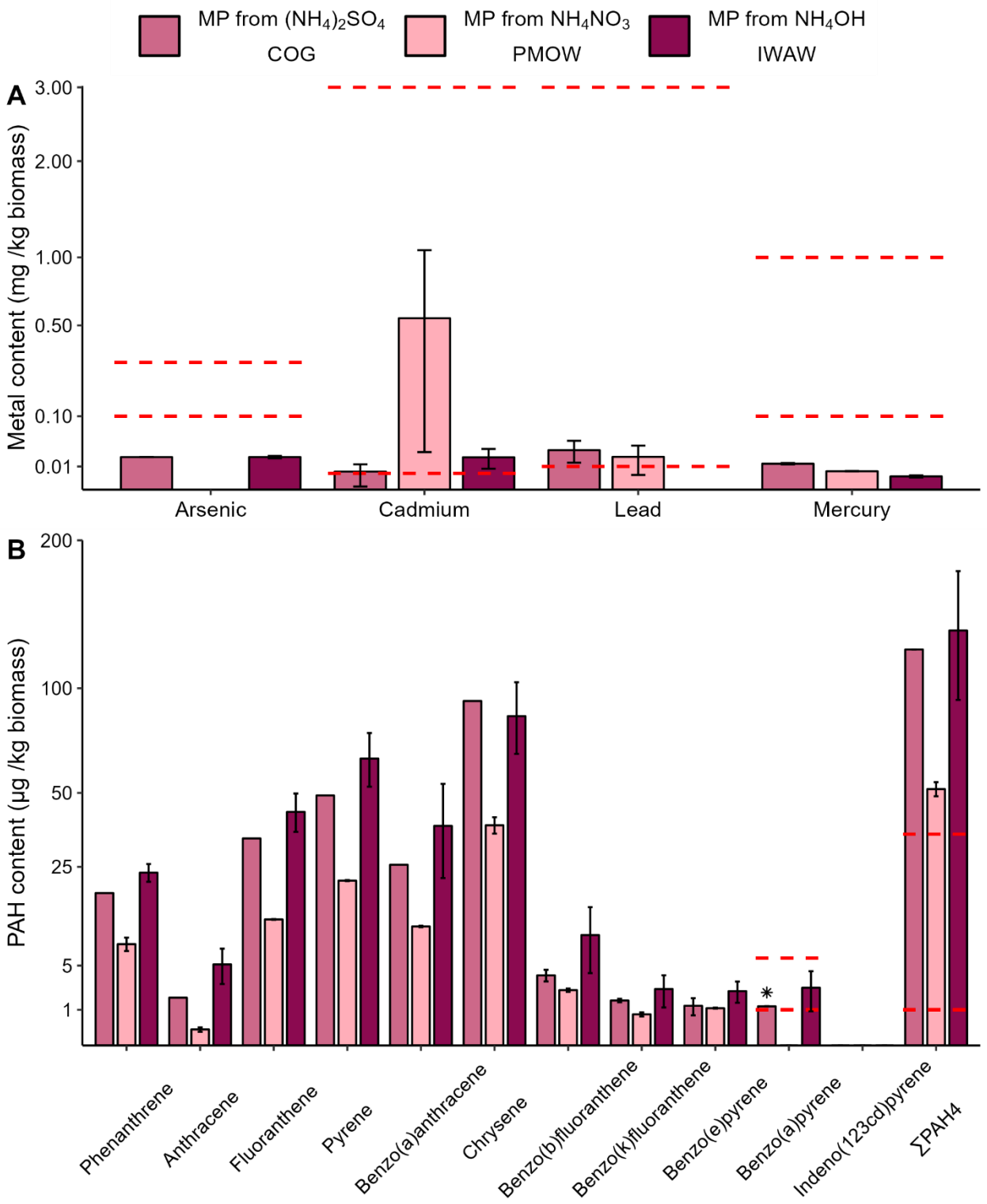

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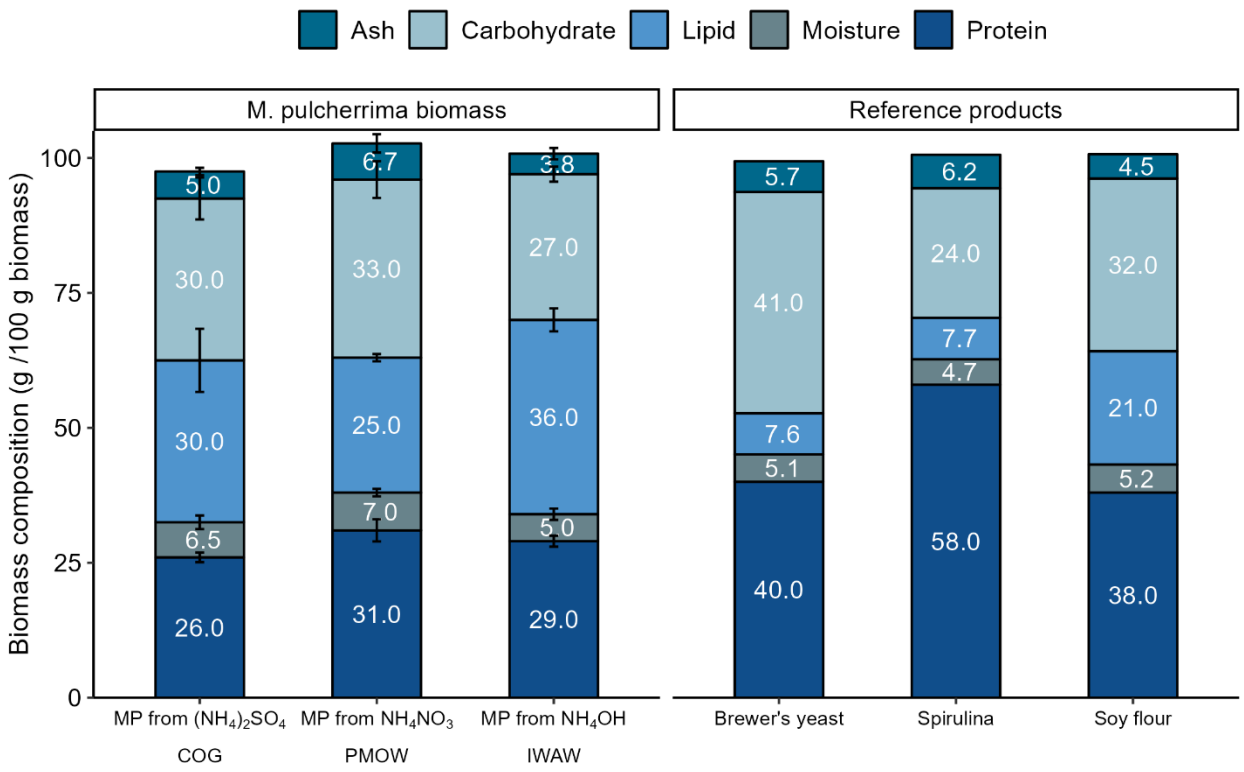

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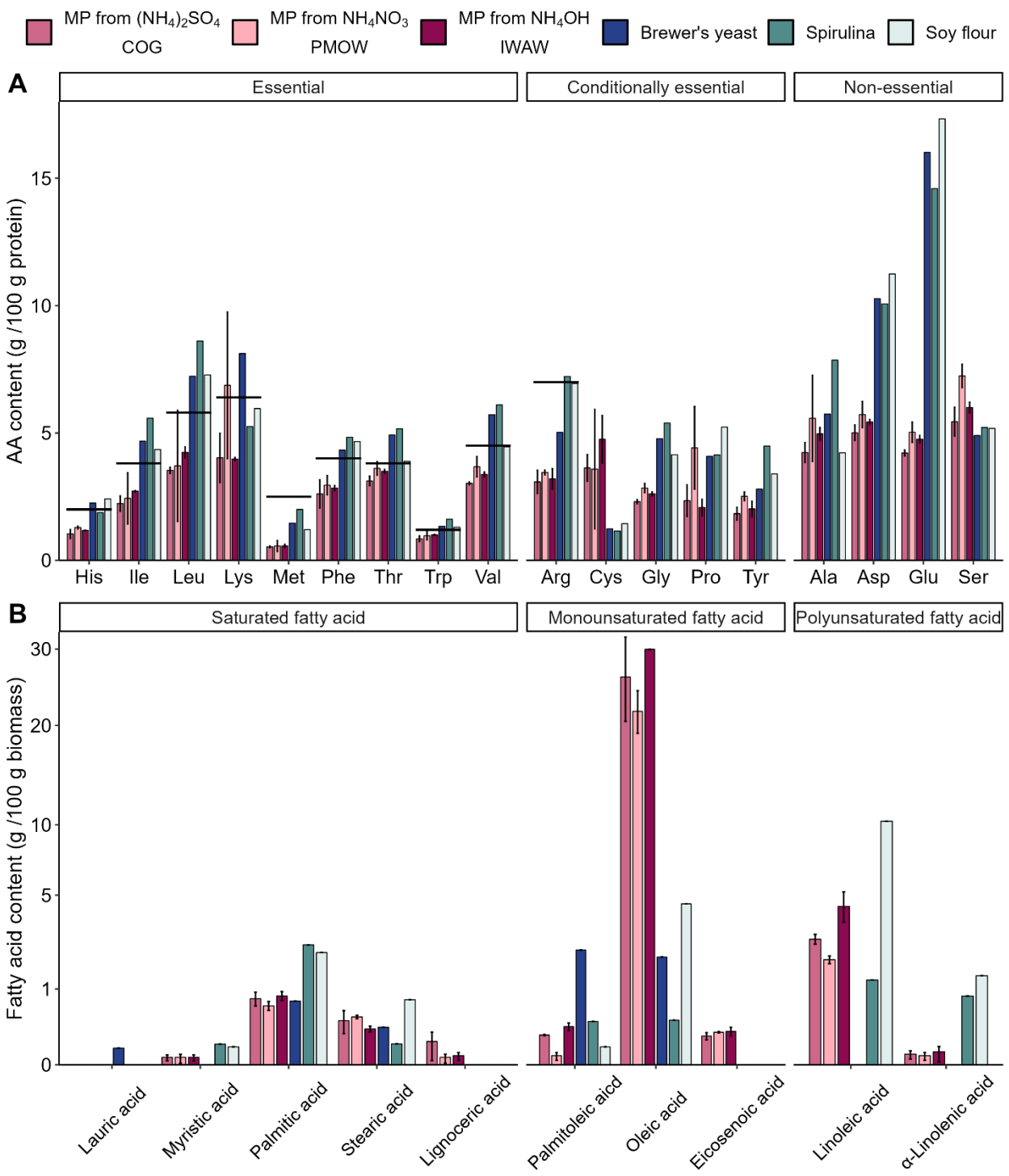

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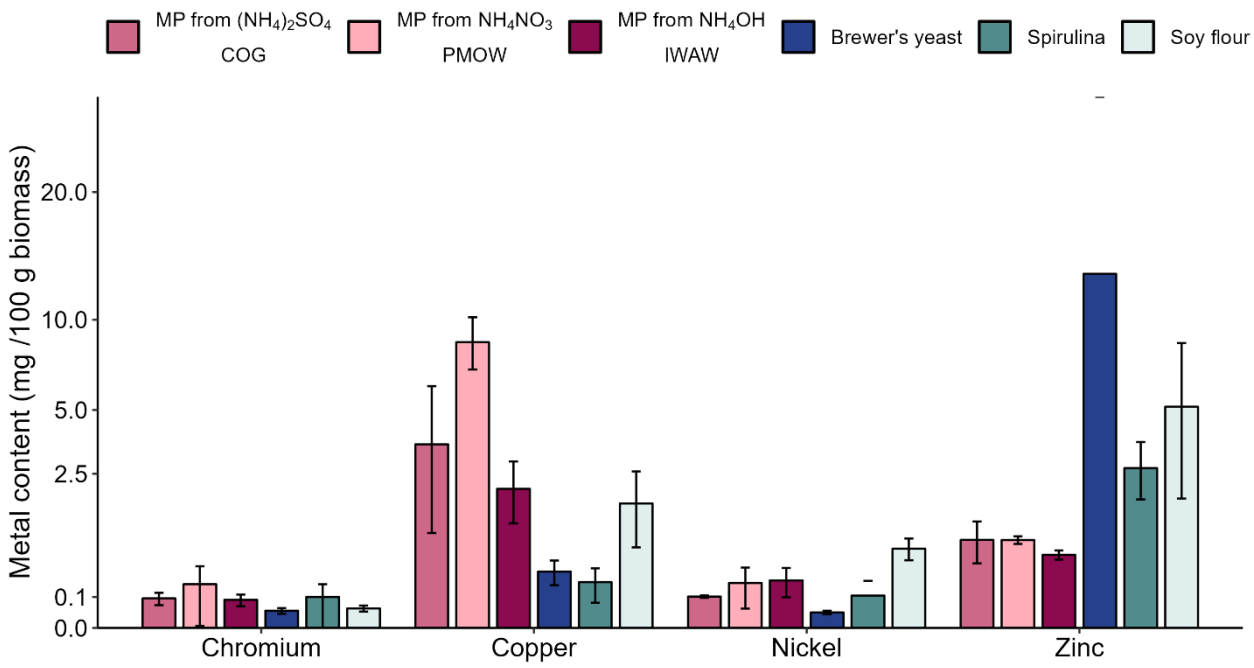
1100 Figure 6



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1102 Figure 7