

1 Valorisation of heat treatment centrate from nucleic acid reduction in microbial protein 2 production and its implications for process efficiency and nutritional quality

3 Abstract

4 The global demand for more sustainable protein has intensified the interest in alternative
5 sources such as microbial protein (MP). Although MP is a promising protein source in that
6 regard, its nucleic acid content is much higher than conventional proteins like meat,
7 necessitating reduction downstream. Industrial processes typically employ heat treatment
8 (HT) to degrade nucleic acids and thereby mitigate health risks. However, this step generates
9 a nutrient-rich liquid fraction containing proteins, nucleic acids, and residual nitrogen, which
10 is typically discarded resulting in nutrient loss and added costs. This study assesses the
11 potential of recycling HT centrate as a nitrogen source for three MP candidates, *Candida utilis*,
12 *Bacillus subtilis*, and *Ogataea polymorpha* comparing its performance against ammonium
13 chloride (NH₄Cl), purified nucleic acids, and yeast extract. Controlled bioreactor cultivations
14 with *C. utilis* were performed to evaluate specific growth rate, biomass yield, COD and
15 nitrogen consumption, and settleability of the biomass. HT centrate supported *C. utilis* growth
16 without negatively affecting macromolecular composition or key process parameters
17 compared to NH₄Cl. In contrast, nucleic acids alone resulted in lower growth rates, biomass
18 yields, nutrient uptake, and settling performance. Species-dependent differences were
19 observed, with *B. subtilis* showing a 16 % higher optical density compared to inorganic
20 nitrogen, highlighting the potential for enhanced performance in strains with high proteolytic
21 and nucleolytic capacity. Further characterization of the HT centrate and targeted
22 pretreatments are needed to improve bioavailability. While recycling this stream could reduce

23 nutrient and water inputs and lower operational costs, long-term continuous operations are
24 required to confirm industrial feasibility.

25 **Keywords:**

26 nucleic acid removal; process performance; resource recovery; sidestream valorisation;
27 single-cell protein

28 **1 Introduction**

29 The search for sustainable protein sources has become increasingly urgent as global
30 population growth and resource limitations put pressure on conventional agriculture.
31 Traditional animal and plant proteins require substantial land, water, and energy inputs, and
32 their production is often linked to environmental degradation (Dury et al., 2019). Meeting
33 future demand is particularly challenging, with global protein requirements projected to rise
34 by up to 78% by 2050 compared to 2017, driven by population growth and shifts toward
35 protein-rich diets (Henchion et al., 2017). This trajectory threatens the resilience of existing
36 food production systems and risks overexploitation of already constrained resources.
37 Microbial protein (MP)—the biomass of bacteria, yeasts, filamentous fungi, or microalgae—
38 offers a promising alternative. Its production is independent of agricultural land and climate
39 variability (Leger et al., 2021). Moreover, microbial biomass provides high-quality protein,
40 positioning MP as a key contributor to future sustainable food systems.

41 One of the key bottlenecks in MP production is its elevated nucleic acid content, which can
42 account for up to 25% of the cell dry weight (Mydland et al., 2008). This is primarily attributed
43 to the high growth rates of microbial cells, as rapid cellular multiplication requires increased
44 synthesis of nucleic acids, particularly ribosomal RNA, to support accelerated protein synthesis
45 (Ritala et al., 2017). High dietary intake of nucleic acids has been associated with adverse

46 health outcomes, including nephrolithiasis, cardiovascular disease, and diabetes (Ishikawa et
47 al., 2013; Ritala et al., 2017). Consequently, the World Health Organization recommends a
48 maximum daily total nucleic acid consumption (DNA and RNA) of 4 g (Larsen and Joergensen,
49 1996). Without further treatment, the MP consumption is limited to only cover 10-42 % of the
50 recommended daily protein intake, because the safe nucleic acid limit would be reached first
51 (Larsen and Joergensen, 1996). Various downstream processes—most notably heat treatment
52 (HT)—can reduce nucleic acid content by up to 80 % (Larsen and Joergensen, 1996; Ohta et
53 al., 1971; Ritala et al., 2017), enabling a MP-based diet to surpass (up to double) the daily
54 protein requirements without exceeding safe nucleic acid thresholds. However, this HT step
55 is energy-intensive—accounting for 15–49 % of the total energy demand in MP production—
56 and often relies on natural gas for steam generation, imposing significant environmental and
57 economic burdens (Van Peteghem et al., 2022a).

58 After HT, the MP is typically separated through centrifugation, while the resulting HT
59 centrate—rich in lysed biomass, proteins, nucleic acids, and nutrients— is conventionally sent
60 to wastewater treatment (Lonchamp et al., 2019; Parsons et al., 2019; Westlake, 1986; Young
61 and Koplove, 1975). **Although reintroducing nucleic acid-containing centrate may appear**
62 **counterintuitive, its reuse as a nitrogen source could be explored as a process-integration**
63 **strategy to improve nitrogen recovery and reduce resource losses, provided that it will not**
64 **have overall negative impacts on the process.** Wastewater treatment facilities remove
65 nutrients from the fermentation broth to ensure that the effluent can be safely discharged.
66 However, the removal of nutrients from the HT centrate also requires additional resource and
67 energy inputs, thereby imposing both environmental and economic burdens (Van Peteghem
68 et al., 2022a), while the contained nutrients are wasted.

69 To date, efforts to valorise this HT centrate within MP production have been minimal
70 (Lonchamp et al., 2020, 2019), even though it could bring potential benefits. Partially recycling
71 the centrate directly into MP production could reclaim critical nutrients (Yano et al., 1980)
72 (nitrogen, phosphorus, trace-elements, and vitamins) that are often supplied in excess in
73 biobased processes (Simpson et al., 2021; Van Peteghem et al., 2022b; Zhang et al., 2023).
74 Nevertheless, any potential application for food (or feed) purposes would require strict
75 evaluation of nucleic acid (and potentially other molecule) levels to comply with safety
76 regulations.

77 This approach could reduce the demand for external raw materials. Additionally, the lysed
78 biomass, and residual proteins present in the HT centrate could enhance MP production by
79 improving growth rate, and biomass yields, especially in MP processes based on mineral
80 nitrogen (Gu et al., 2015; Olufunke O. Ezekiel et al., 2012). The nucleic acids contained in the
81 HT centrate could further contribute as alternative sources of carbon, nitrogen, and
82 phosphorus, thereby increasing resource use efficiency (Antheunisse, 1972; Merchant and
83 Helmann, 2012; Mydland et al., 2008). However, one study has reported that the presence of
84 nucleic acids may impair biomass settling, potentially complicating downstream processing
85 steps such as centrifugation (Westlake, 1986).

86 In addition to process-level considerations, species-specific traits play a critical role in
87 determining the effectiveness of HT centrate reuse. Differences in nitrogen assimilation
88 pathways, enzymatic capabilities (e.g., protease and nuclease activity), and stress tolerance
89 across microbial taxa may result in divergent responses to recycled media (Chen et al., 2023;
90 Gu et al., 2015). Therefore, species screening is essential to identify MP candidates that can
91 thrive under such conditions.

92 Although previous studies have explored the recycling of lysed biomass or spent medium
93 within bioprocesses (Bagul and Annapure, 2020; Depraetere et al., 2015), the reuse of HT
94 centrate within MP production has not, to the authors' knowledge, been systematically
95 assessed. In this study, we explore the potential of HT centrate within MP production itself.
96 The HT centrate is specifically evaluated as a nitrogen source, in a proof of concept to assess
97 its technical feasibility, rather than a full nutritional or safety assessment of the final product.
98 The investigation comprises two parts: (1) a controlled evaluation using *Candida utilis* to
99 assess the impact of HT centrate on process performance, biomass composition, and biomass
100 settling; and (2) a broader screening of alternative MP candidates to account for species
101 differences in tolerance to recycled media. This work aims to provide insights into the
102 feasibility and implications of centrate recycling for a more circular MP production.

103 2 Material and methods

104 2.1 Microbial cultures

105 Four microbial species were used in this work to evaluate the nitrogen sources, specifically
106 *Kluyveromyces marxianus* MUCL 11985, *Candida utilis* IHEM 4005, and *Bacillus subtilis* LMG
107 20037, which were purchased from the Belgian Co-ordinated Collections of Microorganisms
108 (BCCM), and *Ogataea polymorpha* ATTC 34438, obtained from the American Type Culture
109 Collection (ATCC, Virginia, United States). These microbial species were selected based on
110 their Qualified Presumption of Safety (QPS) and Generally Regarded as Safe (GRAS) statute
111 granted by the European Food Safety Authority (EFSA) and U.S. Food and Drug Administration
112 (FDA).

2.2 Experimental design

Four distinct nitrogen sources were assessed for their effect on growth performance and biomass composition in pure cultures of *C. utilis*. The evaluated nitrogen sources included NH₄Cl (Carl Roth®, Germany), purified nucleic acids from *C. utilis* (Roche, Belgium), yeast extract (Carl Roth®, Germany), and HT centrate. To evaluate the effect of specific nitrogen sources on the growth of *C. utilis*, reactor experiments were conducted in ammonium mineral salts (AMS) medium with lactic acid as the sole carbon source (5 g COD·L⁻¹). Nitrogen sources were supplemented in amounts corresponding to a C/N ratio of 5, based on lactic acid and total nitrogen concentrations. The contribution of other carbon-rich components, such as residual carbohydrates, was not included in the calculation, as their concentration was relatively low and comparable across conditions (Table 1), and thus not expected to significantly affect the overall C/N balance. Both phosphate (PO₄³⁻) and sulfate (SO₄²⁻) concentrations were standardized across all media (Tables S2–S3), and each formulation received identical vitamin and trace-element supplementation.

To investigate organism-specific responses, *O. polymorpha* and *B. subtilis* were additionally cultured on the same nitrogen sources. Growth kinetics and final biomass concentrations were assessed in 96-well microplates.

2.3 Pre-culture and media composition

Pre-cultures of *C. utilis*, *B. subtilis*, and *O. polymorpha* were cultivated according to the conditions described in Delmoitié et al (2025). Upon reaching late exponential phase, these cultures were transferred (10 v/v%) into adjusted AMS medium with the different nitrogen sources (Table 1) for bioreactor experiments (*C. utilis*) or 96-well plate experiments (*C. utilis*, *B. subtilis*, and *O. polymorpha*). In the HT centrate (Table 1), the proteins originated from

136 extracellular production during the fed-batch cultivation and hydrolysis of biomass during HT,
137 while the NH_4^+ represents residual inorganic nitrogen from the same process.

138 **Table 1:** Characterisation of the different concentrated nitrogen sources utilised during the
139 cultivation of *Candida utilis*. Each medium was adjusted to a C/N ratio of 5 based on total
140 nitrogen.

Parameter	Yeast extract (18 g/L)	Nucleic acids (10 g/L)	Undiluted heat treatment centrate	Unit
COD	18.1 ± 0.09	7.3 ± 0.18	23.5 ± 0.22	$\text{g}\cdot\text{L}^{-1}$
TN	2.07 ± 0.21	1.22 ± 0.02	6.81 ± 0.12	$\text{g}\cdot\text{L}^{-1}$
Protein	4.57 ± 0.25	0.51 ± 0.01	5.77 ± 0.08	$\text{g}\cdot\text{L}^{-1}$
NH_4^+	0.10 ± 0.01	ND	7.3 ± 0.51	$\text{g}\cdot\text{L}^{-1}$
Carbohydrates	2.0 ± 0.04	2.3 ± 0.0	2.2 ± 0.05	$\text{g}_{\text{glucose-equivalent}}\cdot\text{L}^{-1}$
Electrical conductivity	3.07	1.18	45.3	$\text{mS}\cdot\text{cm}^{-1}$

141 ND: not detected

142
143 **2.4 Production of heat treatment centrate**

144 The HT centrate was obtained from a fed-batch cultivation of a selected microbial community
145 composed of *C. utilis*, *B. subtilis*, *O. polymorpha*, and *K. marxianus* (Delmoitié et al., 2025). The
146 bioreactors were operated in pH-stat mode using the medium presented in Table S1, and the
147 experiments were terminated upon reaching a biomass concentration of $11.7 \pm 1.1 \text{ gCDW/L}$.
148 The resulting biomass reached a protein content of $48.0 \pm 10.6 \%$. Following cultivation, the
149 biomass was subjected to a two-step heat treatment—15 seconds at 68 °C followed by 2 hours

at 55 °C (Ohta et al., 1971)—to reduce the nucleic acid content via the activation of endogenous nucleases. Reduction of nucleic acids was confirmed by RNA quantification before and after heat treatment using RNA extraction with an RNeasy kit (Qiagen, Germany) followed by Quantifluor® RNA fluorescence quantification (Promega, USA). The microbial biomass was then harvested by centrifugation, and the resulting biomass-free centrate was stored at –20 °C. Prior to use, all collected centrate batches were pooled to eliminate the potential effect of variability and sterilized via filtration through polyether sulfone bottle-top filters (Fisherbrand™).

2.5 Evaluating the effect of different nitrogen sources on the performance and nutritional composition of microbial protein

Bioreactor experiments, with *C. utilis*, were conducted as described in Delmoitié et al. (2025) in a 2 L double jacketed bioreactor (Bluesens, Germany) controlled through an in-house software. Temperature of the bioreactors were maintained at 37°C with a heating bath (Huber KISS® 110A heating, Germany). The dissolved oxygen concentration was continuously measured by a Hamilton® VisiFerm DO Arc probe and maintained at a minimum of 30% through increasing the agitation speed (400-800 rpm) at a constant aeration rate of 2 vvm filter sterilized air (0.2 µm pore size, Millipore, Merck). The pH was measured using a Hamilton® EasyFerm Plus pH sensor, and controlled at 5.5 through addition of 2 M NaOH or 2 M HCl. Antifoam 204 (Sigma-Aldrich, Germany) was added to the reactor at a concentration of 0.01% to prevent foaming.

All precultures, grown on AMS, were diluted (0.1 gCDW/L) and subsequently inoculated into sterile bioreactors at 10 % (v/v). After inoculation, 8 mL sample were frequently withdrawn to monitor growth through OD_{600nm} measurements. In addition, online analysis of O₂ and CO₂

173 was performed (BlueVary, Germany) in the off-gas to assess growth rate. Each sample was
174 centrifuged at 6,603 g for 5 min, the pellet was stored at -20°C for determination of protein,
175 carbohydrate, and ash content. The supernatant was filtered (0.2 µm PVDF filters, Chromafil®)
176 and stored at -20°C for quantification of anions, cations, protein concentration, COD, TN and
177 lactic acid concentrations. After the stationary growth phase was reached a sludge volume
178 index (SVI) estimation was performed on the produced biomass.

179 **2.6 Screening of other microbial protein candidates for the re-use of heat treatment**
180 **centrate**

181 To evaluate the effect of the nitrogen sources on the growth kinetics of other MP candidates,
182 a screening experiment was performed on 96-well plates. *C. utilis*, *B. subtilis*, and *O.*
183 *polymorpha* were pre-grown on AMS medium containing lactic acid at 1.5 gCOD/L, buffered
184 with 100 mM MES, and supplemented with the selected nitrogen sources. The screening was
185 conducted in 96-well plates with a working volume of 0.2 mL and a 10% v/v inoculum at six
186 biological replicates. The 96-well plate was incubated at 37°C and 180 rpm in a plate reader
187 (Infinite M200 Pro, Tecan, Switzerland), with OD_{600nm} measurements recorded every 15
188 minutes.

189 **2.7 Analytical techniques**

190 Organic acids such as lactic acid, acetic acid, formic acid, propionic acid, and butyric acid were
191 analysed with a 930 Compact Ion Chromatograph Flex (Metrohm®, Switzerland), using a
192 Metrosep Organic acids 250/7.8 column with inline bicarbonate removal. The column was
193 equipped with a Metrosep Organic acid Guard/4.6. Cations were analysed with a 761 Compact
194 Ion Chromatograph (Metrohm®, Switzerland), equipped with a Metrosep C6-250/4.0 column
195 and a conductivity detector. Anions such as chloride, phosphate, and sulphate were analysed

196 using a Methrom 930 compact Ion Chromatograph, equipped with a conductivity detector and
197 a Metrosep A supp 5-150/4.0 column (Metrohm®, Switzerland). Determination of COD and TN
198 were performed using commercial kits (NANOCOLOR®; MACHEREY-NAGEL GmbH & Co. KG).

199 Microbial growth was monitored through OD measurements at 600 nm using a
200 spectrophotometer (Infinity M200 Pro, Tecan, Switzerland). The CDW and ash content of the
201 biomass were quantified as TSS and VSS with a glass fibre filter of 0.7 µm pore size
202 (AP4004705; Millipore; Merck). Protein content was determined in washed biomass samples
203 using the Pierce™ BCA Protein assay kit (Thermo Scientific™, Belgium), with each
204 measurement performed in technical triplicate. Total carbohydrate content in the biomass
205 was determined with the method of Josefsson (Grasshof et al., 1983), using a
206 spectrophotometer (NANOCOLOR® Advance; MACHEREY-NAGEL GmbH & Co. KG) at 520 nm.

207 **2.8 Data analysis**

208 **2.8.1 Growth rate estimation**

209 For the bioreactor experiments, CO₂ off gas data was used (BlueVary) to calculate the carbon
210 evolution rate (CER) during microbial cultivations (Wechselberger et al., 2013). The off-gas
211 data were analysed in R (version 1.2.4). The CER (mol/L·h) was calculated using the flowrate
212 (F) of air at 4 L/min, volume of bioreactor 2 L (Eq. 1), with M molar mass (44.0 g/mol), P is
213 pressure (kPa), R is the ideal gas constant (8.31 J/mol·K), and T is temperature (310 K). The
214 CER was log transformed in function of the time. The linear section of the growth curve was
215 used to estimate the growth rate (µ) (Eq. 2).

$$216 \quad CER = \frac{F_{air} \times P \times M}{R \times T} (CO_{2-out} - CO_{2-in}) \quad (1)$$

$$217 \quad \ln(CER) = \mu \times t + b \quad (2)$$

For the screening of growth performance of the other MP candidates on the nitrogen sources, the growth rate was calculated through log-transformed of the growth curve and fitted to the Richards equation (Eq. 3) using the nls.lm optimization algorithm from the minpack lm package (version 1.2-4) in R. The specific growth rate for each condition was calculated from this fit as described in (Candry et al., 2018).

$$\ln\left(\frac{\Delta OD}{\Delta OD_{min}}\right) = A * (1 + v + e^{1+v} * e^{\frac{\mu_{max}}{A}(1+v)^{1+\frac{1}{v}}(\lambda-t)})^{-\frac{1}{v}} \quad (3)$$

2.8.2 Assessment of process parameters

The biomass yield was calculated based on the lactic acid consumption and obtained biomass concentrations c_{TSS} (Eq.4). The consumption of COD and TN were calculated as the difference between the initial and final concentrations, divided by the initial concentrations (Eq. 5-6). The unidentified nitrogen balance fraction was calculated (Eq. 7) by was calculated by subtracting the contributions of $N-NH_4^+$ and protein-bound nitrogen—determined using a protein-to-nitrogen conversion factor of 5.35 (Jia et al., 2024)—from the TN.

$$Y_{X/s} \left(\frac{gCDW}{gCOD_{LA-consumed}} \right) = \frac{c_{TSS} \left(\frac{g}{L} \right)}{COD_i - COD_f \left(\frac{g}{L} \right)} \quad (4)$$

$$COD_{\%} = \frac{COD_i - COD_f}{COD_i} \times 100 \quad (5)$$

$$TN_{\%} = \frac{TN_i - TN_f}{TN_i} \times 100 \quad (6)$$

$$Unidentified\ nitrogen = TN \left(\frac{gN}{L} \right) - NH_4^+ \left(\frac{gN}{L} \right) - Protein \left(\frac{gN}{L} \right) \quad (7)$$

2.8.3 Settling potential of microbial biomass

The sludge volume index at 30 minutes (SVI_{30}) was determined to quantify the settling behaviour of *C. utilis* after growth in each selected nitrogen source. SVI_{30} was calculated as the volume of settled biomass ($\text{mL} \cdot \text{L}^{-1}$) after 30 minutes divided by the biomass concentration c_{TSS} (g/L) (Eq. 8).

$$SVI_{30} = \frac{\text{Settled sludge volume } \left(\frac{\text{mL}}{\text{L}}\right)}{c_{TSS} \left(\frac{\text{g}}{\text{L}}\right)} \quad (8)$$

The monovalent–divalent cation ratio was determined to characterise the ionic balance of the medium. It was calculated as the total molar concentration of monovalent cations (e.g., Na^+ , K^+ , NH_4^+) divided by twice the total molar concentration of divalent cations (e.g., Ca^{2+} , Mg^{2+}), reflecting their respective charge contributions (Eq. 9).

$$\frac{\text{Monovalent}}{\text{Divalent}} = \frac{\sum_i C_i \times z_i}{\sum_j C_j \times z_j} \quad (9)$$

2.8.4 Statistical analysis

For the exploration of the species dependent growth performance of the different nitrogen sources, an ANOVA was performed on the optical density, and growth rate if the assumption were met. Followed by a Tukey's Honest Significant Difference (HSD) post hoc tests. When either of the assumptions were violated, a nonparametric Kruskal–Wallis test was used followed by pairwise Wilcoxon rank-sum tests with $\alpha = 0.05$. In all cases, p-values were adjusted to correct for multiple comparisons (Tukey HSD for ANOVA, Bonferroni-corrected Wilcoxon for Kruskal–Wallis).

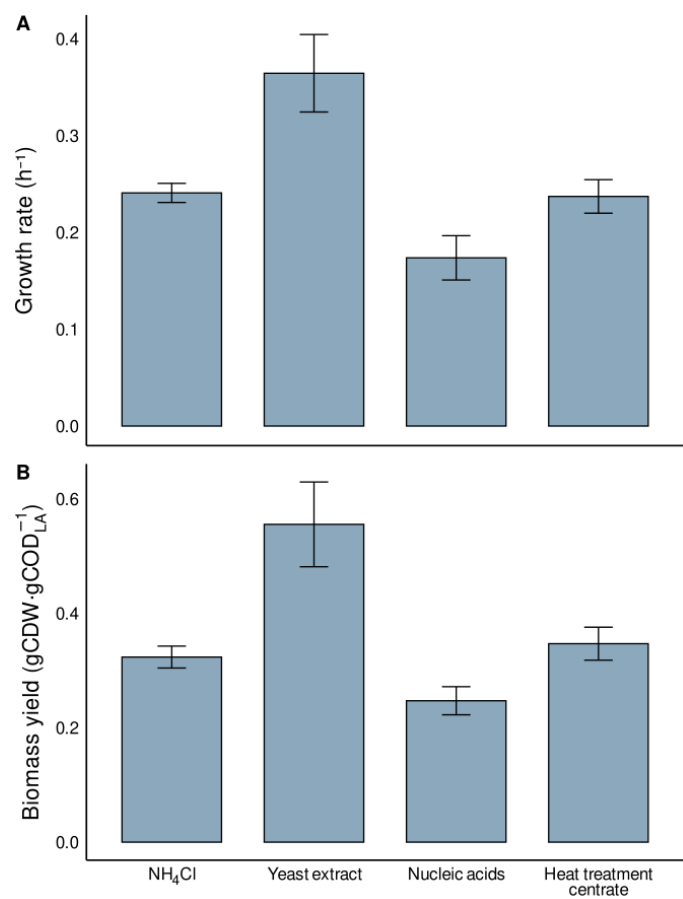
3 Results and discussion

3.1 Growth performance of *Candida utilis* in heat treatment centrate as a recycled nitrogen source

The choice of nitrogen sources profoundly influenced both the specific growth rate, biomass yield, and substrate utilization during MP production using *C. utilis*. Cultures supplemented with yeast extract showed the best growth performance achieving a specific growth rate of $0.36 \pm 0.04 \text{ h}^{-1}$ (**Fig. 1A**), and biomass yield of $0.56 \pm 0.07 \text{ gCDW} \cdot \text{gCOD}_{\text{LA}}^{-1}$ (**Fig. 1B**). In contrast, cultures grown on HT centrate or NH_4Cl exhibited comparable performance, with a specific growth rate of 0.24 h^{-1} and biomass yield ranging between 0.32 and $0.35 \text{ gCDW} \cdot \text{gCOD}_{\text{LA}}^{-1}$. When nucleic acids were supplied as the sole nitrogen source, the specific growth rate and yield declined to $0.17 \pm 0.02 \text{ h}^{-1}$ and $0.25 \text{ gCDW} \cdot \text{gCOD}_{\text{LA}}^{-1}$, respectively. Although minor carbon sources from complex substrates could potentially contribute to microbial growth, our data suggest that their utilization can be considered negligible. Carbohydrate concentrations were measured during the experiments showing no to minimal consumption (Table S4). Thus, lactate was identified as the predominant carbon substrate responsible for the observed carbon yield.

The growth performance observed across nitrogen sources was mirrored in both lactic acid and TN consumption patterns. Cultures supplemented with yeast extract or NH_4Cl fully consumed the supplied lactic acid (5 gCOD/L) within 17 h, whereas those grown on HT centrate or nucleic acids consumed only 82.4-83.5 % (**Table 2**). A similar trend was seen in TN utilization (**Fig. 2A**), with consumption decreasing from yeast extract (54.9 %) to NH_4Cl (46.4 %), HT centrate (29.1 %), and nucleic acids (8.67 %). When yeast extract or nucleic acids were used as nitrogen sources, the majority of assimilated nitrogen could not be specifically identified but was presumed to consist primarily of amino acids (in the case of yeast extract)

279 and nucleotides (in the case of nucleic acids). These unidentified nitrogen fractions accounted
280 for 79.8-86.3 % and the predominant share of nitrogen uptake, respectively (**Fig. 2B**). In
281 contrast, cultures grown on HT centrate primarily assimilated inorganic nitrogen (NH_4^+), with
282 only a minor portion (7.9 %) attributed to unidentified nitrogen compounds. The HT centrate
283 is expected to include partially hydrolysed peptides and nucleotides within the typical range
284 of 6 to 98 kDa, as reported for heat-treated fungal biomass (Lonchamp et al., 2020, 2019),
285 which translates to sufficient nutrient bioavailability. Future studies should include detailed
286 characterizations involving organic nitrogen speciation to refine this characterization.



288 **Fig. 1:** Growth performance of *Candida utilis* cultivated on different nitrogen sources: NH₄Cl,
289 yeast extract, nucleic acids, and heat treatment centrate. (A) Specific growth rate (h⁻¹) and (B)
290 biomass yield (gCDW·gCOD_{LA}⁻¹) are presented for each condition. Error bars represent
291 standard deviations of biological replicates (n=2).

292 The enhanced performance of *C. utilis* or other species when grown on yeast extract was
293 previously observed, with growth rates increased by 45.9-533 % and biomass yields by 28.4-
294 82.4 %, compared to inorganic nitrogen (Chen et al., 2023; Olufunke O. Ezekiel et al., 2012;
295 Ouedraogo et al., 2017). In addition, this increase could also be due to increased vitamin or
296 co-factor concentrations inherently present in YE (Kieliszek et al., 2019; Liu et al., 2018). A
297 more comprehensive compositional analysis covering vitamins, and protein or peptide size
298 distributions, and concentrations of other components is therefore required to clarify these
299 effects and to establish whether the observations in this study are representative of HT
300 centrate more broadly. In contrast, the use of nucleic acids as sole nitrogen source led to a
301 decline in growth rate and biomass yield by 27.8 %, and 23.6 %, respectively. These findings
302 align with previous studies showing that DNA derivatives as sole nitrogen source reduced
303 growth and cell densities in *Bifidobacterium bifidum*, *Shewanella* spp., and *Escherichia coli*
304 (Huang et al., 2022; Pinchuk et al., 2008). However, other reports showed that cultivation of
305 *Komagataella phaffii* on a mixture of RNA oligonucleotides and nucleotides achieved similar
306 biomass yields, compared to when grown on medium containing peptone, and yeast extract,
307 albeit with a reduced growth rate (Liang et al., 2025). In addition, species-specific preferences
308 in nucleotide uptake have been reported, with *E. coli* favouring double stranded DNA, *B.*
309 *bifidum* preferring deoxyribonucleosides, and *K. phaffii* assimilating short RNA
310 oligonucleotides (5-30 nucleotides) more efficiently than longer fragments or
311 mononucleotides (Huang et al., 2022; Liang et al., 2025). These studies suggests that nucleic

acid utilization depends on polymer size and microbial enzymatic capabilities. In our study, the limited nitrogen uptake from nucleic acids likely reflects the restricted nuclease activity of *C. utilis* (Buerth et al., 2011; Cazin et al., 1969; Sen and Komagata, 1979), constraining the nitrogen assimilation and thereby reducing both biomass yield and growth rate.

Table 2: Consumption of organics expressed as chemical oxygen demand of *Candida utilis* cultivated on NH₄Cl, yeast extract, nucleic acids and heat treatment centrate as nitrogen sources.

Nitrogen source	Total chemical oxygen demand consumption (%)	Lactic acid consumption (%)
NH ₄ Cl	77.1 ± 5.6	100
Yeast extract	75.7 ± 5.3	100
Nucleic acids	52.6 ± 0.4	82.4 ± 5.8
Heat treatment centrate	73.5 ± 6.8	83.5 ± 6.1

This similarity in growth performance between HT centrate and NH₄Cl highlights the potential of recycling the centrate in MP production reducing raw materials input. However, the total nitrogen consumption during cultivation on HT centrate was lower, at 37.5 % compared to 46.4 % for NH₄Cl. Moreover, 87.7 % of the consumed nitrogen was inorganic (NH₄⁺) with 7.23 % being related to protein consumption, and 7.79 % to unidentified nitrogen. *C. utilis* predominantly consumed the residual NH₄Cl carried over from the previous cultivation phase, rather than the organic nitrogen fractions released during HT. The HT likely resulted in partial hydrolysis of microbial biomass into peptides, nucleotides, and nucleosides—compounds that constitute the unidentified nitrogen fraction but appear less readily assimilated by *C. utilis*, as

329 also seen in the cultures grown only on nucleic acids. This finding indicates that ammonium
330 was the most abundant and easily assimilable nitrogen form in the HT centrate.

331 Although the HT process partially hydrolyses microbial biomass into nucleic acids and protein
332 fragments—likely constituting the unidentified nitrogen fraction—these compounds appear
333 to be less readily utilized by *C. utilis* as seen when grown solely on nucleic acids. This highlights
334 a clear preference for inorganic nitrogen, particularly NH_4Cl over more complex or polymeric
335 nitrogen sources in the reused centrate. However, the centrate used here was produced
336 through activation of endogenous enzymes (a form of enzymatic hydrolysis), implying that a
337 portion of the organic nitrogen was already converted to smaller, more bioavailable molecules
338 such as peptides, nucleotides and nucleosides. The experiment was also designed to assess
339 whether nucleic-acid-rich stream utilization results in inhibitory effects on growth, as part of
340 exploring the potential of HT centrate reuse for nitrogen reuse. These results therefore
341 demonstrate that HT centrate can serve as an inorganic nitrogen substitute, while targeted
342 pre-treatments could further enhance the utilization of the organic nitrogen fraction.

343

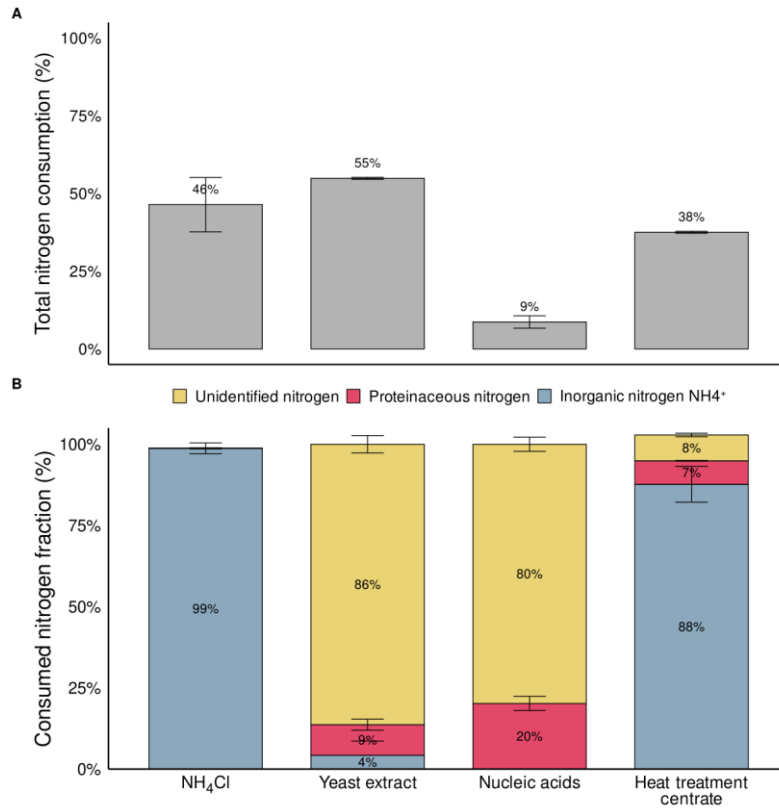


Fig. 2: The total nitrogen consumption (A) and consumed nitrogen fractions (B) of *Candida utilis* grown on different nitrogen sources: NH_4Cl , yeast extract, nucleic acids, and heat treatment (HT) centrate. Error bars represent standard deviations of biological replicates.

Theoretical calculations by Stouthamer (1973) suggest that supplementation of nucleic acids/nucleotides, such as in the HT centrate, could lower the ATP demand for de novo RNA/DNA synthesis by approximately 11.5 %, thereby increasing net biomass yield. Nevertheless, this benefit was not observed when HT centrate was used for the cultivation of *C. utilis*. However, other studies with other organisms (e.g. *E. coli*, *B. bifidum*) have shown enhanced biomass formation with nucleic acid supplementation (Huang et al., 2022). This

354 suggests that the utility of HT centrate may be species-specific and influenced by the presence
355 or activity of hydrolytic enzymes such as DNases, RNases, and proteases. In this context, HT
356 centrate offered growth performance comparable to NH₄Cl despite the limited protease and
357 nuclease activity of *C. utilis*.

358 Further research should focus on characterisation of the nitrogen components that remained
359 unidentified in this work, in both yeast extract and HT centrate and their consumption rates
360 to fully understand their preferential uptake. Furthermore, to improve the nitrogen
361 availability and potentially enhance growth rate and biomass yield, the HT centrate could be
362 further hydrolysed, an approach which could enhance the bioavailable nitrogen and thus
363 improve the biomass yield and growth rate (Dufossé et al., 1997; Leake and Read, 1991).
364 Despite the current absence of growth enhancement, the fact that HT centrate resulted in
365 comparable performance to inorganic nitrogen supports its reuse in MP production while
366 lowering raw material and environmental impact, related to nitrogen addition.

367 **3.2 The nutritional composition of *Candida utilis* grown on different nitrogen sources**

368 The protein content of *C. utilis* remained largely unaffected at 24.4-25.5 % when utilizing
369 NH₄Cl, centrate HT, or nucleic acids as nitrogen sources (**Fig. 3**). Moreover, the carbohydrate
370 content remained similar for HT centrate and NH₄Cl at 34.4-36.8 % while it increased to 45.2 %
371 when *C. utilis* was grown on nucleic acids as sole nitrogen source. In contrast, protein content
372 increased to 35.4 %, and carbohydrate content decreased to 30.0 % when utilizing yeast
373 extract as nitrogen source.

374 The pronounced effect of yeast extract on the nutritional composition aligns with previous
375 studies, reporting two-fold increases in protein content relative to inorganic nitrogen (Garg
376 and Neelakantan, 1982; Gu et al., 2015; Kim et al., 2016; Shanthi et al., 2018). The cultivation

377 of *C. utilis* on nucleic acids as sole nitrogen source did not impact the protein content but did
378 increase the carbohydrate content. These results, together with the low nitrogen
379 consumption observed in that case (Section 3.1), indicate that *C. utilis* experienced a nutrient
380 imbalance, which is known to promote carbohydrate accumulation in some species (Brown
381 and Rose, 1969; François and Parrou, 2001; Kieliszek et al., 2021; Lucca et al., 1995). In
382 addition, different nitrogen sources could lead to an increase in cell wall polysaccharides such
383 as β -glucan, α -glucan, and trehalose in *C. utilis* (Bzducha-Wróbel et al., 2024). However, as no
384 direct species-targeted quantification was performed in our synthetic microbial community,
385 this interpretation remains a plausible hypothesis that should be confirmed in future work.

386 Notably, HT centrate, although containing proteins, nucleic acids, and other unidentified
387 compounds, did not impact the protein content of *C. utilis* relative to the growth on inorganic
388 nitrogen. This is likely attributable to the fact that the majority of the assimilated nitrogen
389 (88 %) was inorganic (NH_4^+). The similar macromolecular composition of *C. utilis* when grown
390 on NH_4Cl and HT centrate highlights the potential for producing a consistent end product
391 through HT centrate recycling.

392 An elevated nucleic acid concentration in the growth medium does not necessarily result in
393 higher nucleic acid content in the microbial biomass, as intracellular nucleic acid levels are
394 largely determined by microbial species-specific responses and growth rate rather than by the
395 extracellular nucleic acid concentration, especially under the non-limiting conditions applied
396 here (François and Parrou, 2001). Any nucleic acids that are not assimilated—or that are only
397 partially hydrolyzed—are expected to remain in the liquid phase and be removed with the
398 supernatant during biomass harvesting. The applied HT followed an established method
399 known to effectively hydrolyse nucleic acids into nucleotides and nucleosides, ensuring

substantial reduction of nucleic acid content in the biomass (Ohta et al., 1971). Moreover, the overall biomass composition after heat treatment was similar to that obtained with ammonium chloride as nitrogen source, indirectly suggesting that the use of this stream had a minimal effect on the nucleic acid content of the microbial biomass. This work therefore serves as a proof of concept demonstrating the suitability of the HT centrate as a nitrogen source for MP production.

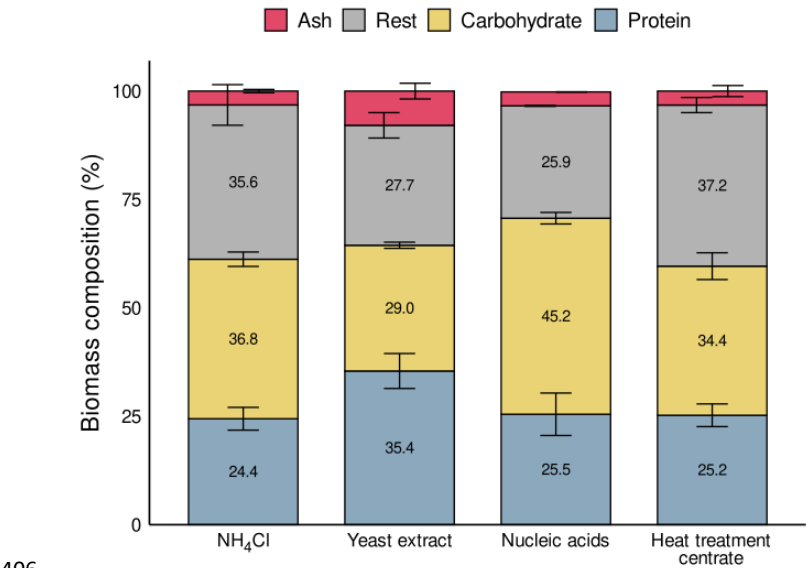
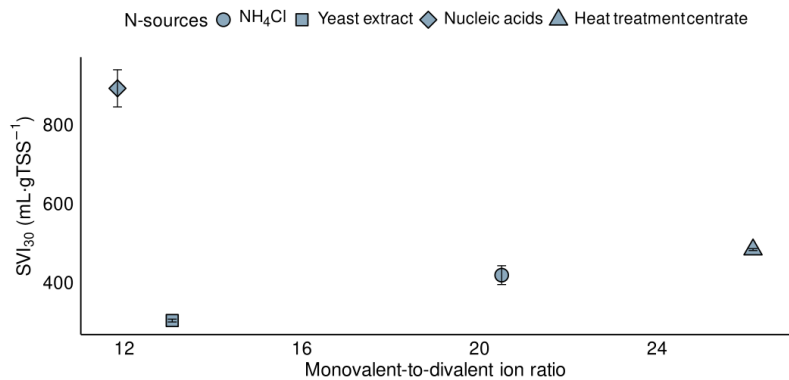


Fig. 3: Biomass composition of *Candida utilis* grown on the different nitrogen sources: NH₄Cl, yeast extract, nucleic acids, and heat treatment centrate. Error bars represent standard deviations of biological replicates.

3.3 Impact of medium composition and nitrogen source on settling characteristics of *Candida utilis*

Although upstream process parameters and nutritional composition were comparable for *C. utilis* grown on NH₄Cl or HT centrate, the settling behaviour (assessed via SVI₃₀) differed

414 between nitrogen sources (**Fig. 4**) which can negatively impact downstream processing. The
 415 lowest SVI₃₀ was achieved when *C. utilis* was cultivated on yeast extract (303 mL·g⁻¹) while the
 416 highest being when cultivated on nucleic acids (892 mL·g⁻¹) as sole nitrogen source.
 417 Settling performance depends on a broad range of parameters such as medium viscosity,
 418 extracellular polymeric substance production, cell wall composition, and ratio of monovalent
 419 (Na⁺, and K⁺) to divalent ions (Ca²⁺ and Mg²⁺) in the medium (Aguilar-Uscanga and François,
 420 2003; Cousin and Ganczarczyk, 1999; Hang and Woodams, 1981; Li and Yang, 2007;
 421 Sivasubramanian et al., 2021; Soares, 2011; Stewart, 2018). For HT centrate, NH₄Cl, and yeast
 422 extract, a clear trend was observed between this ratio and SVI₃₀ (**Fig. 5**). A lower ratio of
 423 monovalent to divalent ions can result in enhanced settling properties, as seen in our study
 424 and others (Sivasubramanian et al., 2021). In contrast, the medium containing nucleic acids as
 425 sole nitrogen source had the lowest ratio of monovalent and divalent ions but resulted in
 426 biomass with a SVI₃₀ 2-3 times higher than the other evaluated nitrogen sources.



427
 428 **Fig. 4:** Sludge volume index (SVI₃₀) of *Candida utilis* cultivated on different nitrogen sources
 429 and its relation to monovalent-to-divalent ion ratio. Error bars represent standard deviations
 430 of biological replicates (n =2).

431 The difference in settling behaviour when growing on nucleic acids led us to believe that other
432 factors, besides monovalent to divalent ion ratio has an impact on the settling behaviour of *C.*
433 *utilis*. The atypical settling behaviour may result from the production of extracellular
434 polymeric substances, triggered by stress associated with limited nitrogen assimilation—
435 despite sufficient nitrogen availability—as only 8.7 % of the supplied nitrogen was consumed.
436 EPS production has been shown to alter biomass settling characteristics (Boonchai et al., 2015;
437 Kronusová et al., 2022; F. Yang et al., 2022; Yang et al., 2023). Furthermore, Westlake (1986)
438 suggested that degraded nucleic acid fragments may adsorb to the cell surface, compounding
439 the reduction in settling efficiency. Although, the precise mechanism is not known, the results
440 point towards worsened SVI₃₀ when *C. utilis* is grown on medium containing nucleic acids as
441 sole nitrogen source. As HT centrate contains nucleic acids and other complex organic
442 components, their contribution to biomass flocculation and settling behaviour remains
443 uncertain. These hypotheses remain speculative, and further work should combine EPS
444 quantification, zeta-potential measurements, and particle-size or floc-structure analysis to
445 determine how nucleic-acid-rich streams alter aggregation and settling dynamics, particularly
446 given the exploratory nature of this study.

447 While the impaired settling observed when using nucleic-acid-rich streams is interesting, the
448 underlying mechanisms remain speculative. Future work should include quantification of
449 extracellular polymeric substances (EPS), zeta potential measurements, and analyses of
450 particle size and floc structure to clarify how nucleic-acid-rich streams influence aggregation
451 and settling behaviour. Given the exploratory nature of this study, these results should be
452 interpreted as indicative trends rather than definitive evidence, providing a basis for more
453 targeted investigations into the physicochemical effects of nucleic acid-rich streams on
454 microbial biomass separation processes.

3.4 Differential growth responses to nitrogen sources among microbial protein candidates

The three microorganisms evaluated (**Fig. 5**), specifically *C. utilis*, *B. subtilis*, and *O. polymorpha*, exhibited distinct growth patterns depending on the supplied nitrogen source. On yeast extract, both *C. utilis* and *B. subtilis* presented significantly increased specific growth rate (17.8–125 %) and optical density (28.0–220 %) compared to NH_4Cl , whereas *O. polymorpha* suffered a 30.3 % decrease in growth rate despite a 49.1 % increase in optical density. Growth on HT centrate showed similar rates compared to NH_4Cl and a 10.4–16.1 % increase in optical densities for all tested species. Only *O. polymorpha* showed a 85.0 % decrease in optical density. Cultivation on pure nucleic acids reduced growth rate and optical density in *C. utilis* and *B. subtilis* (12.2–33.7 % and 18.7–33.1 %, respectively), while *O. polymorpha* uniquely showed a 16.5 % increase in growth rate but a 25.1 % decrease in optical density relative to NH_4Cl .

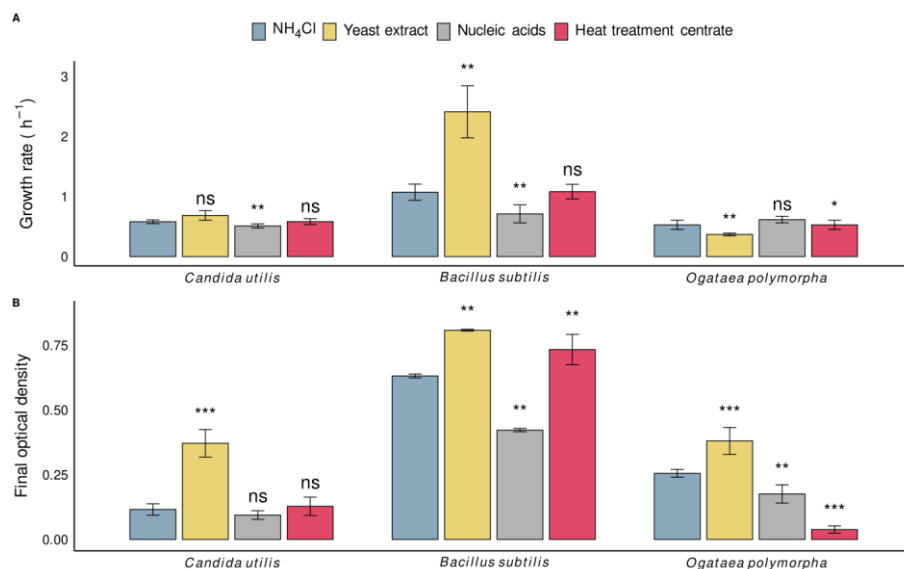


Fig. 5: Comparative growth performance of *Candida utilis*, *Bacillus subtilis*, and *Ogataea polymorpha* on different nitrogen sources: (A) specific growth rate (h⁻¹) and (B) maximum optical density. Error bars represent standard deviations of biological replicates (n=6). For panel (A), differences among nitrogen sources were evaluated using a Kruskal–Wallis test. For panel (B), *C. utilis* and *O. polymorpha* were analysed using one-way ANOVA, whereas *B. subtilis* was analysed using a Kruskal–Wallis test. In all cases, the performance of yeast extract, nucleic acids, and HT-centrate was compared against NH₄Cl as the reference condition. Significance levels are indicated as ns ($p > 0.05$), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

These divergent outcomes likely reflect species-specific capacity to access and utilize complex nitrogen fractions. The relatively good performance of *B. subtilis* on HT centrate may be attributed to its well-documented secretion of proteases and nucleases, facilitating uptake of hydrolysed proteins and nucleic acids (Chen et al., 2023; Condon, 2003; Su et al., 2020). In

contrast, *C. utilis* and *O. polymorpha* showed limited benefit, consistent with their restricted nuclease activity. Notably, the poor growth of *O. polymorpha* on HT centrate cannot be attributed to ionic strength, as both HT centrate and NH_4Cl exhibited similar salt content (Tables S2–S3 in supplementary material), and the species is known to tolerate higher salt levels (Corbu et al., 2021; Riley et al., 2016). This suggests that inhibitory compounds or by-products from HT may negatively affect *O. polymorpha*. Differences in nitrogen assimilation capacity and enzymatic activity (e.g., proteases and nucleases) likely drive the observed species-dependent performance, which is consistent with evidence that microbial nitrogen assimilation and its regulation vary widely across taxa and even among closely related species (Amon et al., 2010; Reed et al., 2025).

3.5 Identifying bottlenecks, opportunities, and knowledge gaps for recycling heat treatment centrate to microbial protein production

Recycling HT centrate as nitrogen source in MP production supported comparable growth and nutritional composition of *C. utilis* relative to conventional inorganic nitrogen (NH_4Cl). Although, the presence of nucleic acids and proteins in HT centrate might enable an increase in biomass yield and protein content (Huang et al., 2022; R. Yang et al., 2022), no such benefits were observed for *C. utilis*. In contrast, *B. subtilis* showed a 16.1 % increase in optical density, suggesting that effective protease and nuclease activity is critical for utilizing the complex nitrogen components in HT centrate. Recycling of spent medium has previously been shown to reduce nutrient supplementation and process water costs by up to 55 % (Arora et al., 2023; Wynne et al., 2025), reinforcing its potential as a cost-saving strategy.

At present, HT centrate is discharged for treatment rather than reused, representing both a loss of recoverable nutrients and a missed opportunity for process integration. However, to

506 fully valorise this stream, detailed characterization of the unidentified organic nitrogen
507 fraction—particularly peptide length distribution and nucleic acid derivatives—is essential.
508 The HT centrate is expected to contain organic nitrogen in the form of short peptides, free
509 amino acids, and nucleic acid derivatives generated through thermal lysis and partial
510 hydrolysis of the microbial biomass (Lonchamp et al., 2020, 2019). These molecules are
511 metabolically relevant, as the bioavailability of peptides or amino acids differs substantially
512 from that of intact proteins or nucleic acids and may influence growth rate, nitrogen utilisation
513 efficiency, and EPS production. A more detailed characterisation—e.g., through LC-MS/MS for
514 amino acid and peptide profiling, size-exclusion chromatography for peptide length
515 distribution, or targeted nucleotide and nucleoside quantification—would enable
516 discrimination between readily assimilable and more recalcitrant fractions. Given the species-
517 dependent responses observed in this and other studies, these analyses would also support
518 the design of targeted pre-treatments to enhance nitrogen uptake.

519 Further investigation is essential before this approach reaches industrial implementation.
520 Particular attention should be given to the potential accumulation of residual organics, or
521 inhibitory compounds during repeated reuse, which may compromise compliance with (food
522 and feed) safety regulations. Nutrient imbalances introduced by secondary metabolites in HT
523 centrate also merit attention, as they may affect metabolism and metabolite formation (e.g.
524 EPS), and settling behaviour. In addition, careful control of nutrient balances is essential when
525 recycling HT centrate. Salt accumulation through repeated reuse can disrupt osmotic balance,
526 potentially reducing biomass concentrations by up to 40.8 % under severe imbalances
527 (Murphy et al., 2024). Interestingly, elevated ionic strength has also been associated with an
528 18.6 % increase in protein content, suggesting a complex trade-off between yield and
529 composition (Paquette et al., 2022). Beyond reducing water and nutrient inputs, HT centrate

530 recycling may also lower medium sterilisation costs by enabling partial reuse medium (Li et
531 al., 2014); however, it concurrently increases the risk of microbial contamination due to the
532 potential introduction of residual biomass or surviving spores from previous cycles (Lu et al.,
533 2020; Wynne et al., 2025), necessitating appropriate monitoring and control strategies.
534 Despite these limitations, our findings provide a valuable indication of how HT centrate could
535 be integrated into MP production.

536 4 Conclusion

537 Reusing HT centrate in MP production offers a promising route to recover valuable nitrogen
538 without compromising *Candida utilis* growth or biomass composition, when compared to
539 conventional inorganic sources such as NH_4Cl , typically employed in bioprocesses. This
540 demonstrates the potential of HT centrate as a cost-effective and more sustainable nitrogen
541 input. Importantly, the impact of HT centrate appears to be microbial species-dependent, and
542 in the case of *B. subtilis* increased the growth performance—potentially due to its strong
543 proteolytic and nucleolytic capacity. This suggests that, beyond safe reuse, HT centrate may
544 even confer a growth advantage in certain microbial species, further reinforcing its potential
545 within MP production systems. Comprehensive profiling of the peptide and nucleotide
546 fractions in HT centrate is essential to guide targeted pretreatment strategies, such as
547 enzymatic hydrolysis or thermal processing, aimed at converting macromolecules into their
548 monomers. These approaches may enhance nutrient bioavailability, particularly for microbial
549 species with limited proteolytic or nucleolytic capacity. When implemented, HT centrate
550 recycling could lower water and nutrient needs and medium-related costs, and improve
551 overall process sustainability, but further research is required to translate these findings to
552 industrial-scale MP-producing systems.

5 References

- Aguilar-Uscanga, B., François, J. m., 2003. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Lett. Appl. Microbiol. 37, 268–274. <https://doi.org/10.1046/j.1472-765X.2003.01394.x>
- Amon, J., Titgemeyer, F., Burkovski, A., 2010. Common patterns - unique features: nitrogen metabolism and regulation in Gram-positive bacteria. FEMS Microbiol. Rev. 34, 588–605. <https://doi.org/10.1111/j.1574-6976.2010.00216.x>
- Antheunisse, J., 1972. Decomposition of nucleic acids and some of their degradation products by microorganisms. Antonie Van Leeuwenhoek 38. <https://doi.org/10.1007/BF02328101>
- Arora, N., Lo, E., Legall, N., Philippidis, G., 2023. A Critical Review of Growth Media Recycling to Enhance the Economics and Sustainability of Algae Cultivation. Energies 16, 5378. <https://doi.org/10.3390/en16145378>
- Bagul, V.P., Annapure, U.S., 2020. Effect of sequential recycling of spent media wastewater on docosahexaenoic acid production by newly isolated strain *Aurantiochytrium* sp. ICTFD5. Bioresour. Technol. 306, 123153. <https://doi.org/10.1016/j.biortech.2020.123153>
- Boonchai, R., Kaewsuk, J., Seo, G., 2015. Effect of nutrient starvation on nutrient uptake and extracellular polymeric substance for microalgae cultivation and separation. Desalination Water Treat. 55, 360–367. <https://doi.org/10.1080/19443994.2014.939501>
- Brown, C.M., Rose, A.H., 1969. Effects of Temperature on Composition and Cell Volume of *Candida utilis*. J. Bacteriol. 97, 261–272. <https://doi.org/10.1128/jb.97.1.261-272.1969>
- Buerth, C., Heilmann, C.J., Klis, F., Koster, C., Ernst, J., Tielker, D., 2011. Growth-dependent secretome of *Candida utilis*. Microbiol. Read. Engl. 157, 2493–503. <https://doi.org/10.1099/mic.0.049320-0>
- Bzducha-Wróbel, A., Farkaš, P., Bieliková, S., Čížová, A., Sujkowska-Rybkowska, M., 2024. How do the carbon and nitrogen sources affect the synthesis of β -(1,3/1,6)-glucan, its structure and the susceptibility of *Candida utilis* yeast cells to immunolabelling with β -(1,3)-glucan monoclonal antibodies? Microb. Cell Factories 23, 28. <https://doi.org/10.1186/s12934-024-02305-4>
- Candry, P., Van Daele, T., Denis, K., Amerlinck, Y., Andersen, S.J., Ganigué, R., Arends, J.B.A., Nopens, I., Rabaey, K., 2018. A novel high-throughput method for kinetic characterisation of anaerobic bioproduction strains, applied to *Clostridium kluyveri*. Sci. Rep. 8, 9724. <https://doi.org/10.1038/s41598-018-27594-9>
- Cazin, J., Kozel, T., Lupan, D., Burt, W., 1969. Extracellular Deoxyribonuclease Production by Yeasts. J. Bacteriol. 100. <https://doi.org/10.1128/jb.100.2.760-762.1969>
- Chen, T., Brul, S., Hugenholtz, J., 2023. Exploring the potential of *Bacillus subtilis* as cell factory for food ingredients and special chemicals. Microb. Cell Factories 22, 200. <https://doi.org/10.1186/s12934-023-02208-w>
- Condon, C., 2003. RNA Processing and Degradation in *Bacillus subtilis*. Microbiol. Mol. Biol. Rev. 67, 157–174. <https://doi.org/10.1128/MMBR.67.2.157-174.2003>
- Corbu, V., Petrut, S., Vassu, T., Pelinescu (maiden name Smarandache), D., Avram, I., Elena, R., Csutak, O., 2021. Environmental stress responses in yeasts and lactic acid bacteria strains isolated from dairy traditional Romanian fermented products. Romanian Biotechnol. Lett. 26, 2548–2559. <https://doi.org/10.25083/rbl/26.2/2548.2559>

599 Cousin, C.P., Ganczarczyk, J.J., 1999. The Effect of Cationic Salt Addition on the Settling and
600 Dewatering Properties of an Industrial Activated Sludge. *Water Environ. Res.* 71, 251–
601 254. <https://doi.org/10.2175/106143098X121725>

602 Delmoitié, B., Regueira, A., De Wever, H., Ganigué, R., 2025. To ferment or not to ferment?
603 Evaluating the impact of pre-fermentation of cheese whey permeate on microbial
604 protein production with designed synthetic communities. *J. Environ. Chem. Eng.* 13.
605 <https://doi.org/10.1016/j.jece.2025.117831>

606 Depraetere, O., Pierre, G., Noppe, W., Vandamme, D., Foubert, I., Michaud, P., Muylaert, K.,
607 2015. Influence of culture medium recycling on the performance of *Arthrospira*
608 *platensis* cultures. *Algal Res.* 10, 48–54. <https://doi.org/10.1016/j.algal.2015.04.014>

609 Dufossé, L., D., D., Guérard, F., 1997. Fish protein hydrolysates as nitrogen sources for
610 microbial growth and metabolite production, in: *Recent Res. Dev. Microbiol.* pp. 365–
611 381.

612 Dury, S., Bendjebbar, P., Hainzelin, E., Giordano, T., Bricas, N., 2019. Food Systems at risk: new
613 trends and challenges. Rome, Italy.

614 Garg, S.K., Neelakantan, S., 1982. Effect of nutritional factors on cellulase enzyme and
615 microbial protein production by *Aspergillus terreus* and its evaluation. *Biotechnol.*
616 *Bioeng.* 24, 109–125. <https://doi.org/10.1002/bit.260240110>

617 Grasshof, K., Liebezeit, G., Josefsson, B., Dawson, R., 1983. Methods of seawater analysis.

618 Gu, H., Nagle, N., Pienkos, P.T., Posewitz, M.C., 2015. Nitrogen recycling from fuel-extracted
619 algal biomass: Residuals as the sole nitrogen source for culturing *Scenedesmus acutus*.
620 *Bioresour. Technol., Advances in biofuels and chemicals from algae* 184, 153–160.
621 <https://doi.org/10.1016/j.biortech.2014.11.095>

622 Hang, Y.D., Woodams, E.E., 1981. Rapid removal of lactic acid from wastewater by a flocculent
623 yeast. *J. Food Sci.* 46, 1498–1499. <https://doi.org/10.1111/j.1365-2621.1981.tb04206.x>

625 Henchion, M., Hayes, M., Mullen, A.M., Fenelon, M., Tiwari, B., 2017. Future Protein Supply
626 and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods* 6, 53.
627 <https://doi.org/10.3390/foods6070053>

628 Huang, L., Zhang, Y., Du, X., An, R., Liang, X., 2022. *Escherichia coli* Can Eat DNA as an Excellent
629 Nitrogen Source to Grow Quickly. *Front. Microbiol.* 13, 894849.
630 <https://doi.org/10.3389/fmicb.2022.894849>

631 Ishikawa, T., Aw, W., Kaneko, K., 2013. Metabolic Interactions of Purine Derivatives with
632 Human ABC Transporter ABCG2: Genetic Testing to Assess Gout Risk. *Pharmaceuticals*
633 6, 1347–1360. <https://doi.org/10.3390/ph6111347>

634 Kieliszek, M., Bierla, K., Jiménez-Lamana, J., Kot, A.M., Alcántara-Durán, J., Piwowarek, K.,
635 Błażej, S., Szpunar, J., 2020. Metabolic Response of the Yeast *Candida utilis* During
636 Enrichment in Selenium. *Int. J. Mol. Sci.* 21, 5287.
637 <https://doi.org/10.3390/ijms21155287>

638 Kieliszek, M., Błażej, S., Bzducha-Wróbel, A., Kot, A.M., 2019. Effect of selenium on growth
639 and antioxidative system of yeast cells. *Mol. Biol. Rep.* 46, 1797–1808.
640 <https://doi.org/10.1007/s11033-019-04630-z>

641 Kim, G., Mujtaba, G., Lee, K., 2016. Effects of nitrogen sources on cell growth and biochemical
642 composition of marine chlorophyte *Tetraselmis* sp. for lipid production. *ALGAE* 31,
643 257–266. <https://doi.org/10.4490/algae.2016.31.8.18>

644 Kronusová, O., Kaštánek, P., Koyun, G., Kaštánek, F., Brányik, T., 2022. Factors Influencing the
645 Production of Extracellular Polysaccharides by the Green Algae *Dictyosphaerium*

646 *chlorelloides* and Their Isolation, Purification, and Composition. *Microorganisms* 10,
647 1473. <https://doi.org/10.3390/microorganisms10071473>

648 Larsen, J., Joergensen, L., 1996. Reduction of RNA and DNA in *Methylococcus capsulatus* by
649 endogenous nucleases. *Appl. Microbiol. Biotechnol.* 45, 137–140.
650 <https://doi.org/10.1007/s002530050661>

651 Leake, J.R., Read, D.J., 1991. Proteinase activity in mycorrhizal fungi III. *New Phytol.* 117, 309–
652 317. <https://doi.org/10.1111/j.1469-8137.1991.tb04912.x>

653 Leger, D., Matassa, S., Noor, E., Shepon, A., Milo, R., Bar-Even, A., 2021. Photovoltaic-driven
654 microbial protein production can use land and sunlight more efficiently than
655 conventional crops. *Proc. Natl. Acad. Sci.* 118, e2015025118.
656 <https://doi.org/10.1073/pnas.2015025118>

657 Li, T., Chen, X., Chen, J., Wu, Q., Chen, G.-Q., 2014. Open and continuous fermentation:
658 Products, conditions and bioprocess economy. *Biotechnol. J.* 9, 1503–1511.
659 <https://doi.org/10.1002/biot.201400084>

660 Li, X.Y., Yang, S.F., 2007. Influence of loosely bound extracellular polymeric substances (EPS)
661 on the flocculation, sedimentation and dewaterability of activated sludge. *Water Res.*
662 41, 1022–1030. <https://doi.org/10.1016/j.watres.2006.06.037>

663 Liang, X., Zhuang, J., An, R., Liang, X., 2025. Yeast Eats RNA Oligonucleotides as Excellent
664 Nitrogen Source Via Endocytosis, Autophagy, and RNA Degradation.
665 <https://doi.org/10.20944/preprints202504.1194.v1>

666 Liu, J., Li, H., Zhao, G., Caiyin, Q., Qiao, J., 2018. Redox cofactor engineering in industrial
667 microorganisms: strategies, recent applications and future directions. *J. Ind. Microbiol.*
668 *Biotechnol.* 45, 313–327. <https://doi.org/10.1007/s10295-018-2031-7>

669 Lonchamp, J., Akintoye, M., Clegg, P.S., Euston, S.R., 2020. Functional fungal extracts from the
670 Quorn fermentation co-product as novel partial egg white replacers. *Eur. Food Res.*
671 *Technol.* 246, 69–80. <https://doi.org/10.1007/s00217-019-03390-1>

672 Lonchamp, J., Clegg, P., Euston, S., 2019. Foaming, emulsifying and rheological properties of
673 extracts from a co-product of the Quorn fermentation process. *Eur. Food Res. Technol.*
674 245. <https://doi.org/10.1007/s00217-019-03287-z>

675 Lu, Z., Loftus, S., Sha, J., Wang, W., Park, M.S., Zhang, X., Johnson, Z.I., Hu, Q., 2020. Water
676 reuse for sustainable microalgae cultivation: current knowledge and future directions.
677 *Resour. Conserv. Recycl.* 161, 104975.
678 <https://doi.org/10.1016/j.resconrec.2020.104975>

679 Lucca, M.E., Romero, M.E., Callieri, D.A.S., 1995. Continuous culture of *Candida utilis*:
680 influence of medium nitrogen concentration. *World J. Microbiol. Biotechnol.* 11, 515–
681 518. <https://doi.org/10.1007/BF00286365>

682 Merchant, S., Helmann, J., 2012. Elemental Economy: Microbial Strategies for Optimizing
683 Growth in the Face of Nutrient Limitation. *Adv. Microb. Physiol.* 60, 91–210.
684 <https://doi.org/10.1016/B978-0-12-398264-3.00002-4>

685 Murphy, L., Lynch, C., O'Connell, D., 2024. Valorisation of Spent Yeast Fermentation Media
686 through Compositional-Analysis-Directed Supplementation. *Appl. Microbiol.* 4, 959–
687 971. <https://doi.org/10.3390/applmicrobiol4020065>

688 Mydland, L.T., Frøyland, J.R.K., Skrede, A., 2008. Composition of individual nucleobases in diets
689 containing different products from bacterial biomass grown on natural gas, and
690 digestibility in mink (*Mustela vison*). *J. Anim. Physiol. Anim. Nutr.* 92, 1–8.
691 <https://doi.org/10.1111/j.1439-0396.2007.00674.x>

Ohta, S., Maul, S., Sinskey, A.J., Tannenbaum, S.R., 1971. Characterization of a Heat-Shock Process for Reduction of the Nucleic Acid Content of *Candida utilis*. Appl. Microbiol. 22. <https://doi.org/10.1128/am.22.3.415-421.1971>

Olufunke O. Ezekiel, Ogugua C. Aworh, James C. Du Preez, Laurinda Steyn, 2012. Cultivation of *Candida utilis* on Cassava Peel Hydrolysates for Single-cell Protein Production. J. Food Sci. Eng. 2. <https://doi.org/10.17265/2159-5828/2012.08.004>

Ouedraogo, N., Savadogo, A., Somda, M.K., Tapsoba, F., Zongo, C., Traore, A.S., 2017. Effect of mineral salts and nitrogen source on yeast (*Candida utilis* NOY1) biomass production using tubers wastes. Afr. J. Biotechnol. 16, 359–365. <https://doi.org/10.5897/AJB2016.15801>

Paquette, A.J., Vadlamani, A., Demirkaya, C., Strous, M., De la Hoz Siegler, H., 2022. Nutrient management and medium reuse for cultivation of a cyanobacterial consortium at high pH and alkalinity. Front. Bioeng. Biotechnol. 10. <https://doi.org/10.3389/fbioe.2022.942771>

Parsons, S., Abeln, F., McManus, M.C., Chuck, C.J., 2019. Techno-economic analysis (TEA) of microbial oil production from waste resources as part of a biorefinery concept: assessment at multiple scales under uncertainty. J. Chem. Technol. Biotechnol. 94, 701–711. <https://doi.org/10.1002/jctb.5811>

Pinchuk, G.E., Ammons, C., Culley, D.E., Li, S.-M.W., McLean, J.S., Romine, M.F., Nealson, K.H., Fredrickson, J.K., Beliaev, A.S., 2008. Utilization of DNA as a Sole Source of Phosphorus, Carbon, and Energy by *Shewanella* spp.: Ecological and Physiological Implications for Dissimilatory Metal Reduction. Appl. Environ. Microbiol. 74, 1198–1208. <https://doi.org/10.1128/AEM.02026-07>

Reed, K., Dang, C., Walkup, J., Purcell, A., Hungate, B., Morrissey, E., 2025. Comparing field and lab quantitative stable isotope probing for nitrogen assimilation in soil microbes. Appl. Environ. Microbiol. 91, e0184924. <https://doi.org/10.1128/aem.01849-24>

Riley, R., Haridas, S., Wolfe, K.H., Lopes, M.R., Hittinger, C.T., Göker, M., Salamov, A.A., Wisecaver, J.H., Long, T.M., Calvey, C.H., Aerts, A.L., Barry, K.W., Choi, C., Clum, A., Coughlan, A.Y., Deshpande, S., Douglass, A.P., Hanson, S.J., Klenk, H.-P., LaButti, K.M., Lapidus, A., Lindquist, E.A., Lipzen, A.M., Meier-Kolthoff, J.P., Ohm, R.A., Otiilar, R.P., Pangilinan, J.L., Peng, Y., Rokas, A., Rosa, C.A., Scheuner, C., Sibirny, A.A., Slot, J.C., Stielow, J.B., Sun, H., Kurtzman, C.P., Blackwell, M., Grigoriev, I.V., Jeffries, T.W., 2016. Comparative genomics of biotechnologically important yeasts. Proc. Natl. Acad. Sci. U. S. A. 113, 9882–9887. <https://doi.org/10.1073/pnas.1603941113>

Ritala, A., Häkkinen, S.T., Toivari, M., Wiebe, M.G., 2017. Single Cell Protein—State-of-the-Art, Industrial Landscape and Patents 2001–2016. Front. Microbiol. 8. <https://doi.org/10.3389/fmicb.2017.02009>

Sen, K., Komagata, K., 1979. Distribution of Urease and Extracellular Dnase in Yeast Species. J. Gen. Appl. Microbiol. 25, 127–135. <https://doi.org/10.2323/jgam.25.127>

Shanthi, G., Premalatha, M., Anantharaman, N., 2018. Effects of l-amino acids as organic nitrogen source on the growth rate, biochemical composition and polyphenol content of *Spirulina platensis*. Algal Res. 35, 471–478. <https://doi.org/10.1016/j.algal.2018.09.014>

Simpson, S.D., Gao, A.H., Conrado, R.J., Winkler, J.D., Mueller, A.P., Brown, S., Tran, L.P., Koepke, M., Mihalcea, C.D., 2021. Secondary acetate fermentation. WO2021016523A1.

738 Sivasubramanian, R., Chen, G., Mackey, H.R., 2021. The effectiveness of divalent cation
 739 addition for highly saline activated sludge cultures: Influence of monovalent/divalent
 740 ratio and specific cations. *Chemosphere* 274, 129864.
 741 <https://doi.org/10.1016/j.chemosphere.2021.129864>
 742 Soares, E.V., 2011. Flocculation in *Saccharomyces cerevisiae*: a review: Yeast flocculation: a
 743 review. *J. Appl. Microbiol.* 110, 1–18. [https://doi.org/10.1111/j.1365-](https://doi.org/10.1111/j.1365-2672.2010.04897.x)
 744 [2672.2010.04897.x](https://doi.org/10.1111/j.1365-2672.2010.04897.x)
 745 Stewart, G.G., 2018. Yeast Flocculation—Sedimentation and Flotation. *Fermentation* 4, 28.
 746 <https://doi.org/10.3390/fermentation4020028>
 747 Stouthamer, A.H., 1973. A theoretical study on the amount of ATP required for synthesis of
 748 microbial cell material. *Antonie Van Leeuwenhoek* 39, 545–565.
 749 <https://doi.org/10.1007/BF02578899>
 750 Su, Y., Fang, H., Zhang, D., 2020. *Bacillus subtilis*: a universal cell factory for industry,
 751 agriculture, biomaterials and medicine | Microbial Cell Factories | Full Text. *Microb.*
 752 *Cell Factories* 19. <https://doi.org/10.1186/s12934-020-01436-8>
 753 Van Peteghem, Sakarika, M., Matassa, S., Pikaar, I., Ganigué, R., Rabaey, K., 2022a. Towards
 754 new carbon-neutral food systems: Combining carbon capture and utilization with
 755 microbial protein production. *Bioresour. Technol.* 349, 126853.
 756 <https://doi.org/10.1016/j.biortech.2022.126853>
 757 Van Peteghem, Sakarika, M., Matassa, S., Rabaey, K., 2022b. The role of microorganisms and
 758 carbon-to-nitrogen ratios for microbial protein production from bioethanol. *Appl.*
 759 *Environ. Microbiol.* 88. <https://doi.org/10.1128/aem.01188-22>
 760 Wechselberger, P., Sagmeister, P., Herwig, C., 2013. Real-time estimation of biomass and
 761 specific growth rate in physiologically variable recombinant fed-batch processes.
 762 *Bioprocess Biosyst. Eng.* 36, 1205–1218. <https://doi.org/10.1007/s00449-012-0848-4>
 763 Westlake, R., 1986. Large-scale Continuous Production of Single Cell Protein. *Chem. Ing. Tech.*
 764 58, 934–937. <https://doi.org/10.1002/cite.330581203>
 765 Wynne, E., Yoon, J., Park, D., Cui, M., Morris, C., Lee, J., Wang, Z., Yoon, S., Han, J., 2025.
 766 Regeneration of Spent Culture Media for Sustainable and Continuous mAb Production
 767 via Ion Concentration Polarization. *Biotechnol. Bioeng.* 122, 373–381.
 768 <https://doi.org/10.1002/bit.28888>
 769 Yang, F., Huang, J., Xu, S., Huang, X., Guo, J., Fang, F., Chen, Y., Yan, P., 2022. Influence of
 770 nitrogen-poor wastewater on activated sludge aggregation and settling: Sequential
 771 responses of extracellular proteins and exopolysaccharides. *J. Clean. Prod.* 359,
 772 132160. <https://doi.org/10.1016/j.jclepro.2022.132160>
 773 Yang, F., Wang, S., Li, H., Wang, G., Wang, Y., Yang, J., Chen, Y., Yan, P., Guo, J., Fang, F., 2023.
 774 Differences in responses of activated sludge to nutrients-poor wastewater: Function,
 775 stability, and microbial community. *Chem. Eng. J.* 457, 141247.
 776 <https://doi.org/10.1016/j.cej.2022.141247>
 777 Yang, R., Chen, Z., Hu, P., Zhang, S., Luo, G., 2022. Two-stage fermentation enhanced single-
 778 cell protein production by *Yarrowia lipolytica* from food waste. *Bioresour. Technol.*
 779 361, 127677. <https://doi.org/10.1016/j.biortech.2022.127677>
 780 Yano, T., Mori, H., Kobayashi, T., Shimizu, S., 1980. Reusability of Broth Supernatant as
 781 Medium. *J. Ferment. Technol.* 58, 259–266.
 782 Young, T.B., Koplove, M., 1975. Recycle fermentation process. US3886046A.
 783 Zhang, B., Ren, D., Liu, Q., Liu, X., Bao, J., 2023. Coproduction of single cell protein and lipid
 784 from lignocellulose derived carbohydrates and inorganic ammonia salt with soluble

785 ammonia recycling. Bioresour. Technol. 384, 129345.
786 <https://doi.org/10.1016/j.biortech.2023.129345>
787