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Heterotrophic growth of *Galdieria sulphuraria* on residues from aquaculture and fish processing industries

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HIGHLIGHTS

- *G. sulphuraria* cultivation occurred in a medium made from fish processing streams.
- Proteolytic treatment of rainbow trout residues resulted in a superior nitrogen source.
- In a non-sterile fed-batch culture a biomass concentration of 80 g L^{-1} was achieved.
- Produced biomass did show littles microbial contaminants.

GRAPHICAL ABSTRACT

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ABSTRACT

The study aimed at zero-waste utilization of fish processing streams for cultivation of microalgae *Galdieria sulphuraria*. Wastewater from a fish processing facility, slam (mix of used fish feed and faeces), and dried pellet (sediments after enzymatic hydrolysis of rainbow trout) were investigated as potential sources of carbon, nitrogen, and phosphate for cultivation of *G. sulphuraria*. The pellet extract was found to support the growth of *G. sulphuraria* when appropriate diluted, at concentrations below 40 % (v/v). It was revealed that wastewater does not impact the growth negatively, however free amino nitrogen and carbon sources need to be supplied from another source. Therefore, only proteolyzed pellet extract (20 %, v/v) was selected for upscaling and a biomass concentration of 80 g L^{-1} (growth rate was 0.72 day^{-1}) was achieved in a non-sterile fed-batch culture. Even though biomass was produced under non-sterile conditions no pathogens such as *Salmonella* sp. could be detected.

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1. Introduction

Residues from food and feed processing but also organic waste streams have been intensively investigated as nutrient sources in biotechnological processes (Koutinas et al., 2014). The overall goal was to utilize organic material instead of disposing it. Biotechnological processes allow for the formation of a broad range of products such as organic acids (Son et al., 2022), bioplastics (Zhao et al., 2023), proteins (Khan et al., 2023), or single cell biomass (Spalvins et al., 2018). The different organic material that should undergo a utilization, may vary in composition. There are materials which are rich in carbohydrates (Kazemi Shariat Panahi et al., 2022), proteins (Khan et al., 2023), or lipids (Abomohra et al., 2022), or have a more balanced composition (Thygesen et al., 2021). Depending on the composition, different utilization approaches need to be developed. In the past, the focus was mainly on carbon sources to be recovered and used in biotechnological processes (Ginni et al., 2021; Koutinas et al., 2014; Prado et al., 2016). Nowadays, the focus has been shifted to the recovery of nitrogen compounds such as proteins and amino acids (Thielemann and Pleissner, 2023). This is due to environmental issues linked by the uncontrolled decomposition of these compounds if not properly handled, but also due to the value of organic nitrogen compounds and the urgency of using it responsibly in circular concepts (UNEP, 2019).

Global fish production is expected to achieve the production volume of 200 metric tons (MT) by 2029, making up a 14% increase (25 MT) compared to the pre-COVID period of 2017–2019 (OECD/FAO, 2022). However, the fish industry processes only 30–40% of fish raw material into high value products for direct human consumption such as fish fillets, steaks, or medallions. The remaining part of the fish discards and side streams obtained after the primary production (from 25% to 70%) and including fins, heads, skin, and viscera, are mostly used for low-value applications such as fish meal and oil (Peñarubia, 2021). Annually, discards from the world's fisheries exceed 9 million tons equivalent to about 10% of the annual catch (FAO, 2020). However, if the quality of fish discards or side streams does not correspond to the quality requirements for these categories of products according to the EU Waste Framework Directive (Directive 2006/12/EC), the generated fish residuals are directed towards the production of biogas, compost, or even incineration or landfilling (Venugopal, 2021). Despite the low value traditionally assigned to fish side streams, they could be used for extraction of essential nutrients such as vitamins, omega-3 rich fish oils or small bioactive peptides/hydrolysates (Bartolomei et al., 2023).

From a utilization and application perspective, it can make sense to separate proteins and amino acids from organic material streams and to use both directly (Pleissner, 2022). For the use in chemical products, for instance, such an approach is rather unproblematic. However, if the focus is on the utilization of organic waste streams for feed or even food purposes, then a conversion is needed to allow the formation of products which are microbially and chemically safe and specific legislative restriction are to be considered (Cho et al., 2022; Delsignore and Siddiqui, 2022). In this respect, the conversion of organic waste streams via microalgae could be a path towards a supply of safe biomass.

Studies have been published considering the use of phototrophic microalgae such as the diatom *Synedra* sp. for the utilization of fish pond wastewater and the nutrients within (Li et al., 2017), *Chlorella sorokiniana* and *Scenedesmus acuminatus* for removal of nitrogen-compounds in shrimp wastewater (Li et al., 2022), fish processing wastewater treatment using microalgae-containing microbiota (Riaño et al., 2011), and for utilizing fish residues as organic nitrogen source for *Arthrospira platensis* (Shanthi et al., 2021). Furthermore, Vidya et al. studied the microalgal strains *Chroococcus* sp., *Haematococcus pluvialis*, *Dunaliella* sp., *Coelastrella saipanensis*, and *Chlorella* sp. in dairy wastewater and fish waste as nutrients (Vidya et al., 2023). Even though the cultivations were carried out under illumination the authors found a heterotrophic nutrient absorption. However, it is assumed here that a growth of contaminations cannot be ruled out when fish waste with high microbial load is applied and a treatment to reduce contaminations might be necessary.

Recently published results have shown, that the conversion of digestate via the heterotrophically grown microalga *Galdieria sulphuraria* results in biomass which is low in microbial contaminations (Pleissner and Händel, 2023). *G. sulphuraria* was grown at a pH of 2 and 45 °C. Those conditions did not only prevent contaminations from taking over the non-sterile carried out cultivation, but also diminished most of the bacteria incl. pathogens like *Salmonella* sp. (Dauda et al., 2019). However, it should be admitted that spore forming bacteria were still present after a seven days long treatment at pH 2 and further treatment is needed. Nevertheless, even the reduction of the microbial load alone is a promising start for the development of novel utilization strategies.

G. sulphuraria has been shown to grow on various hydrolysates of organic waste streams (di Cicco et al., 2021; Henkanatte-Gedera et al., 2017; Julius Pahmeyer et al., 2022; Pleissner and Händel, 2023; Pleissner et al., 2021; Russo et al., 2021; Scherhag and Ackermann, 2020; Sloth et al., 2017). Thus, it was hypothesized that *G. sulphuraria* might also be suitable to convert waste streams appearing from aquaculture. Waste streams from aquaculture can be heterogenous in term of composition and integrated waste management strategies are required to limit environmental degradation (Dauda et al., 2019; Siddiqui et al., 2023). Optimally, the waste management strategy prevents the waste from uncontrolled degradation, diminishes pathogens, and creates biomass which can potentially be used as aquaculture feed. It might be expected that a waste management strategy via *G. sulphuraria* cultivation can fulfil these points.

In order to prove this strategy, in the present study wastewater from a fish processing facility, a mix of used fish feed and faeces collected from aquaculture ponds, and sediments obtained after enzymatic hydrolysis of fish residues have been investigated as nutrient source in non-sterile *G. sulphuraria* fed-batch cultivation. The aim was to recover carbohydrates as carbon sources, amino acids as nitrogen sources as well as phosphate to establish a *G. sulphuraria* cultivation. The outcomes of this study are expected to contribute towards an efficient utilization of aquaculture waste streams and a circular aquaculture economy.

2. Material and methods

2.1. Galdieria sulphuraria

G. sulphuraria strain 21.92 was purchased from the Culture Collection of Algae (SAG, University of Göttingen, Germany) and maintained in 100 mL flasks containing 20 mL cyanidium medium consisting of 2.5 g L^{-1} glucose, 1 g L^{-1} $(NH_4)_2SO_4$, 0.02 g L^{-1} K_2HPO_4 and 0.02 g L^{-1} $MgSO_4 \cdot 7H_2O$ at pH 2, 45 °C and shaken at 130 rpm on an orbital shaker. Subcultivation occurred once per week by adding 50 µl of algae suspension to 20 mL fresh cyanidium medium.

2.2. Residues

Residual streams used for *G. sulphuraria* cultivation were pellet (dried sediments obtained after enzymatic hydrolysis of rainbow trout raw material), slam (a mix of used fish feed and faeces), and wastewater, and were supplied by Norwegian University of Science and Technology (NTNU). Pellet was obtained after enzymatic hydrolysis of rainbow trout (*Oncorhynchus mykiss*) raw material. After centrifugation of the hydrolysate, the pellet was collected, freeze-dried for storage purpose, and subsequently used in experiments.

Wastewater was collected from the production line of fish processing factory (MOWI, Norway), and consisted of fish blood and water. Fish processing wastewater was autoclaved and kept at room temperature until used in experiments.

Slam was collected from the end of a Salsnes filter system at a hatchery plant (Hofseth Aqua, Tafjord, Norway). The slam contained fish faeces and feed remainder etc. It was either stored frozen at –20 °C

until use, or solid material was separated from liquid by centrifugation, freeze-dried for storage purpose, and subsequently used in experiments.

2.3. Preparation of extracts

With the freeze-dried residues, an aqueous extraction was prepared. Freeze-dried pellet powder was mixed with ultra-pure water (Mili-Q) or fish processing wastewater to give a 10 % (w/v) suspension. This suspension was incubated on an orbital shaker at 120 rpm and 22 °C for 24 h. After incubation, the mixture was centrifuged (10 min, 20,000*g*) and the supernatant was filtrated over a GF/F glass fibre filter. The pH of the filtrate was adjusted to 2 with H_2SO_4 and autoclaved (15 min, 121 °C) to give the final pellet extract for use in *G. sulphuraria* cultivation.

The preparation of slam-extract was performed in the same way as pellet-extract, but the initial suspension in water contained only 5 % (w/v) slam powder. When frozen slam was used as starting material, a suspension with a 5 % (w/w) dry matter content was likewise prepared with Mili-Q water.

2.4. Hydrolysis of residues

To further assess the enzyme-digestibility of residues and thereby release nutrients, pellet-extract, slam-extract and fish processing wastewater were treated with 2 mL L^{-1} of protease (Protease S-02, ASA Spezialenzyme GmbH), *gluco*-amylase (Glucoamylase AN, ASA Spezialenzyme GmbH), and cellulase (enzyme blend "Cellic CTec2", Sigma-Aldrich). Incubation was done at pH 5 and 50 °C for 48 h. Sampling occurred regularly. After centrifugation of samples for 5 min at 9,800*g*, the supernatant was frozen at −20 °C until analysis.

For use in cultivation experiments, pellet and slam were digested with only protease or *gluco*-amylase, respectively. The hydrolysis was performed in the incubation step during extract preparation: The 10 % (w/v) pellet suspension was supplemented with 2 mL L^{-1} of enzyme solution (Protease S-02, ASA Spezialenzyme GmbH) and incubated for 24–48 h on an orbital shaker at 130 rpm and 60 °C and pH 3. After incubation the mixture was centrifuged (10 min, 20,000*g*) and the supernatant was filtrated over a GF/F glass fibre filter. The pH of the filtrate was adjusted to 2 with H_2SO_4 and autoclaved (15 min, 121 °C) to give the final hydrolysed pellet extract.

To release carbon sources such as glucose from slam, the 5 % (w/v) slam suspension was treated with 1 % (v/v) H_2SO_4. After 3 h of incubation on a rotary shaker, the mixture was autoclaved (15 min, 121 °C). After adjustment of the pH to 4 with NaOH, the mixture was supplemented with 1 mL L^{-1} *gluco*-amylase (Glucoamylase AN, ASA Spezialenzyme GmbH) and incubated at 65 °C on an orbital shaker for 24 h. After centrifugation of the mixture and filtration of the supernatant over glass fibre filters (GF/F, Whatman), the pH of the filtrate was adjusted to 2 with H_2SO_4 to give the final slam-extract for use in *G. sulphuraria* cultivation.

Since wastewater consisted of fish processing residues a proteolytic treatment was carried to reveal if organic nitrogen compounds can be recovered. Before hydrolysis, wastewater was well shaken to get a homogenous suspension of undissolved constituents. Hydrolysis was carried out with protease S2, glucoamylase and cellulase as described above.

2.5. Cultivation of Galdieria sulphuraria

Pellet extract as well as hydrolyzed pellet extract were diluted to concentrations of 20, 40, 60, or 80 % (v/v) using filtrated (0.45 μM syringe filter, Minisart RC) but not hydrolyzed fish processing wastewater. The mixtures were supplemented with 2.5 g L^{-1} glucose and inoculated with 5×10^6 *G. sulphuraria* cells per mL. Cultures were grown in flasks using 50 mL medium on an orbital shaker at 45 °C, 130 rpm, and pH 2.

Glycerol was further tested as a carbon source for *G. sulphuraria*. In these experiments, protease-digested pellet extract was diluted to a concentration of 20 % (v/v) with fish processing wastewater. Glycerol was added at concentrations of 0, 5, 10, 20, and 50 g L^{-1}. Inoculation and growth condition were as described above.

Slam-extract from protease-digestion or acid-enzyme-digestion was diluted with hydrolysed pellet extract in the ratio of 80:20 (v/v). As control, 20 % (v/v) hydrolyzed pellet extract in fish processing wastewater was supplemented with 0.9 g L^{-1} of glucose. Inoculation and growth condition were as described above.

Cultivation of *G. sulphuraria* at larger scale was carried out in an aerated 5 L bioreactor (Sartorius, Germany) under controlled pH and temperature conditions. Cultivation was started with 2.5 L medium consisting of 20 % (v/v) hydrolyzed pellet extract and 20 g L^{-1} glycerol. The medium was inoculated with 5×10^6 *G. sulphuraria* cells per mL. The bioreactor was aerated with air and stirring occurred at 400 rpm. No regulation of pH by the addition of base or acid was needed. In order to overcome inhibitions by too high extract and glycerol concentrations the cultivation was carried out as fed-batch culture and nutrients in the form of new extract or glycerol were added regularly when depleted.

Sampling occurred regularly. After centrifugation of samples for 5 min at 9,800*g*, the supernatant was frozen at −20 °C until analysis and used in experiments. Cultivations have been carried in duplicate and mean values are presented in Results and discussion section.

2.6. Microbial analysis

The investigation of the microbiological status of the produced *G. sulphuraria* biomass focused on the determination of aerobic, mesophilic microbes, yeasts/molds, enterobacteria, enterococci, *Escherichia coli* and *Salmonella* sp. The standardized test methods were carried out as described earlier (Pleissner and Händel, 2023).

2.7. Analytics

Number of cells was counted manually using a Neubauer counting chamber. Furthermore, to follow the increase in biomass, optical density was determined at 750 nm using a Cary 6000i UV–VIS-NIR spectrometer (Agilent).

To determine the dry matter of applied residues as well as produced *G. sulphuraria* biomass a halogen moisture analyzer (HR 83, Mettler Toledo) was used.

Biochemical composition and ash content were quantified in dried pellet and slam biomasses as well as *G. sulphuraria* biomass by near infrared spectroscopy (Unity Scientific GmbH, Germany).

Nitrate was determined using a NO3-1 TC cuvette test set (WTW, Germany). The test was performed according to the manufacturer's instructions using a pHotoFlex STD device (WTW, Germany) for photometric measurements.

Phosphate, free amino nitrogen (FAN), glucose, and glycerol concentrations were determined as described earlier (Pleissner et al., 2021).

3. Results and discussion

3.1. Residues characterization

In this study two solid materials namely pellet and slam as well as the liquid downstream fish processing wastewater were used for the cultivation of the microalga *G. sulphuraria*. In order to be useful as nutrient source for *G. sulphuraria,* streams should be rich in carbohydrates and/or proteins in order to make carbon and/or nitrogen sources available. Pellet material contained more than 65 % (w/w) proteins, around 12 % (w/w) fat but no carbohydrates. A similar composition has also been found in an earlier study (Ghaly et al., 2013). This result was expected, as pellet material consist predominantly of fish residues made up of head, tails, skin, gut, fins, and frames, which are rich in proteins and amino acids (Ghaly et al., 2013). Contrarily, slam contained around 20

% (w/w) carbohydrates, 29 % (w/w) proteins and around 10 % (w/w) fat. Furthermore, the ash content was 10 times higher in slam compared to pellet (Table 1). Slam basically consists of unused feed and commercial feed contains (w/w) 18 to 50 % proteins, 10 to 25 % lipids, 15 to 20 % carbohydrates, and below 8.5 % ash as well as trace amounts of vitamins and minerals (Craig and Helfrich, 2019). Thus, composition of the slam is in accordance with and determined by the composition of feed used.

While the solid materials pellet and slam were rich in organic compounds, the fish processing wastewater was not. No free carbohydrates could be detected, and the FAN concentration was with 0.02 g L^{-1} rather low. Nitrate was found in wastewater in concentrations of 12.5 mg L^{-1}. Phosphate was not determined.

3.2. Pellet, slam, and wastewater hydrolysis

It was concluded from the analysis of composition (Table 1) that the hydrolysis of slam would result in a hydrolysate rich in carbohydrates and FAN, while the hydrolysis of pellet would give a hydrolysate which is rich in FAN. Recovery of nutrients from pellet and slam was initiated by the preparation of extracts. Pellet extract was prepared from a 10 % (w/v) suspension and slam extract from 5 % (w/v) suspension after incubation on an orbital shaker at 120 rpm and 22 °C for 24 h. The extracts were not only rich in organic compounds which can be further hydrolyzed but also contained various ions. Pellet extract contained 35 mg L^{-1} nitrate and 1,250 mg L^{-1} phosphate. Slam extract contained 37 mg L^{-1} nitrate and 475 mg L^{-1} phosphate.

The 5 % (w/v) slam suspension was first investigated as source for carbohydrates (e.g., glucose from starch) but neither an enzymatic nor a subsequently carried out acidic and enzymatic hydrolysis did reveal in considerable amounts of glucose. Concentrations obtained did not exceed glucose concentrations above 1.2 g L^{-1}, indicating that only 10 % (w/w) of the max. glucose amount could be obtained. FAN did only slightly increase from 0.24 g L^{-1} to 0.29 g L^{-1} after 1 day and did not further increase afterwards when slam was treated with a protease. Nevertheless, a FAN concentration of 0.29 g L^{-1} was expected to be decent to carry out a cultivation of *G. sulphuraria*. It remains unknown from the results why only 10 % (w/w) of the carbohydrates could be hydrolyzed. It might be speculated that carbohydrates that passed the digestive tract of fish or underwent a partly degradation by microorganisms in the water are not in accessible form for the applied enzymes. Even though starch is, beside sugars, considered as source for carbon with nutritional value for fish (Kamalam et al., 2017) it might be assumed that the present carbohydrates were not in the form starch However, it should also be admitted once more that even an acidic hydrolysis beforehand did not result in an increase of the concentration of released glucose.

Since pellet did not contain carbohydrates (Table 1) it was not further treated to make them available as caron source. However, pellet suspension was treated with a protease and this treatment resulted in a considerable increase in FAN from 0.36 g L^{-1} at the beginning to 0.80 g L^{-1} after 1 day and 0.90 g L^{-1} after 2 days (Fig. 1). This result was expected as the pellet remained from the hydrolysis of protein-rich material (Ghaly et al., 2013) and various studies revealed the potential of this material as source for organic nitrogen compounds (Benhabiles et al., 2012; Ghaly et al., 2013; Korkmaz and Tokur, 2022; Siddiqui et al., 2023). Even if the pellet material used in the present was already hydrolyzed once, it still contained hydrolysable material which gave a decent high FAN concentration of 0.90 g L^{-1} to carry out the cultivation of *G. sulphuraria* even in a fed-batch culture.

Table 1
Biochemical composition of pellet, obtained after protein hydrolysis, and slam residues (n.d. = not determined) as well as *Galdieria sulphuraria* biomass produced in a fed-batch culture using 20 % (v/v) hydrolyzed pellet extract and glycerol as nutrients.

Component [%, w/w]	Pellet	Slam	*G. sulphuraria* biomass
Carbohydrates	n.d.	19.9	44.2
Proteins	65.6	29.2	18.9
Fat	11.6	9.6	0.9
Ash	2.1	21.8	2.6

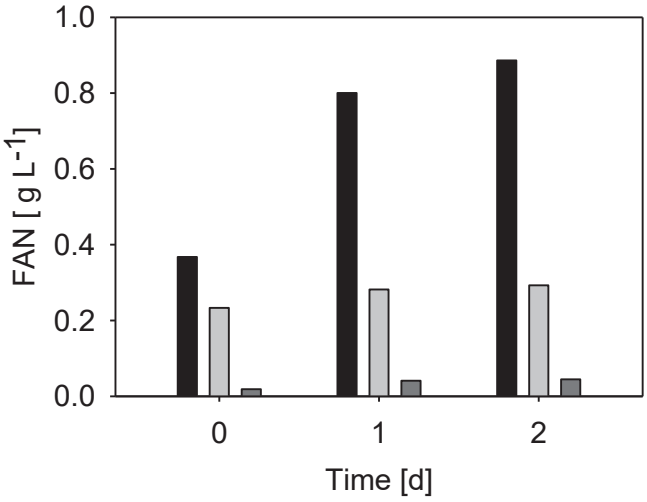

Fig. 1. Release of free amino nitrogen by proteolytic treatment of pellet extract (black bar), slam extract (light grey bar), and wastewater (dark grey bar).

A proteolytic treatment of wastewater only resulted in a slight increase from 0.02 g L^{-1} to 0.04 g L^{-1} FAN (Fig. 1). It should be admitted here that the hydrolysis of pellet and slam was highly comparable between batches diluted with demineralized water and fish processing wastewater. Even though the concentration of nutrients in wastewater was low, it was considered in the present study as source for water to produce extracts and to carry out dilutions.

3.3. Growth performance of Galdieria sulphuraria

3.3.1. Growth in presence of extract

The cultivation of *G. sulphuraria* was tested in untreated and hydrolyzed pellet extract. As shown in Fig. 1 and described above the hydrolysis did result in an increase in FAN and this was expected to contribute to the growth performance of *G. sulphuraria* (Pleissner et al., 2021). Pellet extract as well as hydrolyzed pellet extract were diluted to concentrations of 20, 40, 60, or 80 % (v/v) using filtrated but not hydrolyzed fish processing wastewater, supplemented with 2.5 g L^{-1} glucose, and inoculated with *G. sulphuraria* cells. Since wastewater was used for dilution, it was of interest to reveal whether it causes any negative impact on the growth.

In Fig. 2A–C the cultivation of *G. sulphuraria* in cyanidium medium and cyanidium medium mixed with wastewater in a ratio of 50:50 (v/v) is shown. Growth of *G. sulphuraria* was in both cultivations highly comparable and more than 1.0×10^8 cells were found after 5 days long growth period (Fig. 2A). Glucose was completely consumed (Fig. 2B) and further around 0.2 g L^{-1} FAN was utilized in both cultivations (Fig. 2C). The obtained growth rates were with 0.79 and 0.77 day^{-1} for cyanidium medium (as control medium) and the mixture of cyanidium medium and wastewater, respectively, comparable (Table 2). Even in pure wastewater cells grew and consumed glucose (Fig. 2D–F). However, growth performance was not comparable to the other cultures and rather linear than exponential as cells were limited in FAN already from the beginning. Thus, it can be concluded that wastewater does not impact the growth of *G. sulphuraria* negatively, but FAN and carbon source need to be supplied from another source.

A negative impact on growth of *G. sulphuraria* was found when concentrations of 40 % (v/v) and higher of hydrolyzed or untreated

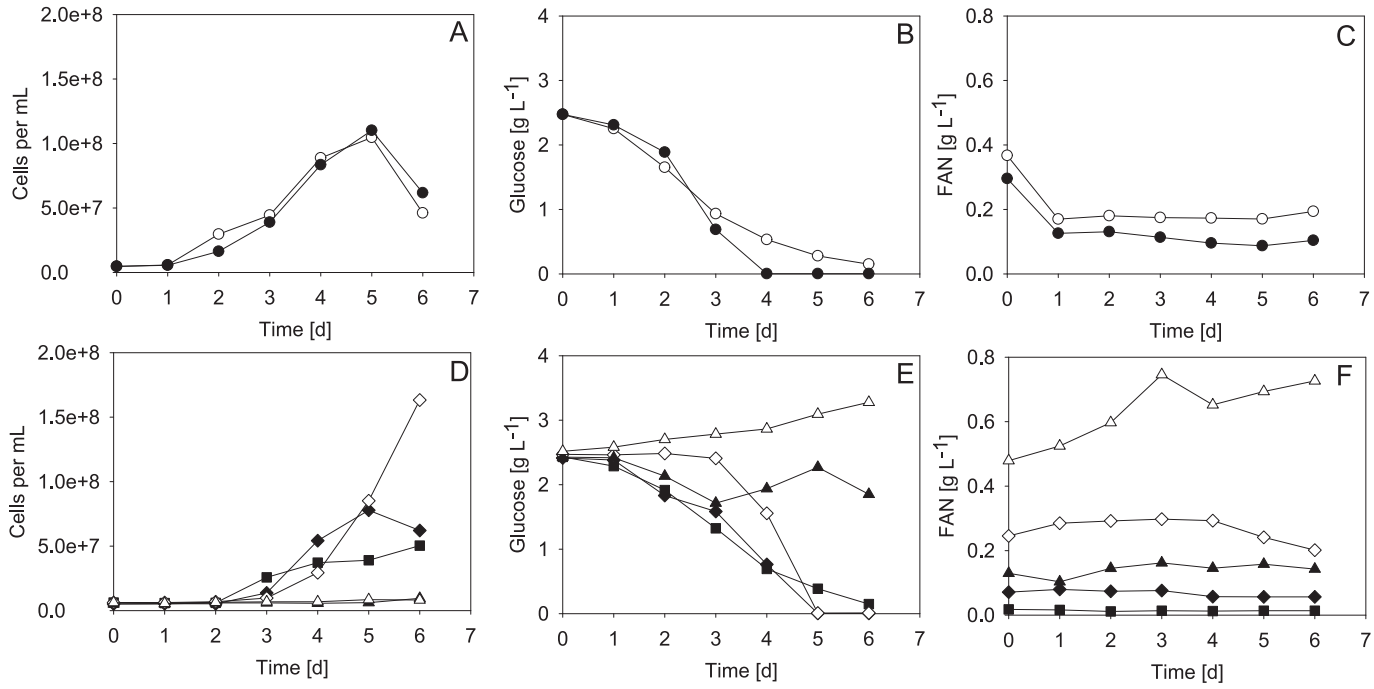

Fig. 2. Growth of *Galdieria sulphuraria* (A) and consumptions of glucose (B) and free amino nitrogen (FAN, C) in cyanidium medium (open circles) as control and a mixture of cyanidium medium and wastewater (50:50, v/v, closed circle). Inserts D, E, and F show the growth of *G. sulphuraria* and consumptions of glucose as well as FAN, respectively, when wastewater (closed square), 20 % (v/v) pellet extract (closed diamond), 40 % (v/v) pellet extract (closed triangle), or 20 % (v/v) hydrolyzed pellet extract (open diamond), or 40 % (v/v) hydrolyzed pellet extract (open triangle) were used.

Table 2
Obtained growth rates from *Galdieria sulphuraria* cultivations carried out in presence of recovered nutrients from fish processing wastewater and waste as well as glucose or glycerol as carbon source.

Medium	Growth rate [day^{-1}]
2.5 g L^{-1} glucose, cyanidium medium	0.79
2.5 g L^{-1} glucose, cyanidium medium and wastewater (50:50, v/v).	0.77
2.5 g L^{-1} glucose, 20 % (v/v) untreated pellet extract	0.68
2.5 g L^{-1} glucose, 20 % (v/v) hydrolyzed pellet extract	0.59
0 g L^{-1} glycerol, 20 % (v/v) hydrolyzed pellet extract	0.42
5 g L^{-1} glycerol, 20 % (v/v) hydrolyzed pellet extract	0.72
10 g L^{-1} glycerol, 20 % (v/v) hydrolyzed pellet extract	0.63
20 g L^{-1} glycerol, 20 % (v/v) hydrolyzed pellet extract	0.47
Larger scale fed-batch cultivation using 20 g L^{-1} glycerol, 20 % (v/v) hydrolyzed pellet extract	0.72

extract was applied. In Fig. 2D–F examples for cultivations with hydrolyzed and untreated 40 % (v/v) pellet extract are shown. No growth occurred during the 6 days cultivation period and neither glucose nor FAN was consumed. Further investigations have shown that a high phosphate concentration up to 4 g L^{-1} does not inhibit growth (results not shown). An effect of glucose can be ruled out, since all cultures contained a comparable concentration. However, an effect neither of FAN nor other hydrolysate constituents such as proteins can be ruled out. It was expected that a hydrolysis and degradation of proteins improves growth performance (Pleissner et al., 2021), however, this was not the case in the present study. Thus, a dilution of the extracts was necessary. On the other hand, the 20 % (v/v) pellet extract did not inhibit, and a good growth performance could be seen (Fig. 2D–F). The growth rates were 0.68 day^{-1} and 0.59 day^{-1} for untreated and hydrolyzed pellet extract. Glucose was completely consumed in both approaches during the 6 days long cultivation period (Fig. 2E). The higher FAN concentration in hydrolyzed compared to untreated extract resulted in a considerably higher cell number. While maximum 7.8 × 10^{7} cells were measured in the cultivation using untreated extract, 1.6 × 10^{8} cells were found using treated extract. However, it should be admitted that FAN was not limited in both cultivations (Fig. 2F).

Since the yield of glucose, that can be recovered from the tested waste and residues streams, was rather low, another carbon source namely glycerol was tested. Glycerol can potentially be obtained after treatment of lipid-rich fish waste (Alfio et al., 2021), and thus represents a valuable carbon source for the purpose described here. Furthermore, it has been shown already that glycerol is a good carbon source for *G. sulphuraria* (Perez Saura et al., 2022). In Fig. 3A–P cultivations of *G. sulphuraria* in presence of hydrolyzed 20 % (v/v) pellet extract supplemented with different glycerol concentrations are shown. Glycerol concentrations from 0 to 50 g L^{-1} have been tested. However, no growth was observed at 50 g L^{-1}. Furthermore, a decrease in growth rate from 0 to 20 g L^{-1} could be seen (Table 2). Growth rate decreased from 0.72 day^{-1} in presence of 5 g L^{-1} glycerol, to 0.63 day^{-1} and 0.47 day^{-1} in presence of 10 and 20 g L^{-1}, respectively. In all cultures glycerol was depleted after at latest 10 days of cultivation. The different glycerol concentrations resulted in different optical densities and the higher the glycerol concentration the higher the optical density measured. An optical density of close to 9 AU was obtained even when no glycerol was supplied and an optical density of 14, 20, and 38 AU when 5, 10, 20 g L^{-1} were used (Fig. 3A, B, E, F, I, J, M, and N). The higher the OD the more FAN was consumed (Fig. 3C, G, K, O). Interestingly, the 0.25 g L^{-1} supplied phosphate were sufficient to reach an optical density of 38 AU. However, this amount was also completely consumed only when an OD of 14 AU was reached (Fig. 3A, D, E, H, I, L, M, and P). This result may indicate a luxury consumption of phosphate by *G. sulphuraria* when supplied in excess. Only in the culture with 0 g L^{-1} glycerol 0.1 g L^{-1} phosphate remained in the medium.

The growth performance in terms of growth rates is comparable to the results published earlier where 0.65, 0.69 and 0.60 day^{-1} were found in defined mediums as well as in restaurant and bakery waste in the presence of glucose (Sloth et al., 2017). However, below the growth rates found in untreated and hydrolyzed digestate (0.9–1.2 day^{-1}), and

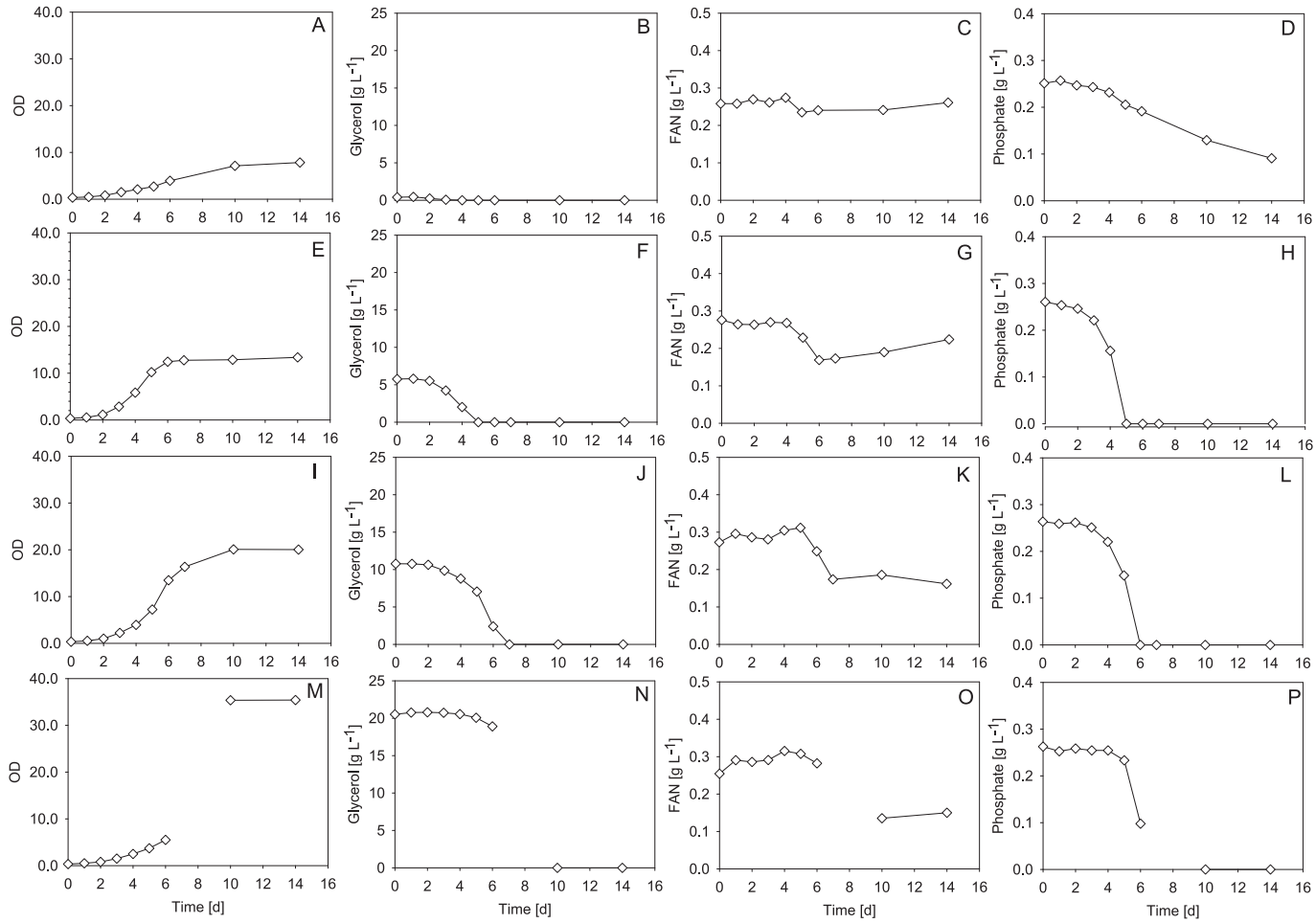

Fig. 3. Growth of *Galdieria sulphuraria* in the presence of 20 % (v/v) hydrolyzed pellet extract supplemented with 0 g L^{-1} (A-D), 5 g L^{-1} (E-H), 10 g L^{-1} (I-L), or 20 g L^{-1} (M–P) glycerol. Development of optical density (OD, A, E, I, M) and consumptions of glycerol (B, F, J, N), free amino nitrogen (FAN, C, G, K, O), and phosphate (D, L, H, P) are shown in inserts.

in presence of glucose as well as in the cyanidium medium as control (Pleissner et al., 2021). Earlier published studies revealed that growth in cultures containing glucose or glycerol at concentrations below 5 g L^{-1} was not impacted. At higher concentrations, however, an inhibition of growth may occur (Perez Saura et al., 2022; Rahman et al., 2020). This was also the main conclusion from the growth rates shown in Table 2.

While pellet extract has been found to support the growth of *G. sulphuraria* when appropriately diluted, slam extract was not. No growth, even down to a dilution of 20 % (v/v), could be seen (results not shown). Thus, for upscaling studies only proteolyzed pellet extract was tested.

3.4. Larger scale fed-batch cultivation

The larger scale cultivation was carried out as fed-batch using hydrolyzed pellet extract (20 %, v/v) and glycerol (Fig. 4A–D), and following the results obtained from experiments shown in Figs. 2 and 3. The culture did show a rather long lag-phase of 7 days (Fig. 4A). Afterwards growth started to proceed exponentially at a rate of 0.72 day^{-1} until Day 8 (Table 2). All nutrients were steadily consumed (Fig. 4B–D). After 8 days of cultivation 0.5 L of new hydrolysate and 20 g L^{-1} glycerol were added and after 12 days 1 L new hydrolysate as well as 20 g L^{-1} glycerol. From Day 13 onwards 20 g L^{-1} glycerol was added daily. The steady supply of new nutrients resulted in a steady increase in optical

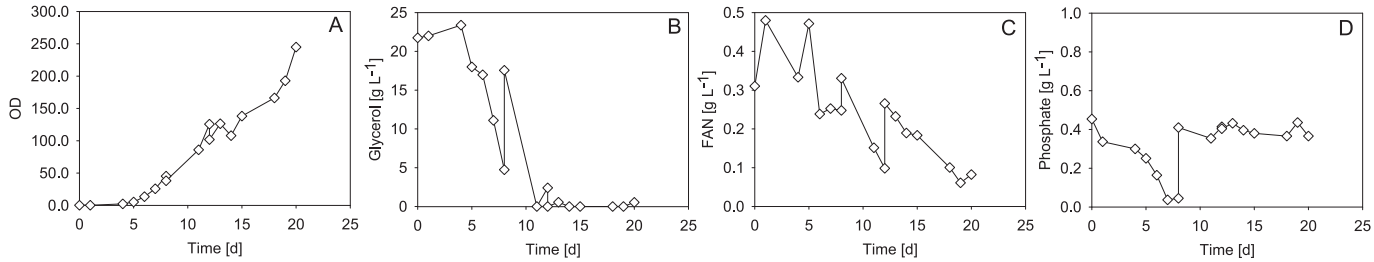

Fig. 4. Fed-batch culture of *Galdieria sulphuraria* in the presence of 20 % (v/v) hydrolyzed pellet extract supplemented with 20 g L^{-1} glycerol. Development of optical density (OD, A) and consumptions of glycerol (B), free amino nitrogen (FAN, C), and phosphate (D) over time are shown. New hydrolysate and 20 g L^{-1} glycerol were added after Days 8 (0.5 L hydrolysate) and 12 (1 L hydrolysate). From Day 13 onwards 20 g L^{-1} glycerol was added daily.

density and around 250 AU was measured after 20 days. This high optical density stands for a biomass concentration of around 80 g L^{-1}. From the results it can be seen that glycerol was clearly limited and completely consumed. FAN and phosphate were still present (Fig. 4B–D). It has already been shown that *G. sulphuraria* can be cultured at high density and in fed-batch cultures using defined mediums and biomass concentrations between 80 and 120 g L were obtained (Graverholt and Eriksen, 2007; Schmidt et al., 2005). A biomass concentration of 80 g L^{-1}, however, has not been achieved in earlier studies using a complex hydrolysate as nitrogen and phosphorous source in a fed-batch culture.

The obtained *G. sulphuraria* biomass was rich in carbohydrates 44.2 % (w/w) and proteins 18.9 % (w/w). However, the fat content (0.9 % (w/w)) was rather low (Table 1). This composition is different to what has been found earlier where the carbohydrate content was half and the protein content twice as high (Pleissner et al., 2021). In another study an even higher protein content of more than 50 % (w/w) was obtained (Dandamudi et al., 2021). Graziani et al. found a protein content of 26.5 % (w/w), a lipid content of 11.4 % (w/w) and a carbohydrate content of 69.1 % (w/w) when *G. sulphuraria* cells were heterotrophically grown, but a protein content of 32.5 % (w/w) when autotrophically grown (Graziani et al., 2013). Massa et al. found a protein of around 20 % (w/w) in nitrogen limited *G. sulphuraria* cells (Massa et al., 2019), and thus one might conclude that *G. sulphuraria* cells cannot utilize FAN once it drops below a certain concentration. This might be a concentration below 0.1 g L^{-1} as this has been observed in all cultivation (Figs. 2-4). Consequently, cells were limited in FAN and utilized intracellular proteins.

Neither *Salmonella* sp. nor *Escherichia coli* could be found in the produced biomass. Furthermore, no species belonging to Enterococcus and only 9 counts were found for species belonging to Enterobacteriacae. Furthermore, only 10 counts belonging to moulds were found. Aerobic spore formers were counted with 100 counts per g biomass, and thus rather little. Most of the aerobic mesophilic counts were formed by yeasts (26,000 to 9,500 counts per g biomass), which are known to tolerate acidic pH-values. Thus, applying *G. sulphuraria* for residues and waste utilization does not only result in valuable biomass but also contributes to a hygenization of substrates with a high microbial load.

The hygenization of fish waste in combination with its utilization is considered here as the major perspective. However, the utilization of *G. sulphuraria* biomass itself still needs further development. In particular the carbohydrate fraction in *G. sulphuraria* in form of glycogen is currently underutilized but has intersting propertise which make it a slowly digestible and resistent polymer (Martinez-Garcia et al., 2017). Studies have dealt with the hydrothermal liquefaction of *G. sulphuraria* biomass for biofuels generation (Cheng et al., 2019; Cui et al., 2020) and even with its use as gold and palladium absorber (Ju et al., 2016). However, a realistically high value product which can be extracted from *G. sulphuraria* is the pigment phycocyanin (Ju et al., 2016). Even though the strain used in this study did not accumulate phycocyanin, the results can be transfered to another strain, which is known to accumulate phycocyanin even under heterotrophic conditions (Sloth et al., 2017). Furthermore, a potential use of *G. sulphuraria* biomass might be as source for stable and high-quality proteins for future food applications (Canelli et al., 2023).

Even though *G. sulphuraria* biomass potentially offers a couple of valuable products, it can be economically challenging to produce it from fish waste streams. Such a utilization appraoch needs to be directly linked to the current use of fish waste in order to limit process steps to a minimum. For instance, hydrolysate from fish residues is a valuable product. When the remaining fraction is immediatelly used to further extract nutrients for *G. sulphuraria* then drying and transportation can be ommited. A definite advantage of using *G. sulphuraria* and its acidic growth conditions is that sterilization can be skipped. It should further be noted that fish wastes are no proper source for carbon compounds. Thus, an extra carbon source such as glycerol was tested which appears as side stream from biodiesel production. Adding glycerol as carbon source does not only contribute to the growth of *G. sulphuraria* but also to a higher phycocyanin content compared to glucose as carbon source (Perez Saura et al., 2022).

4. Conclusions

This study revealed the potential of *G. sulphuraria* to utilize fish processing streams and to form a valuable biomass. Considering the potential of fish processing streams in terms of nutrient sources, *G. sulphuraria* can contribute to add value wherever these streams appear. Furthermore, in a fed-batch culture high biomass concentrations free of pathogens can be reached even under non-sterile conditions, making this approach more economically attractive as energy intensive sterilization can be skipped.

CRediT authorship contribution statement

Daniel Pleissner: Conceptualization, Methodology, Resources, Visualization, Supervision, Writing – original draft. **Stephanie Schönfelder:** Methodology, Writing – review & editing. **Nicole Händel:** Methodology, Writing – review & editing. **Julia Dalichow:** Methodology, Writing – review & editing. **Judith Ettinger:** Methodology, Writing – review & editing. **Kristine Kvangarsnes:** Methodology, Writing – review & editing. **Egidijus Dauksas:** Methodology, Writing – review & editing. **Turid Rustad:** Methodology, Writing – review & editing. **Janna Cropotova:** Conceptualization, Methodology, Resources, Visualization, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Janna Cropotova reports article publishing charges was provided by Norwegian University of Science and Technology.

Data availability

Data will be made available on request.

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