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Published in:
Bioresource Technology

DOI:
10.1016/j.biortech.2021.125477

Publication date:
2021

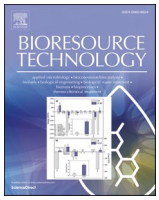
Document Version
Publisher's PDF, also known as Version of record

Bioresource Technology 337 (2021) 125477

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech



Heterotrophic cultivation of *Galdieria sulphuraria* under non-sterile conditions in digestate and hydrolyzed straw

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HIGHLIGHTS

- *G. sulphuraria* cultivation occurred on medium made from agricultural residues.
- Digestate was revealed as superior nitrogen source for *G. sulphuraria*.
- Proteolytic treatment of digestate led to increased yield of biomass and growth rate.
- Produced biomass was with around 40% (w/w) rich in protein.

ARTICLE INFO

Keywords:
Straw
Digestate
Anaerobic digestion
Hydrolysis
Agricultural residues

ABSTRACT

Non-sterile heterotrophic cultivation of *Galdieria sulphuraria* in presence of digestate as well as straw after hydrolysis was investigated. *G. sulphuraria* can be grown in pure digestate at rates of 0.9 day^{-1} with glucose. However, a proteolytic treatment of digestate resulted in increased growth rates (1.2 day^{-1}) and doubled cell concentrations. Furthermore, *G. sulphuraria* can utilize glucose obtained after straw hydrolysis. Biomass yields in glucose limited cultures were around 0.9 g per g glucose, while only 0.2 g biomass was formed per g glucose in glucose sufficient cultures. Biomass composition (w/w) of *G. sulphuraria* grown in digestate supplemented with straw hydrolysate consisted of 20% carbohydrates, 37% proteins and 3% lipids. This study revealed the potential to utilize agricultural waste streams to form algal biomass rich in proteins and may pave the way to novel utilization strategies to be implemented in rural areas.

1. Introduction

Waste streams from food and agriculture processing appear in considerable amounts globally. To the 1.3 billion tons of food (Gustavsson et al., 2013, 2011) wasted annually come in addition billion tons of agricultural residues (Bentsen et al., 2014) of non-edible lignocellulosic biomass (e.g., straw) as well as liquid waste streams (e.g., digestate and wastewater) with a high nutrient load. Digestate, for instance, contains phosphorous and nitrogen and is needed as fertilizer in agricultural biomass production (Praveen et al., 2018; Sayedin et al., 2020). A direct application as fertilizer, however, is challenged by environmental regulations. In Germany, the use of digestate as fertilizer and its spraying onto agricultural areas requires an intensive monitoring of the released quantities of nitrogen and phosphorous compounds to prevent a eutrophication of surface and ground waters, and thus is no feasible option for many farmers anymore. Collected food waste from food processing industries, restaurants and grocery stores is often energetically used and sent to anaerobic digestion for biogas production (Negri et al., 2020; Rusín et al., 2021). The potential as nitrogen source, however, remains unused and proteins accumulate in the remaining digestate. On the other hand, lignocellulosic biomass such as straw is rich in carbon, and sugars obtainable by its hydrolysis are useable in biotechnological processes (Okonkwo et al., 2021; Ouyang et al., 2020). However, due to missing alternatives or infrastructure, lignocellulosic biomass is often incinerated in rural areas even though incineration contributes not only to a loss of resources but also to environmental issues caused by emissions (Pongpiachan et al., 2017). Because of the efforts needed to make efficiently use of waste streams in an

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https://doi.org/10.1016/j.biortech.2021.125477
Received 26 May 2021; Received in revised form 25 June 2021; Accepted 26 June 2021
Available online 1 July 2021

environmentally benign way, processes which not only allow a recovery, but also a recycling of nutrients (Sayedin et al., 2020) are urgently needed.

The cultivation of microalgae on digestate (Henkanatte-Gedera et al., 2017; Tchinda et al., 2019) and/or hydrolyzed organic waste (Haske-Cornelius et al., 2020; Lau et al., 2014; Pleissner et al., 2013, 2017, 2015; Stiles et al., 2018) has been shown a strategy to utilize waste streams and to produce algal biomass rich in proteins, lipids and unsaturated fatty acids as well as carbohydrates. Particularly farmers who frequently produce solid and liquid waste streams rich in carbon, nitrogen and phosphorous compounds are increasingly interested in algal-based utilization strategies and utilizing the value of algal biomass as source of pigments, antioxidants, proteins, and lipids etc. The heterogeneity of waste streams and possible appearance of microbial contamination during algae cultivation, however, may hinder the implementation of such processes. The problems associated with microbial contaminations can be overcome when algal strains are applied which grow under extremophilic conditions. For instance, the microalga *Galdieria sulphuraria* is one of the species growing under strong acidic conditions and temperatures of 40–50 °C, conditions which were found to significantly reduce the cell number of *Escherichia coli* (Munasinghe-Arachchige et al., 2019). *G. sulphuraria* is further of interest as its biomass contains proteins and the high value pigment phycocyanin, and due to its ability to grow in presence of complex substrates (Cheng et al., 2019; Henkanatte-Gedera et al., 2017; Sloth et al., 2017). Sloth et al. cultured *G. sulphuraria* under mixo- and heterotrophic conditions with the aim to improve the accumulation of phycocyanin (Sloth et al., 2006). In carbon-limited continuous flow cultures using glucose or glycerol as carbon source and an illumination of 65 μmol photons m^{-2} s^{-1} contents between 15 and 28 mg g^{-1} were obtained. *G. sulphuraria* is relevant for industrial processes as it grows not only on standardized culture medium, but also on agricultural residues. Massa et al. have shown the feasibility of spent cherry brine, a stream rich in carbon and nitrogen, as nutrient source. Growing *G. sulphuraria* in batch cultures resulted in a yield of 0.22 g biomass per g reducing sugar in spent cherry brine (Massa et al., 2019). The biomass harvested after 12 days contained (w/w) 58.4% carbohydrates, 22.0% proteins and 4.6% lipids. Nevertheless, further research is needed to extent the substrate portfolio of *G. sulphuraria* and to convert residues of low value to high value biomass. Due to the ability of *G. sulphuraria* to grow under extremophilic conditions and to utilize various complex residual biomass it is hypothesized here that this might be a promising candidate to establish simple and robust decentralized process in rural areas. This approach would allow for the transformation of organic wastes into useful higher value products (Tsui & Wong, 2019). Furthermore, the conversion of complex and not defined organic streams into defined algal biomass is an option to upcycle agricultural residues with low nutritional value to protein-rich biomass with an application in feed production. This approach would outcompete the conventional processes anaerobic digestion, composting and incineration, which revealed their potential as decentralized processes but are associated with a loss in functionalized organic molecules (Pleissner, 2018).

The aim of this study was an investigation of the cultivation of *G. sulphuraria* in presence of nutrients recovered from digestate obtained after anaerobic digestion of cattle manure as well as straw after hydrolysis. Firstly, digestate and straw have been characterized and based on their composition, secondly, enzymatically digested using proteases and/or cellulases. Thirdly, the growth of *G. sulphuraria* in mixtures of hydrolyzed digestate (as nitrogen source) and straw hydrolysate (as carbon source) has been determined and bioreactors studies under controlled environmental conditions were carried out. Fourthly, *G. sulphuraria* biomass was characterized regarding protein, lipid and starch contents. Particular attention has been paid on the non-sterile cultivation of *G. sulphuraria* to provide an approach, which not only allows an efficient use of waste streams but is also simple enough to be implemented decentralized in rural areas.

2. Material and methods

2.1. Digestate and straw

In August 2020, digestate was obtained from an anaerobic digestion plant using cattle manure as substrate and stored at 4 °C until used in experiments. Dry wheat straw was obtained in September 2020 from a farmer and kept under dry conditions and 20 °C until used in experiments.

2.2. 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 4 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 4, 45 °C and shaken at 130 rpm in an orbital shaker. Subcultivation occurred once per week by adding 50 μL of algae suspension to 20 mL fresh cyanidium medium.

Inocula for experiments were grown in digestate supplemented with 4 g L^{-1} glucose at pH 2, 45 °C and shaken at 130 rpm in an orbital shaker.

2.3. Hydrolysis of digestate and straw

To make nutrients available from digestate and straw an enzymatic treatment has been carried out. All hydrolytic treatments were carried in an EloFerm bioreactor (Biotronix GmbH, Germany) equipped with a 1 L cylindrical glass container under controlled temperature conditions. Samples were taken before and after the hydrolytic treatment, centrifuged at 10,000 × g for 5 min and the supernatant kept frozen at −20 °C until analysis. Hydrolysates were stored at 4 °C until used in experiments.

To 1 L of digestate, 1 mL of CellicCtec2 (Novozymes, Denmark) was added and the hydrolysis carried out at pH 5 and 50 °C for 5 h followed by the addition of 1 mL Protease S-02 (ASA-Enzyme, Germany) and repeated incubation at pH 5 and 50 °C for 5 h.

To hydrolyze straw, 23.5 g was soaked in 1 L demineralized water. Afterwards the suspension was homogenized at 24,000 rpm for 5 min using an Ultra-Turrax T25 Homogenizer (IKA-Labortechnik, Germany) at 20 °C. To the homogenized straw, 1 mL of CellicCtec2 (Novozymes, Denmark) was added and the hydrolysis carried out at pH 5 and 50 °C for 24 h.

Hydrolysates obtained from hydrolytic treatments were stored at 4 °C until used in cultivation experiments.

2.4. Cultivation of Galdieria sulphuraria in flasks

Because of the complex composition of digestate its effect on the growth of *G. sulphuraria* was first assessed in 250 mL flasks containing 50 mL cyanidium medium (see section 2.2) or digestate of different concentrations (25%, 50%, and 100%, v/v), obtained by diluting with demineralized water, at pH 2, 45 °C and shaken at 130 rpm in an orbital shaker.

Secondly, to make use of most of the nutrients, to 50 mL of digestate of different concentrations (25%, 50% and 100%, v/v) 0.06 mL, 0.12 mL, or 0.18 mL of Protease S-02 (ASA-Enzyme, Germany) was added, inoculated with *G. sulphuraria* and incubated at pH 2, 45 °C and shaken at 130 rpm in an orbital shaker.

All cultivations were carried out in triplicate using 1.0×10^7 cells mL^{-1} inoculum and glucose (4–6 g L^{-1}) as carbon source. Samples were taken regularly, and an aliquot was immediately used for the determination of cell numbers. The remainder was centrifuged at 10,000 × g for 5 min and the supernatant kept frozen at −20 °C until analysis.

Statistically significant difference between cell concentrations obtained in untreated and treated digestate was investigated with student *t*-test using the software SigmaPlot Version 11.

2.5. Cultivation of Galdieria sulphuraria in bioreactor

Bioreactor cultivations were conducted in an EloFerm bioreactor (Biotronix GmbH, Germany) equipped with a 1 L cylindrical glass container. With automatic temperature and pH control, a temperature of 45 °C and pH 2 were maintained. The pH was regulated using 2 M NaOH or 2 M H_2SO_4. Stirring occurred by a magnetic bar at 100 rpm and aeration was provided by air at a flow rate of 0.1 mL minute^{-1}. Cultivations were carried out non-sterile in 400 mL medium and started with 4.6 × 10^6 cells mL^{-1}. In a first approach, *G. sulphuraria* was grown in hydrolyzed digestate (100%, v/v) and straw hydrolysate mixed in a ratio of 1:4 (v/v). Due to the expected carbon limitation when straw hydrolysate was used as carbon source, *G. sulphuraria* was grown in a second approach in hydrolyzed digestate supplemented with 50 g L^{-1} glucose to reveal the potential of digestate as nitrogen and phosphorous source. All cultivations were carried out in duplicate. Samples were taken regularly, and an aliquot was immediately used for the determination of cell numbers. The remainder was centrifuged at 10,000 × g for 5 min and the supernatant kept frozen at −20 °C until analysis.

G. sulphuraria biomass was harvested after cultivation by centrifugation at 10,000 × g for 5 min. The obtained biomass was resuspended with demineralized water to remove remaining culture broth, centrifuged again at 10,000 × g for 5 min and the pellet kept frozen at −20 °C until analysis.

2.6. Analytics

Number of cells was counted manually using a Thoma counter chamber.

To determine the dry matter of applied residues an aliquot was weighed and dried at 105 °C until constant weight.

Organic matter was quantified by heating 1 g of dry residues for 4 h at 575 °C in a muffle furnace and weighing the remainder.

Protein, cellulose, lignin, hemicellulose and starch contents were quantified in dried residues and *G. sulphuraria* biomass by near infrared spectroscopy (Unity Scientific GmbH, Germany).

Ammonium quantification was based on the phenol hypochlorite assay (Berthelot reaction) described in (Vega-Mas et al., 2015).

Free amino nitrogen (FAN) was determined following the modified EBC-ninhydrin method. First, two reagents were prepared. For reagent A, 1 g Na_2HPO_4*$12H_2O$, 0.6 g KH_2PO_4, 0.05 g ninhydrin, and 0.03 g fructose were dissolved in 10 mL demineralized water. Reagent B contained 0.2 g KIO_3, 60 mL demineralized water, and 40 mL absolute ethanol. For analysis, 20 µL sample, 50 µL A, and 30 µL demineralized water were combined and heated at 90 °C for 5 min. Then 900 µL of B were added and absorption at 570 nm was measured. A calibration curve with glycine as standard was used as reference.

Glucose, glycerol, and phosphate concentrations were determined using HPLC (Shimadzu: LC-10AD pump, SIL-10AD auto-sampler, CTO-10AD oven, refractive index detector RID-20A, CBM-20A communication module): 10 µL of sample was injected on an Aminex HPX-87H column (300 mm × 7.8 mm) and eluted isocratically with 1.0 mL minute^{-1} 5 mM H_2SO_4 at 27 °C. A calibration curve was generated with pure solutions of known concentration.

3. Results and discussion

3.1. Characterization of substrates before and after hydrolytic treatment

The characterization of substrates was aimed on illuminating the potential of straw and digestate as sources of nutrients for *G. sulphuraria*. Contrarily to straw with a solid content of 90.6% (w/w), digestate was rather liquid and contained 6.1% (w/w) solids. Both substrates were rich in cellulose and hemicellulose with contents between 20 and 36% (w/w) as well as 10 to 15% (w/w) lignin (Table 1). Digestate revealed its potential as nitrogen source by a protein content of almost 30% (w/w), while in straw 5% (w/w) protein was determined. Fat, starch and free sugars were not detected in both substrates. The liquid phase of digestate before hydrolysis contained predominantly ammonium and FAN at concentrations of 55.5 mg L^{-1} and 87.1 mg L^{-1}, respectively. Resuspending 23.5 g straw in 1 L water resulted in a washing out of phosphate and 45.9 mg L^{-1} was detected. Digestate contained 7.1 mg L^{-1} phosphate.

Table 1
Characterization of straw and digestate before (B) and after (A) hydrolytic treatment (n. d. = not detected).

Parameter	Straw		Digestate	
	B	A	B	A
	Solid fraction			
Ash [%, w/w]	6.8	3.7	17.8	11.9
Lignin [%, w/w]	10.7	11.7	14.3	n. d.
Hemicellulose [%, w/w]	19.2	36.4	28.0	6.6
Protein [%, w/w]	5.0	4.9	29.5	47.7
Cellulose [%, w/w]	30.2	26.5	24.7	26.7
Fat [%, w/w]	n. d.	n. d.	n. d.	n. d.
Starch [%, w/w]	n. d.	n. d.	n. d.	n. d.
Free sugars [%, w/w]	n. d.	1.2	n. d.	n. d.
	Liquid fraction			
Solid-to-liquid ratio [%, w/w]	2.4	2.2	6.1	0.8
Glucose [g L^{-1}]	n. d.	4.5	n. d.	0.5
Ammonium [mg L^{-1}]	n. d.	1.9	55.5	23.8
Free amino nitrogen [mg L^{-1}]	n. d.	19.7	87.1	290.7
Phosphate [mg L^{-1}]	45.9	46.0	7.1	7.1

To make nutrients for *G. sulphuraria* cultivation available from straw and digestate an enzymatic treatment with cellulase or cellulase and protease, respectively, has been carried out. The hydrolysis of straw was started with a solid-to-liquid ratio of 2.4% (w/w). After hydrolysis, 2.2% (w/w) remained (Table 1). The hydrolysis resulted in a release of 4.5 g glucose, 1.9 mg ammonium and 19.7 mg FAN per liter. After hydrolysis, the cellulose and ash contents were slightly reduced, while the hemicellulose content increased from 19 to 36% (w/w). This increase might be associated to homogenization of the straw prior to hydrolysis and an increased exposure of hemicellulose to NIR radiation.

When digestate was treated first with CellicCtec2 and second with Protease-02 the hemicellulose content decreased from 28% (w/w) to 6.6% (w/w), while the protein content increased from 29.5% (w/w) to 47.7% (w/w). This increase might be a result of the reduced hemicellulose and cellulose contents, which also caused a decrease in the solid-to-liquid ratio from 6.1% (w/w) to 0.8% (w/w), and the increased exposure of proteins to NIR radiation. The ammonium, FAN, phosphate and glucose concentrations in the hydrolysate were 23.8 mg L^{-1}, 290.7 mg L^{-1}, 7.1 mg L^{-1}, 0.5 g L^{-1}, respectively.

From the results obtained it seems not necessary to carry out a hydrolysis of digestate prior to cultivation to supply the needed nutrients. However, the increased FAN concentration in hydrolysates and the reduced protein content, and thus complexity, may benefit to the performance of *G. sulphuraria* cultivation. Contrarily, straw needs to be liquefied and carbon compounds released. The yield of glucose obtained per g straw in this study was ca. 20% and a tougher pretreatment than homogenization (e.g., acid pretreatment) (Pleissner & Venus, 2014) is recommended to enable an increased hydrolytic performance.

3.2. Growth of Galdieria sulphuraria in presence of agricultural residues

It was assumed that the complex composition of digestate may inhibit the growth of *G. sulphuraria*. Thus, to investigate the effect of digestate cultivations have been carried out in cyanidium medium as control and in hydrolyzed or untreated digestate of different concentrations (Fig. 1). The respective growth rates are shown in Fig. 2. In all cultivations growth was exponential from day 0 to day 4. In the control cultivation the provided 6.5 g L^{-1} glucose and almost 80 mg L^{-1} phosphate were fully consumed after 4 days and a cell concentration of 5 ×

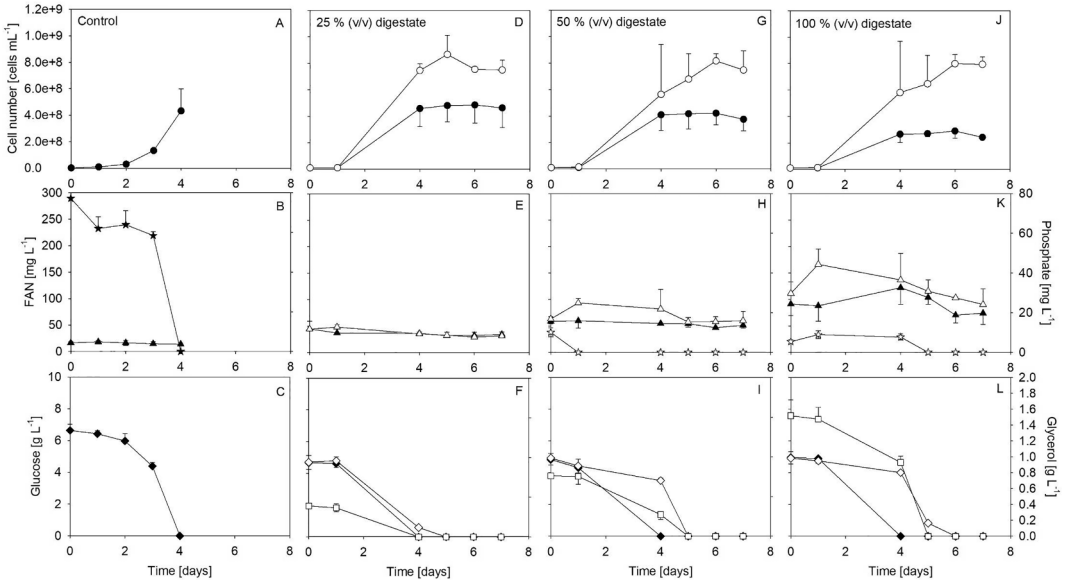

Fig. 1. Time profiles of cell number (circle), free amino nitrogen (triangle, FAN), phosphate (star), glucose (diamond) and glycerol (square) concentrations in cultures of *G. sulphuraria* grown in triplicate in cyanidium medium as control (A-C), and digestate of different concentrations (25%, D-F, 50%, G-I, and 100%, J-L, v/v). The digestate solutions were either used after treatment with Protease S-02 (open symbol) or untreated (closed symbol).

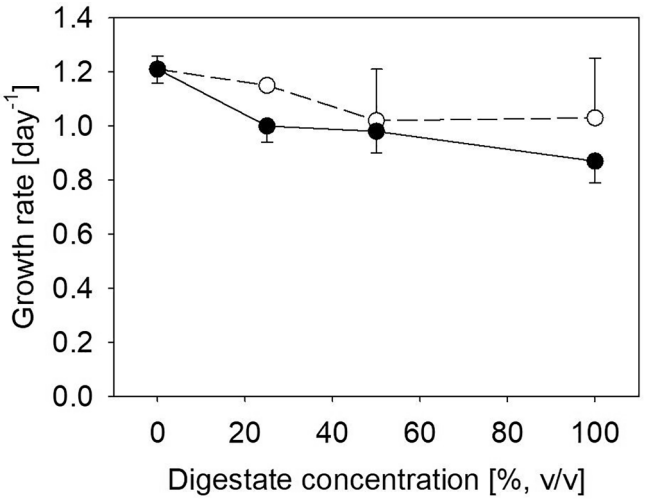

Fig. 2. Exponential growth rate of *G. sulphuraria* grown in cyanidium medium as control (0%, v/v) and digestate of different concentrations (25%, 50% and 100%, v/v) in presence of 4–6 g L^{-1} glucose (the digestate solutions were either used after treatment with Protease S-02, open circles, or untreated, closed circle).

10^8 cells mL^{-1} was reached (Fig. 1A-C). After day 4 the cultivations carried out in presence of untreated digestate reached a stationary phase due to glucose and phosphate limitation (Fig. 1 D-L). As visible in Fig. 1 the maximum cell number reached at day 4 decreased from 5×10^8 to 3×10^8 cells mL^{-1} with increasing digestate concentration. The highest cell number reached at day 4 in cyanidium medium was comparable with the cell number reached in 25% (v/v) digestate. The highest growth rate of 1.2 day^{-1} was found in cyanidium medium, followed by 1.0 day^{-1} in 25 and 50% (v/v) digestate. The growth rate in 100% (v/v) digestate was 0.9 day^{-1} (Fig. 2).

Growth rates found are higher than the one found for *G. sulphuraria* strain 074G in defined medium, food and bakery wastes (Sloth et al., 2017). The authors found growth rates of 0.65, 0.69 and 0.60 day^{-1} in defined medium, restaurant waste and bakery waste, respectively, supplemented with 5 g L^{-1} glucose and 0.5 g L^{-1} ammonium. Rahman et al. found for the same strain growth rates of 0.3–0.4 day^{-1} in presence of corn and potato starch, respectively (Rahman et al., 2020). In comparison, the doubling time calculated from the growth rates 1.2, 1.0 and 0.9 day^{-1} obtained in this study were 18.5 h, 16.6 h and 13.9 h in cyanidium medium, 25 and 50% (v/v) as well as 100% (v/v) digestate, respectively, which lies in the range of doubling times reported earlier (Graziani et al., 2013). Scherhag and Ackermann measured growth rates as a function of substrate concentration and temperature (Scherhag & Ackermann, 2021). As substrate a mixture of sucrose, glucose, and fructose in the range from 6 to 60 g L^{-1} was tested. At 30 °C and 36 g L^{-1} substrate the maximum growth rate was 1.1 day^{-1}, at 42 °C and 36 g L^{-1} substrate the maximum growth rate was 1.5 day^{-1} and at 50 °C and 36 g L^{-1} substrate the growth rate was 0.7 day^{-1}. The authors further used wastewater from fruit processing containing 15 g L^{-1} sugars and obtained at 42 °C a growth rate of 1.2 day^{-1}. However, other than in the present study Scherhag and Ackermann supplemented the wastewater with micronutrients and ammonium (Scherhag & Ackermann, 2021). It should generally be admitted that different *G. sulphuraria* strains do grow differently under heterotrophic conditions and doubling times from 16 to 95 h have been shown (Graziani et al., 2013). Nevertheless, the ability of the used *G. sulphuraria* strain to grow in digestate was clearly revealed.

Regarding the nitrogen sources, the digestate contained ammonium and FAN, while the cyanidium medium did contain only traces of FAN and 0.13 g L^{-1} ammonium. In 25%, 50% and 100% (v/v) of untreated digestate, the FAN concentrations were between 50 and 100 mg L^{-1} (Fig. 1B, E, H and K). Ammonium was also present at concentrations between 50 and 100 mg L^{-1} (not shown). By comparing the concentrations of FAN and ammonium at the beginning and the end of cultivations only a small fraction of both was used by *G. sulphuraria* when cyanidium or untreated digestate was used. As the digestate is rich in proteins (Table 1) and a simple proteolytic treatment has been carried out simultaneously to the cultivation, amino acids were released as nitrogen source. Interestingly, applying a proteolytic treatment did extend the growth phase to 5 to 6 days which resulted in twice as many cells per mL and in increased growth rates compared to the untreated digestate (Fig. 1 D, G, J and Fig. 2). Generally, cell concentrations in untreated and treated digestate were statistically different (P less than 0.05). Due to the proteolytic treatment, the FAN concentration was twice as high compared to untreated digestate and decreased over time for 50% (v/v)

and 100% (v/v) digestates (Fig. 1 H and K) and 0.15 g L^{-1} and 0.3 g L^{-1} were metabolized, respectively. In 25% (v/v) digestate, the FAN concentration did increase only slightly after proteolytic treatment and only traces of FAN were consumed (Fig. 1 E). However, even in 25% (v/v) digestate, the cell concentration doubled in treated digestate (Fig. 1 D). It should be noted that in the present study a simultaneous proteolysis and cultivation has been carried out. Thus, the released FAN during the first 24 h was most likely higher than shown in Fig. 1 as some of released FAN was already consumed by *G. sulphuraria*. The applied FAN-assay includes ninhydrin, which is known for its reaction with amino acids, peptides, proteins, and even ammonium (Friedman, 2004). Thus, it can be ruled out that considerable amounts of nitrogen compounds have been released which do not react with ninhydrin but serve as protein source. Furthermore, no change in ammonium was noted (not shown).

The effect of decreased growth rates with increasing digestate concentration has been found for a mixed microalgal culture under phototrophic conditions and related to increasing ammonium concentrations (Uggetti et al., 2014) and an associated uncoupling effect on photosynthetic processes (Crofts, 1966). In this study, at pH 2, ammonia has been converted completely to ammonium (Salbitani & Carfagna, 2021) and *G. sulphuraria* has been shown to tolerate ammonium concentrations of more than 7.5 g L^{-1} (Schmidt et al., 2005). It can therefore be concluded that the high concentration of ammonium in digestate does not negatively affect the heterotrophic cultivation of *G. sulphuraria*, which is contrarily to many microalgae (Praveen et al., 2018).

The ability of *G. sulphuraria* to remove nutrients has been shown in wastewater (Selvaratnam et al., 2014), primary effluent (Tchinda et al., 2019), landfill leachate (Pan et al., 2021) and produced water (Rahman et al., 2021). In the four studies ammonium and phosphate were efficiently removed. A quick removal of almost 80 mg L^{-1} phosphate was also found in this study (Fig. 1 B, E, H and K), however, ammonium concentration did not significantly change over time. It might be concluded from the results obtained that the presence of amino acids inhibits the consumption of ammonium or that *G. sulphuraria* prefers amino acids over ammonium. However, this conclusion is contradictory to Sloth et al., who concluded that ammonium must be preferred over organic nitrogen source by *G. sulphuraria* 074G (Sloth et al., 2017).

Cultivation carried out with hydrolyzed digestate were limited in glucose after 4 to 5 days. However, it should be admitted that glycerol, which was added with the protease, was present as additional carbon source. The enzyme formulation Protease S-02 (ASA-Enzyme, Germany) contained 360 g L^{-1} glycerol which resulted in a glycerol concentration of 0.4 g L^{-1}, 0.8 g L^{-1} and 1.6 g L^{-1} in 25% (v/v), 50% (v/v) and 100% (v/v) hydrolyzed digestate (Fig. 1F, I and J), respectively. Interestingly,

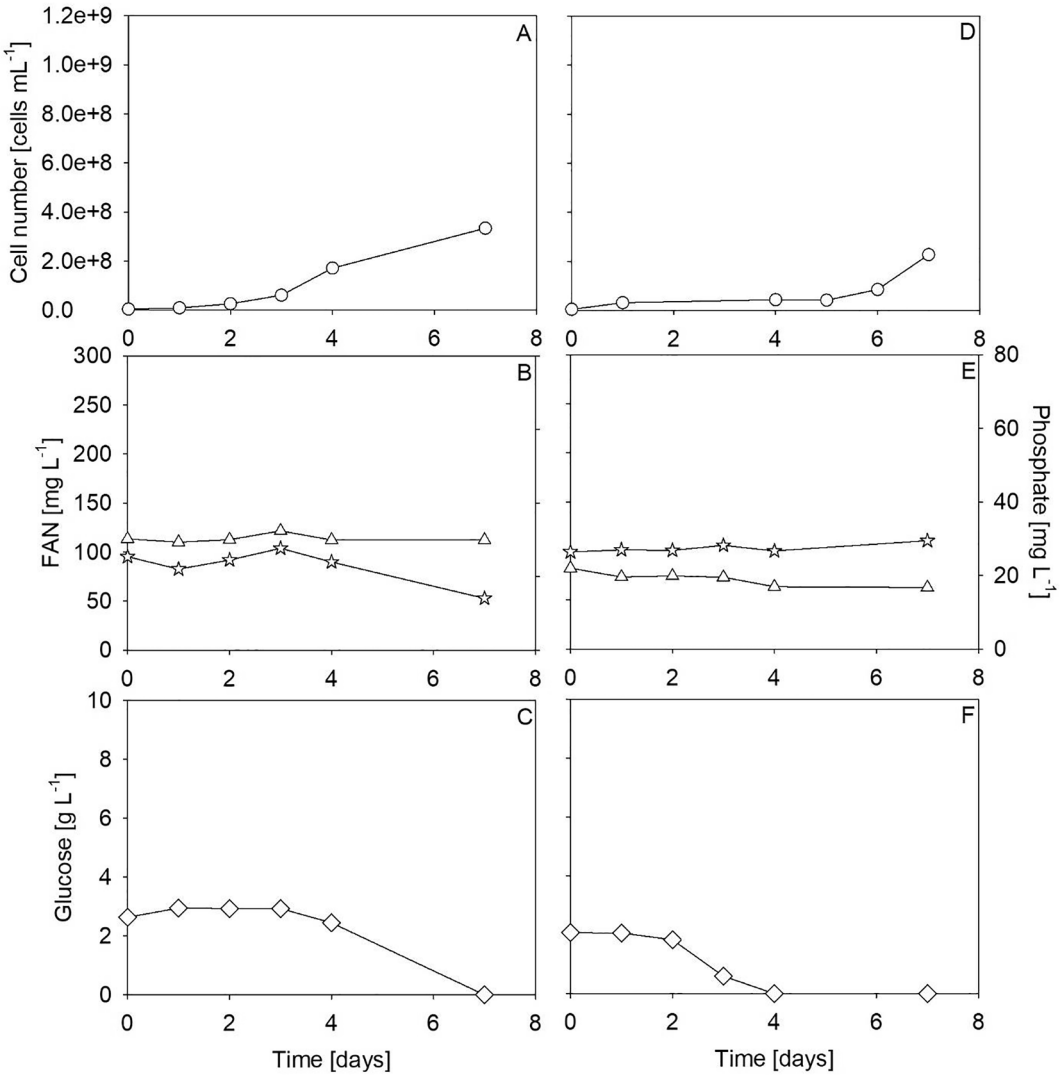

Fig. 3. Time profiles of cell number (A and D), free amino nitrogen (FAN) and phosphate (B and E) as well as glucose (C and F) concentrations in cultures of *G. sulphuraria* grown in duplicate in a medium consisting of 100% (v/v) hydrolyzed digestate and hydrolyzed straw mixed in a ratio of 1:4 (v/v). In average 2.06 g L^{-1} (A = 2.41 g L^{-1} and D = 1.71 g L^{-1}) biomass has been formed after 7 days. For biomass composition see Table 2.

G. sulphuraria did simultaneously consume glucose and glycerol, which may additionally explain the higher cell numbers reached compared to the cultivations with untreated digestate (Fig. 1D, G and J). Furthermore, with the enzyme formulation also 10–20 mg L^{-1} phosphate has been added, which additional contributed to the better growth performance (Fig. 1E, H and K).

It is an asset for the implementation of a utilization process based on *G. sulphuraria* that this organism can consume not only a wide range of carbon sources (Barbier et al., 2005) but also nitrogen sources (found for *Cyanidium caldarium*) (Rigano et al., 1976). A preliminary experiment carried out with only glutamic acid as nitrogen and carbon source in cyanidium medium clearly revealed the ability of *G. sulphuraria* to grow on amino acids (not shown), and thus it can be concluded that amino acids released by the hydrolysis of proteins in digestate may have served not only as nitrogen source but also as carbon source.

When 100% (v(v) hydrolyzed digestate supplemented with straw hydrolysate in a ratio of 4:1 was used as nutrient sources the growth rate was reduced. In Fig. 3 two cultivations carried out in a stirred and aerated bioreactor are shown. In one cultivation (Fig. 3A) the cell number increased at a rate of 0.9 day^{-1} to around 3×10^8 cells mL^{-1} within 7 days. In the second cultivation (Fig. 3D) a growth rate of 0.6 day^{-1} was determined and the cell number after 7 days was 2×10^8 cells mL^{-1}. In both cultivations neither FAN (present at 100–125 mg L^{-1}) nor phosphate (present at 20–30 mg L^{-1}) was totally consumed (Fig. 3B and E). The approximately 2 g L^{-1} glucose provided by hydrolyzed straw was consumed after 4 days (Fig. 3C and F). Only 0.1 g L^{-1} glycerol were detected in both cultivations (not shown). The yield of biomass per gram glucose was 0.92 g and 0.81 g, respectively, which is similar to the yields found for *G. sulphuraria* grown on bakery waste (Sloth et al., 2017) but twice as high the yield of 0.42 g biomass per g glucose found by Henkanatte-Gedera et al. (Henkanatte-Gedera et al., 2017). The high yield obtained may underline the conclusion that not only glucose was used as carbon source, but also amino acids.

The application of straw hydrolysate is not only an approach to convert a material of low value into high-value microalgal biomass (Zhang et al., 2019), but also an approach to convert a material low in proteins into biomass which is rich in proteins. It could be shown that *Chlorella* sp. and *Scenedesmus* sp. adapted well to straw hydrolysate and grew fast (Zhang et al., 2019). Contrarily, *G. sulphuraria* did show at least in one culture a reduced growth rate of 0.6 day^{-1} compared to the

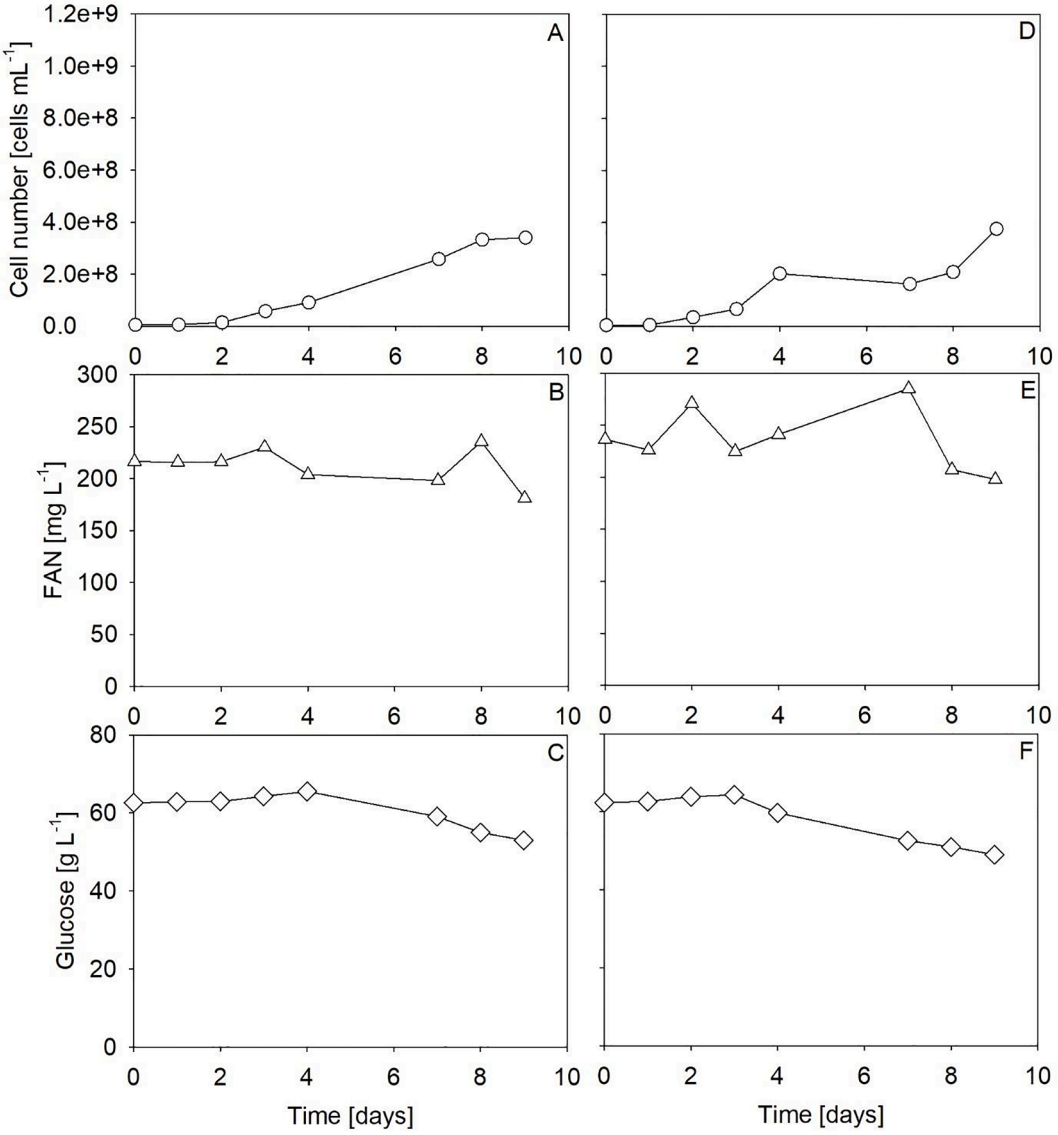

Fig. 4. Time profiles of cell number (A and D), free amino nitrogen (B and E, FAN) and glucose (C and F) concentrations in cultures of *G. sulphuraria* grown in duplicate in a medium consisting of 100% (v/v) hydrolyzed digestate supplemented with 50 g L^{-1} glucose. In average 1.91 g L^{-1} (A = 1.80 g L^{-1} and D = 2.02 g L^{-1}) biomass has been formed after 9 days. For biomass composition see Table 2.

cultivations shown in Figs. 1 and 4. Studies considering the use of straw hydrolysates as nutrient source for *G. sulphuraria* could not be identified, and thus further studies carried out at higher hydrolysate concentrations are needed.

Since all cultivations were limited in carbon, it was of interest to reveal to what extent *G. sulphuraria* can make nutrients available in form of nitrogen and phosphorous from digestate when sufficient carbon is provided. Thus, two cultivations have been carried out in presence of 100% (v/v) hydrolyzed digestate supplemented with 50 g L^{-1} glucose (Fig. 4). After 9 days a cell concentration of around 3×10^8 cells mL^{-1} was obtained. Growth was exponential during the first 4 days and the growth rate for the cultivation shown in Fig. 4A was 0.8 day^{-1} while 1.0 day^{-1} was found for the cultivation shown in Fig. 4D. Phosphate was not detectable, and FAN did not decrease during cultivation (Fig. 4B and E). Less than 10 g L^{-1} glucose was consumed by *G. sulphuraria* in both cultivations. The yield of biomass per gram glucose was 0.19 g and 0.15 g, respectively, and similar to the yield reported in literature in cherry-brine-liquid (Massa et al., 2019). It seems that *G. sulphuraria* does not efficiently use glucose when available in excess. It can further be concluded from this experiment that the limited nutrient in digestate is phosphate when carbon is supplied in excess. Thus, for an implementation with focus on high biomass production an external source of phosphate needs to be supplied.

3.3. Biochemical composition of Galdieria sulphuraria biomass

The biochemical composition of *G. sulphuraria* grown on hydrolyzed digestate mixed with hydrolyzed straw (1:4, v/v) and hydrolyzed digestate supplemented with 50 g L^{-1} glucose shown in Figs. 3 and 4, respectively, is listed in Table 2. In all cultivations a carbohydrate content of 18–23% (w/w) was found, while the protein content was 35 and 41% (w/w). Lipid content was influenced by carbon limitation in the batches with hydrolyzed digestate and straw and 2–4% (w/w) was determined. Under carbon sufficient conditions, as obtained in the batch cultures with hydrolyzed digestate and 50 g L^{-1} glucose, however, 7–9% (w/w) was found. The ash content was between 2 and 6 % (w/w) and slightly higher in carbon limited cultures.

The biomass composition determined by Massa et al. for *G. sulphuraria* was (w/w) 58.4% carbohydrates, 22.0% proteins and 4.6% lipids (Massa et al., 2019). The lipid content is comparable to the one obtained in this study (Table 2), however, the carbohydrate and protein contents found here are 50% less and 100% higher, respectively. Graziani et al. determined a protein content of 26.5% (w/w), a lipid content of 11.4% (w/w) and carbohydrate content of 69.1% (w/w) when *G. sulphuraria* was grown under heterotrophic conditions. However, under autotrophic conditions the protein content of 32.5% (w/w) was determined (Graziani et al., 2013), which is closer to the content obtained in this study. The difference between the reported and observed biomass compositions might be due to different species and culture conditions used in both studies. A low protein content may also indicate a limitation in nitrogen and continuous utilization of intracellularly stored proteins. *G. sulphuraria* has shown to degrade pigments under nitrogen starvation (Sinetova et al., 2006). The degradation of proteins might be another reaction to it.

4. Conclusions

This study revealed the potential of *G. sulphuraria* to utilize agricultural residues and to form biomass with a protein content of around 40% (w/w). Considering the possibilities to implement decentralized processes, *G. sulphuraria* can contribute to add value to straw and digestate in rural areas. This approach makes residue utilization not just more economically attractive, but also presents new opportunities for feedstock production for food, fine chemical, pharma and material sectors.

Table 2
Biochemical composition of *G. sulphuraria* biomass from the batch cultures grown on hydrolyzed digestate mixed with hydrolyzed straw (1:4, v/v) and hydrolyzed digestate supplemented with 50 g L^{-1} glucose shown in Figs. 3 and 4, respectively.

	Hydrolyzed digestate and straw (1:4, v/v)	Hydrolyzed digestate and 50 g L^{-1} glucose
	Fig. 3A-C / Fig. 3D-F	Fig. 4A-C / Fig. 4D-F
Carbohydrate [%, w/w]	21.74 / 17.51	20.53 / 23.29
Proteins [%, w/w]	39.40 / 35.09	40.99 / 39.82
Lipids [%, w/w]	4.12 / 2.26	9.05 / 7.22
Ash [%, w/w]	3.53 / 5.91	3.04 / 2.09

CRediT authorship contribution statement

Daniel Pleissner: Conceptualization, Methodology, Resources, Visualization, Supervision, Writing - original draft. **Astrid Victoria Lindner:** Methodology, Writing - review & editing. **Nicole Händel:** Methodology, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors acknowledge the German Federal Ministry of Education and Research for providing funding within the Era-Net Cofund “FACCE SURPLUS” Program (031B0934B).

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