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SHORT COMMUNICATION

Reduction of the Microbial Load of Digestate by the Cultivation of *Galdieria sulphuraria* Under Acidic Conditions

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Abstract

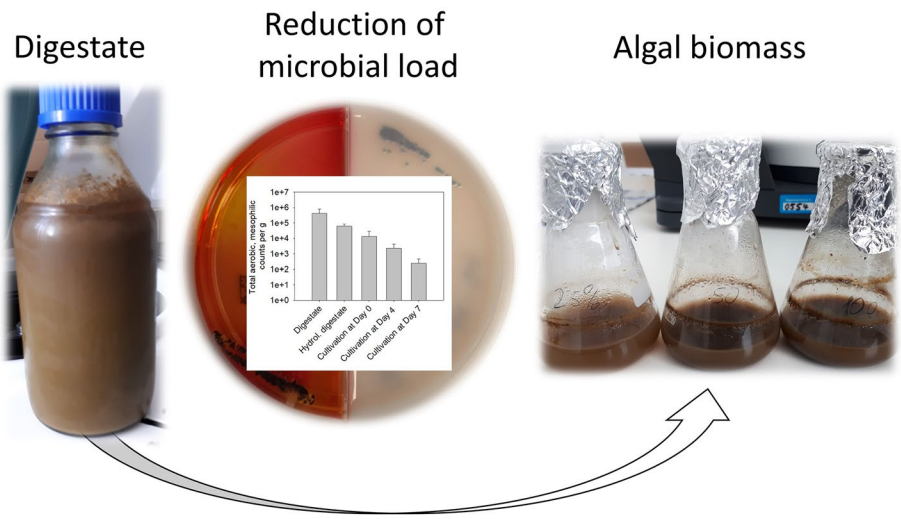
Purpose Organic waste streams with a high microbial load have been used as nutrient sources in the cultivation of heterotrophic microalgae. However, it remains unclear whether an alga-based organic waste utilization can also result in a reduction of the microbial load, and thus in a combined waste utilization and hygenization process.

Methods The heterotrophic cultivation of the microalga *Galdieria sulphuraria* at a pH of 2 and 45 °C as hygenization method for digestate, which serves as nitrogen source in algae cultivation, was investigated. Attention has been paid to *Salmonella* sp. and spores, coming from the organic waste stream digestate and its reduction during hydrolysis and cultivation of *G. sulphuraria* in the resulting hydrolysate.

Results Digestate contained 0.5×10^6 counts per g material, predominantly formed by aerobic, mesophilic organisms. *Salmonella* sp., yeast and molds, enterobacteriaceae as well as enterococci were diminished within 24 h of hydrolysis or cultivation. During hydrolysis of digestate and cultivation of *G. sulphuraria* the counts of aerobic, mesophilic organisms could be subsequently reduced by a log reduction factor of 3. The remaining microorganisms were almost exclusively spore forming ones which were reduced by a log reduction factor of 2 during cultivation under acidic conditions.

Conclusion It is suggested here to include the cultivation of *G. sulphuraria* in future waste management as hygenization process.

Graphical Abstract



Keywords Hydrolysis · *Spores* · *Salmonella* sp. · Algae

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Statement of Novelty

This study investigated the reduction of the microbial load in digestate in a purely carried out heterotrophic culture of *Galdieria sulphuraria.*

Introduction

The cultivation of microalgae has been considered a strategy to produce biomass for food and feed formation [1]. This strategy has been evolved from the fact that microalgae accumulate more than 40% (w/w) of valuable and nutritious proteins as well as essential polyunsaturated fatty acids [2]. The cultivation of microalgae has been predominantly carried out under phototrophic conditions using carbon dioxide and (sun)light. Even though microalgal biomass is rich in valuable compounds, establishing and operating of economically feasible cultivation plants is challenging. The challenges arise, among others, from the difficulties to achieve sufficiently high volumetric biomass concentrations [3]. To overcome this drawback, conversion strategies of organic wastes have been in focus of research activities and strategies dealing with the use of heterotrophic microalgae were successfully investigated over the last years [4]. Using heterotrophic microalgae, organic wastes can be converted into biomass and high volumetric biomass concentrations exceeding the one from phototrophic cultivations can be obtained [5–8]. Such a path can basically be considered an approach to reduce and efficiently utilize organic wastes, and even allows the implementation of decentralized utilization processes where organic material appears in greater amounts [9].

Even though the produced algal biomass obtainable by organic waste conversion can be rich in proteins and essential fatty acids [6, 10–12], and thus can potentially be considered as alternative protein source for food and feed purpose, its application is hindered by legislation. Whenever an organic material, which does not have food quality, is used in microalgae cultivation as nutrient source, produced biomass is banned from being used in food and feed formation [13]. However, there are enormous amounts of solid waste appearing [14]. Some of the organic material is just landfilled or energetically used, and more holistic utilization processes are needed to proceed with an efficient circular bioeconomy [15].

Currently, agriculture is under pressure due to the limited availability of arable land, water scarcity and the increasing need for fertilizer and pesticides [16]. To reduce this pressure, it is expected that novel food systems will be established based, for instance, on products from algal biomass [17] and eventually organic waste may attract more attention to cover the demand for nutrients for its production. However, to overcome legal restrictions and to make use of organic waste materials proper data on their safety is urgently needed [17]. Data should provide information on the presence of contaminations. Particularly the appearance of microbial contaminations in organic waste can be, depending on the storage and period, critical to processing and using it in algal biomass production [18]. In classic biotechnological processes sterilization is often applied to eliminate microbial contaminants. However, due to economic reasons sterilization should be skipped, and thus alternative measures are required allowing a reduction of the microbial load and in particular pathogens in organic waste either beforehand or during processing.

Conventional organic waste treatment processes such as composting and anaerobic digestion include a hygenization step to reduce the microbial load [19, 20]. To transfer the hygenization approach to algal cultivation, *Galdieria sulphuraria* was studied under non-sterile conditions in presence of digestate. Digestate was selected as waste stream as it contains substantial amounts of nitrogen compounds, which serve as nitrogen compounds in algae cultivation [21]. *G. sulphuraria* is an extremophilic microalgae which does grow at a pH of 2 and 45 °C [12]. The low pH was expected to reduce the microbial load in way that *G. sulphuraria* cultivation can be considered an alternative organic waste hygenization process. This assumption has been tested by determining the change of microbial load during hydrolysis of digestate and cultivation. Furthermore, the effect of culture conditions on *Salmonella* sp. and spore forming microorganisms has been assessed. The novelty of this study lies in connecting organic waste treatment with even food and feed production if the microbial load can be substantially reduced and particularly pathogens diminished.

Materials and Methods

Digestate

Digestate was obtained from an anaerobic digestion plant using cattle manure as substrate and stored at 4 °C until used in experiments.

Galdieria sulphuraria

Galdieria 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 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.

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

Salmonella enterica

Salmonella enterica subsp. enterica DSM 11320 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) as a lyophilizate. The lyophilizate was resuspended in a 15 mL centrifuge tube with 10 mL buffered peptone water and incubated at 37 °C for 24 h. Subsequently, spatted out onto specific agar (XLD/Brilliance Salmonella) and incubated according to the manufacturer's instructions. From the grown colonies, a strain collection was made in the Mast CRYOBANK® system and frozen at − 20 °C.

Hydrolysis of Digestate

To make nutrients available from digestate for *G. sulphuraria* cultivation an enzymatic treatment has been carried out. To 1 L of digestate, 1 mL of CellicCtec2 (Novozymes, Denmark) and 1 mL Protease S-02 (ASA-Enzyme, Germany) were added and the hydrolysis carried out at pH 5 and 50 °C for 12 h. Hydrolysate obtained from hydrolytic treatment was stored at 4 °C until used in cultivation experiments.

Cultivation of *Galdieria sulphuraria*

Cultivations of *G. sulphuraria* were carried out under heterotrophic conditions in triplicate in 250 mL flasks containing of 100 mL hydrolyzed digestate supplemented with 10 g L^{-1} glucose and using 5.0×10^6 *G. sulphuraria* cells mL^{-1} as inoculum. Flasks were incubated at a temperature of 45 °C and a pH of 2 and shaken at 150 rpm on an orbital shaker. Samples were taken regularly and used for microbial investigations.

Effect on *Salmonella enterica*

To investigate the effect of hydrolytic treatment on the presence of *S. enterica*, the digestate was prepared with a defined concentration of 106 colony forming units per ml (cfu mL^{-1}). After 0 and 24 h of hydrolytic treatment, the recovery rate of *S. enterica* in the samples was determined. The experiment was carried out in duplicate.

The investigation of the effect of pH 2 and 45 °C (conditions of *G. sulphuraria* cultivation) on the presence of *S. enterica* was carried out in duplicate. To the culture medium, as described in "Cultivation of *Galdieria sulphuraria*" section, first 5.1×10^6 and second 5.7×10^6 *S. enterica* cfu mL^{-1} were added. Each investigation was carried out for 7 days. Samples were taken regularly and used for microbial investigations.

Effect on Spore Forming Microorganisms

The investigation of the presence of aerobic spore forming microorganisms in untreated (pH 7) and hydrolyzed digestate (pH 5) as well as in hydrolyzed digestate inoculated with *G. sulphuraria* (pH 2) was performed. For the latter samples were taken after 0, 4 and 7 days and used for microbial investigations. Each experiment was carried out in duplicate.

Microbial Analysis

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

The investigation of the microbiological status of the samples focused on the determination of aerobic, mesophilic microbes, yeasts/molds, enterobacteria, enterococci, *Escherichia coli* and *Salmonella* sp. The standardized test methods according to § 64 of the German Food and Feed Code (LFGB) (Amtliche Sammlung von Untersuchungsverfahren—ASU) were used as basis for the performed methods. The pH of samples was adjusted to 7 using 1 M NaOH if necessary.

For determination of aerobic, mesophilic plate count (ASU L 00.00-88/2: 2015-06), yeasts/molds (ASU L 01.00-37:1991-12) and enterobacteria (ASU L 00.00-133/2:2018-03), 10 mL sample was added to a stomacher bag with 90 mL dilution solution (buffered NaCl peptone solution) and mixed well for 60 s. A 1:10 dilution series was then prepared with the same dilution solution. 1 mL or 0.1 mL from each dilution was spat out on plate count agar (aerobic, mesophilic plate count) or Sabouraud dextrose agar (yeasts/molds) using the surface method, and for enterobacteria overlaid with crystal violet neutral red bile agar using the plate pour method. Incubation and enumeration of cfu mL^{-1} were performed according to the respective specifications of the method.

Enrichment of *E. coli* (following ASU L 01.00-25:1997-09), enterococci (modified according to ASU L 02.07-2) and *Salmonella* sp. (following ASU L 00.00-20: 2018-03) was prepared by adding 1 mL to 9 mL enrichment medium for *E. coli* and enterococci, and 25 mL to 225 mL enrichment

medium for *Salmonella* sp. Incubation was performed for 24 h at 37 °C for enterococci and *Salmonella* sp., and 48 h at 30 °C for *E. coli*. Fractionated smear was then performed from the sample using an inoculating loop on selective culture media. Subsequent incubation was on xylose-lysine-deoxycholate agar for *Salmonella* sp. and on coliform agar for *E. coli* for 24 h at 37 °C. Enterococci agar plates according to Slanetz and Bartley [22] were incubated at 37 °C for 48 h. Evaluation was performed on counting typical colonies.

For the determination of aerobic spore forming microorganisms, the samples were treated in a water bath at 80 °C for 10 min to deactivate vegetative cells. Further procedure for determination of aerobic, mesophilic plate count and aerobic spore forming microorganisms was performed according to the protocol (ASU L 00.00-88/2: 2015-06). Samples were spread on agar and plates were incubated at 30 °C for 72 h.

Chemical Analysis

Ammonium quantification was based on the phenol hypochlorite assay (Berthelot reaction) described earlier [23].

Free amino nitrogen (FAN) was determined following the modified EBC-ninhydrin method. First, two reagents were prepared. For reagent A, 1 g $Na_2HPO_4 \cdot 12H_2O$, 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 and phosphate concentrations were determined using HPLC (Shimadzu: LC-10AD pump, SIL-10AD autosampler, CTO-10AD oven, refractive index detector RID-20 A, CBM-20 A communication module): 10 µL of sample was injected on an Aminex HPX-87 H column (300 mm × 7.8 mm) and eluted isocratically with 1.0 mL min^{-1} 5 mM H_2SO_4 at 27 °C. A calibration curve was generated with pure solutions of known concentration.

Results and Discussion

The liquid fraction of the hydrolyzed digestate consisted of a solid-to-liquid ratio of 0.8% (w/w), 0.5 g L^{-1} glucose, 23.9 mg L^{-1} ammonium and 290.7 mg L^{-1} FAN. Due to the low glucose concentration, which is used as carbon source by *G. sulphuraria*, the hydrolozed digestate was supplemented with 10 g L^{-1}.

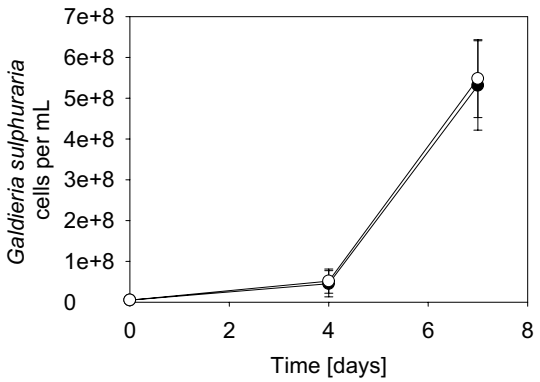

Fig. 1 Growth of *Galdieria sulphuraria* in two independently performed cultivations (carried out in triplicate). The exponential growth rate was in average 0.66 day^{-1}

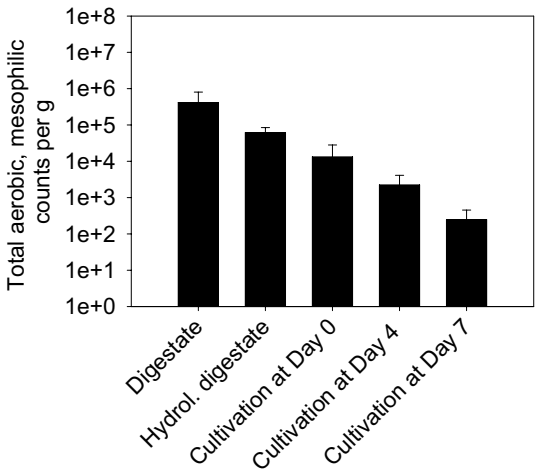

Fig. 2 Total counts of aerobic, mesophilic microorganisms per gram material found in digestate, after hydrolysis and during cultivation of *Galdieria sulphuraria* on hydrolyzed digestate

The cultivation of *G. sulphuraria* on hydrolyzed digestate supplemented with 10 g L^{-1} glucose resulted in an exponential growth rate of 0.66 day^{-1}, which is in the range of growth rates found earlier [24]. Exponential growth was found from the beginning to the end of cultivation after 7 days (Fig. 1). *G. sulphuraria* was not the only microorganism present. As digestate was used as nutrient source, the background microbial load was around 0.5×10^6 counts per g material (Fig. 2), which was predominantly formed by aerobic, mesophilic organisms. *Salmonella* sp., yeast and molds, enterobacteriaceae and enterococci were not present or below 100 counts per g. Even though, *S. enterica* has been added to digestate prior to hydrolysis or to the medium prior to cultivation it diminished already after 24 h irrespective the process step and may therefore possess no risk. During hydrolysis of digestate and cultivation of *G. sulphuraria* the

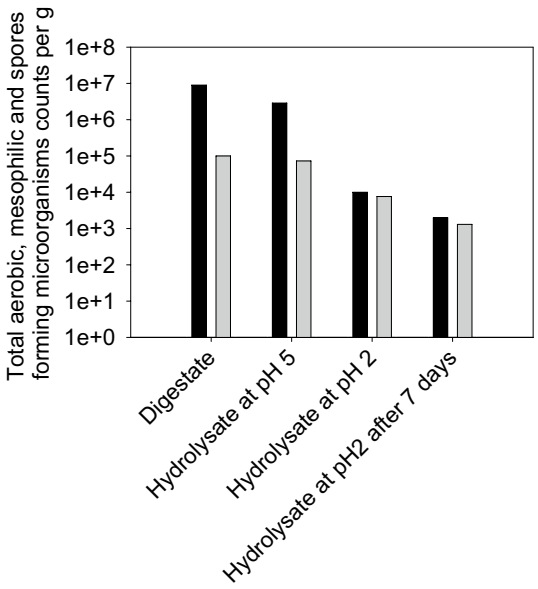

Fig. 3 Total counts of aerobic, mesophilic (black bars) and spore forming (grey bars) microorganisms per gram material found in digestate, after hydrolysis in hydrolysate at pH 5 and 2 as well as in hydrolysate stored at a pH of 2 for 7 days

counts of aerobic, mesophilic organisms could be subsequently reduced. After an *G. sulphuraria* cultivation period of 7 days 245 counts per g remained (Fig. 2), which resulted in a log reduction factor of 3. The reduction in counts was linear over the different process steps and mostly occurred during *G. sulphuraria* cultivation at pH 2.

While it was expected that a pH 2, as used in *G. sulphuraria* cultivation, has an impact on vegetative cells, the impact on spore forming microorganism was unknown. Thus, the contribution of spore forming microorganisms to the total aerobic, mesophilic counts was investigated. As shown in Fig. 3, most of the microorganisms present in digestate or hydrolyzed digestate were vegetative state. Exposing the microorganism in hydrolysate to a pH 2 resulted in a significant decrease in total aerobic, mesophilic counts. The remaining microorganisms after a 7 days long pH 2 treatment were almost exclusively spore forming ones. Interestingly, spore forming microorganism were reduced by a log reduction factor of 2 during cultivation under acidic conditions.

Digestate is basically already a hygenized material when heated to 55 °C for 8 h during or after anaerobic digestion [25]. These are also the conditions where *Salmonella* sp. and *E. coli* diminished below detection limit [26]. Thus, the microbial load of digestate is generally lower compared to fresh manure [27]. Nevertheless, the present study revealed two results of interest. First, processing and using of treated digestate in *G. sulphuraria* cultivation at pH 2 and 45 °C further reduces the aerobic, mesophilic microorganisms by a log reduction factor of 3 and the spore forming ones by a log reduction factor of 2. Second, the hydrolysis and cultivation conditions have a substantial impact on the pathogenic microorganism *Salmonella* sp. within 24 h and this effect was also found when digestate and cultivation medium was enriched with it.

Delanka-Pedige et al. also investigated the reduction of pathogens but in a wastewater treatment system employing *G. sulphuraria* [28]. The authors carried out the cultivation of G. sulphuraria at a pH 4 and a temperature range between 27 and 46 °C as well as a solar radiation from 6.6 to 7.4 kWh m^{-2} day^{-1}. Total coliforms were reduced by a log reduction factor of 3.3 in the wastewater treatment system effluent and no fecal coliforms were detected in the algal effluent. Furthermore, it was found that 98% of the total bacteria and *Enterococcus faecalis* and *E. coli* were removed. In a cultivation of G. sulphuraria carried out at pH 2.5 at larger scale (700 L) using primary effluent, Tchinda et al. confirmed this result and fecal as well as total coliforms were reduced below detection limit within 3 days [29].

From the results and the diminish of potential pathogens it can be concluded that the cultivation of *G. sulphuraria* can be considered as organic waste hygenization process. Therefore, it is suggested here to extent the processes of organic waste hygenization and to include the cultivation of *G. sulphuraria* in future waste management processes. It is of particular interest that the reduction also works for pure heterotrophic processes where organic nutrients are supplied in excess. This is the major difference between the present study and the studies carried out earlier [28, 29]. Munasinghe-Arachchige et al. confirmed the synergetic effects of algal biomass, metabolites, temperature and sunlight as a function of pH on the inactivation of native fecal coliforms. In the present sunlight could be excluded as necessary factor [30]. Also elevated dissolved oxygen levels as factor to reduce *E. coli* [30] could be ruled here as oxygen was rather limited in the carried out heterotrophic cultures.

G. sulphuraria biomass produced in presence of digestate and glucose has been shown to contain around (w/w) 40% proteins, 20% carbohydrates and less than 10% lipids [12]. It is expected that the biomass obtained in this study had a similar biomass composition, and thus is of value for different application. However, since the microbial load coming from digestate was not totally reduced during hydrolysis and cultivation at pH 2 and 45 °C, and due to the possible presence of microbial toxins [30] as well as due to legislative restrictions, the application of *G. sulphuraria* biomass is limited to a material use. Nevertheless, a treatment of biomass to further reduce the spore forming microorganisms and to decrease the microbial load might be an option. Techniques to deactivate spore forming microorganisms are of thermal and non-thermal nature. Since the aim of deactivation is to enter the food and feed sectors, the application of

non-thermal techniques is favored to maintain the nutrition quality [31]. Lv et al., however, also revealed that the combination of sonication and thermal treatment had the most severe impact on *Bacillus cereus* spores [31]. The authors found a log reduction factor of 3.12 after 30 min at 20 kHz, 80 °C and 400 kPa. Another approach is the application of high pressure [32, 33]. After germination of spores is induced, the application of high pressure results in a destruction of it. The pressure applied is usually > 500 MPa and the temperature can be > 60 °C [33]. However, even at high pressure and higher temperature as well as using a combination of sonication and thermal treatment not all spores can be destroyed. Nevertheless, it seems a promising approach to induce spore germination and then expose it to a pH of 2.

Conclusion

Utilization approaches of organic waste streams should not only contribute to the utilization but also to a hygenization. This study has clearly shown that *G. sulphuraria* cultivation carried out at a pH of 2 and 45 °C significantly reduced the microbial load, spore forming microorganisms and pathogens. *Salmonella* sp., yeast and molds, enterobacteriaceae and enterococci were even reduced below detection limit. However, the growth conditions were not tough enough to diminish spores and to potentially allow a use of *G. sulphuraria* biomass even in food and feed production. However, including the cultivation of *G. sulphuraria* in future waste management processes as hygenization process is implied.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nicole Händel. The first draft of the manuscript was written by Daniel Pleissner and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

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Data Availability The datasets generated during and/or analyzed during the current study are not publicly available due to financial reason but are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

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