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Aggregation of purple bacteria in an upflow photobioreactor to facilitate solid/liquid separation: Impact of organic loading rate, hydraulic retention time and water composition

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Abstract

Purple non-sulfur bacteria (PNSB) form an interesting group of microbes for resource recovery from wastewater. Solid/liquid separation is key for biomass and value-added products recovery, yet insights into PNSB aggregation are thus far limited. This study explored the effects of organic loading rate (OLR), hydraulic retention time (HRT) and water composition on the aggregation of *Rhodobacter capsulatus* in an anaerobic upflow photobioreactor. Between 2.0-14.6 gCOD/(L.d), the optimal OLR for aggregation was 6.1 gCOD/(L.d), resulting in a sedimentation flux of 5.9 kgTSS/(m^2.h). For HRT tested between 0.04-1.00 d, disaggregation occurred at the relatively long HRT (1 d), possibly due to accumulation of thus far unidentified heat-labile metabolites. Chemical oxygen demand (COD) to nitrogen (6-35 gCOD/gN) and the nitrogen source (ammonium vs. glutamate) also impacted aggregation, highlighting the importance of the specific wastewater type and its pre-treatment. These novel insights to improve purple biomass separation pave the way for cost-efficient PNSB applications.

Keywords: nutrient recovery, purple phototrophic bacteria, granular sludge, flocculation, granulation

1 Introduction

Purple non-sulfur bacteria (PNSB), a major group of purple phototrophic bacteria, show potential for resource recovery from wastewater, process water, sidestreams and byproducts containing biodegradable organics and nutrients (Alloul et al., 2018; Capson-Tojo et al., 2020; Monroy and Buitrón, 2020). These microbes are metabolically versatile and can use a wide variety of carbon and nitrogen sources, both phototrophically as well as chemotrophically (Imhoff, 2006). In resource recovery, PNSB are typically exploited in photohetero- or -mixotrophic mode where they use light as energy source and organics optionally in combination with inorganic carbon as carbon source(s). Different value-added products have been investigated for resource recovery by PNSB such as microbial protein, fertilizers and polyhydroxyalkanoates (Alloul et al., 2021; Capson-Tojo et al., 2020; Sakarika et al., 2020). Research to efficiently harvest PNSB-rich biomass is, however, limited. Gravitational methods are typically more cost-efficient than membrane separation. In wastewater treatment, gravitational methods can reduce total costs by 38-53% compared to membrane-based systems (Bertanza et al., 2017). Gravitational solid/liquid separating within a feasible amount of time relies on (bio)aggregation of cells into flocs and granules. Aggregation of biomass consists of different mechanisms, where repulsion between cells is to overcome. Polyvalent cations play an important role in the bridging of microbial cells, whereas extracellular polymeric substances allow cells to coagulate by changing the cell surface hydrophobicity, cell surface charge or serve as a microbial glue (Gao et al., 2011; Liu et al., 2003; Suresh et al., 2018). For aerobic activated sludge, the growth of filamentous bacteria helps cells to aggregate as well, forming a backbone for microorganisms to attach to (Verstraete and van Vaerenbergh, 1986). Floc formation or flocculation can be realized by applying a selective pressure on the microbial community through washout of poorly settling biomass (e.g. upflow reactors or sequencing batch reactors), addition of chemical coagulants (e.g. Ca^{2+}, Al^{3+}), adjusting the feeding strategy or changing the chemical oxygen demand to nitrogen (COD/N) ratio in the feed (Suresh et al., 2018). Granulation of the biomass can be achieved through applying increased hydrodynamic shear, regulating the feeding strategy or changing the organic load rate (OLR) in a sequencing batch reactor or upflow reactor systems, such as upflow anaerobic sludge blanket (UASB) reactors (de Sousa Rollemberg et al., 2018; Lim and Kim, 2014).

Limited literature can be found on the aggregation of PNSB biomass in bioreactors, and up to now, only two biotechnological approaches have been explored. Washing out non-settling biomass in a sequencing batch photobioreactor after each run, with increasing OLR from 0.2 to 1.3 gCOD/(L.d) and decreasing hydraulic retention time (HRT) from 2 d to 0.67 d, lead to accumulation of aggregating biomass, forming granules with a sedimentation flux of 4.7 kgVSS/(m^2.h) (Cerruti et al., 2020). Similarly, continuous upflow photobioreactors have proven to be successful for PNSB aggregation. The work by Driessens et al. (1987), for example, achieved aggregation of PNSB-rich sludge with a sedimentation flux of 1-2 kg/(m^2.h). More recently, Stegman et al. (2021) obtained PNSB granules in an upflow reactor testing different upflow rates (2-9 m/h), forming granules with a sludge volume index (SVI_{30}) of 10 mL/g and average setting velocities above 30 m/h. High COD and nitrogen removal efficiencies around 60-90% have been achieved for all these systems. Optimization is, however, still required to reach the effluent discharge limits of 125 mgCOD/L and 15 mgN/L (CEC, 1991). A systematic understanding of the effect of operational tools and wastewater composition, for example, COD/N ratio and nitrogen source, on the aggregation of PNSB-rich biomass are, however, still missing, although these parameters influence aggregation (Suresh et al., 2018).

The main goal of this research was to acquire a systematic understanding of the effect of operational tools and wastewater composition on the aggregation of PNSB-rich biomass. The aggregation mechanism or the structure of the aggregates were not studied, yet are also crucial for process control. In terms of aggregate size, an average diameter of around 100 µm has been observed for PNSB granules and a uniform interior structure with a community dominated by purple bacteria has been detected (Cerruti et al., 2020; Stegman et al., 2021). The research on the structure of PNSB aggregates and the mechanism of aggregation remains nevertheless limited compared to other fields such as aerobic sludge flocculation or anaerobic granulates. For flocculation, for example, it has been shown that a properly balanced ratio of floc formers and filamentous microorganisms is required for good settling biomass. The temperature has also been shown to influence flocculation through a change in filaments population or surface charge (Krishna and van Loosdrecht, 1999; Morgan-Sagastume and Allen, 2005). Divalent cations are also key for both granular and floccular sludge as Ca_2^+, Mg_2^+ and Fe_3^+ will bridge negatively charged functional groups within the extracellular polymeric substances and contribute to adhesion (Gao et al., 2011; Suresh et al., 2018; Tiwari et al., 2006). These mechanistic insights are also important for PNSB. Follow-up research is, therefore, necessary to effectively characterize PNSB aggregates in terms of size distribution, extracellular polymeric substance, and spatial organization (e.g. fluorescence in situ hybridization). Combined with tests on the ionic matrix (mono- and bivalent ratio) and shear, it can provide a deeper understanding of the mechanisms of PNSB aggregation.

2 Materials and methods

2.1 Inoculum and medium composition

Rhodobacter capsulatus ATCC 23782 (available at the American Type Culture Collection), selected as a model organism for PNSB, was precultivated in closed test tubes of 24 mL under axenic conditions with a light intensity of 85-100 µE/(m².s) on synthetic wastewater based on Segers and Verstraete (1983), containing calcium lactate as carbon source (2.4 g/L, hence 2.4 gCOD/L) and sodium glutamate (1.3 g/L, hence 0.12 g N/L and 1.2 g COD/L) as a nitrogen source, and a relatively high dose of $HKPO_4^-$ to act as pH buffer (0.3 gP/L) at a pH of 6.8, enriched with 1 mL of a vitamin solution per liter of medium, containing nicotinic acid (0.1 g/L), biotin (0.015 g/L) and thiamine-HCl 1.0 (g/L). Multiple batch growth tests were conducted with *Rb. capsulatus* at different COD concentrations (0.1, 0.2, 0.4, 0.5, 0.7, 0.8, 1.1, 1.3, 2.5, 4.9 and 9.7 gCOD/L) to characterize the inoculum and derive the Monod kinetics (see supplementary material).

For reactor operations, the nitrogen source was changed to ammonium sulfate, except when glutamate was used to investigate the influence of the nitrogen source (section 3.2.2). In the batch reactor, the medium was sterilized to explore the influence of PNSB metabolites on aggregation (section 3.1.2), while for the continuous upflow reactor, the medium was not sterilized to mimic wastewater conditions.

2.2 Reactor set-ups and operation

The batch reactor consisted out of cylindrical tubes with an inner diameter of 3.5 cm and a volume of 400 mL. All parts of the reactor were autoclaved at 121°C with an overpressure of 1 atm for 20 min and procedures were conducted in the laminar flow to work axenically. The reactor was inoculated at 5 %v/v with axenic inoculum (grown for 2-3 d) and the headspace was flushed for 2 minutes with argon to create an anaerobic environment. The reactor was operated at a temperature of 36 to 38 °C and illuminated with Gro-Lux Fluorescent lamps F40/T12 (Sylvania), supplemented with spotlights of 150 W to reach a light intensity between 85 and 100 µE/(m².s).

The continuous reactor, illustrated in Figure 1, had an inner diameter of 3.5 cm, an illuminated volume of 440 mL and a decanter with an inner diameter of 9.5 cm on top. The carbon- and nitrogen-containing media were dosed separately with peristaltic pumps to minimize microbial contamination and bioconversion in the feed. Feeding was discontinuous for 2 times 5 min every hour yielding flow rates of 0.44, 3.1, 4.4 and 10.6 L/d (corresponding to HRT of 1, 0.3, 0.1 and 0.04 d, respectively). HRT was calculated using the following equation: HRT = $V_{reactor}/Q_{influent}$, with $V_{reactor}$ the volume of the reactor and $Q_{influent}$ the flow rate of the influent. The internal recirculation yielded upflow rates in the reactor and the decanter of 1 m/h and 0.15 m/h, respectively. The reactor was consistently inoculated at 5 %v/v with axenic inoculum of *Rb. capsulatus* (grown for 3 d, more information on growth characteristics available in supplementary material) and illuminated with Gro-Lux Fluorescent lamps F40/T12 (Sylvania), supplemented with spotlights of 150 W. Light intensities were between 150-170 µE/(m².s) because it was expected that aggregation will result in higher biomass concentrations and thus lower light penetration compared to the batch test (85-100 µE/(m².s)).

Before testing different conditions, the reactor was operated at an OLR of 6.1 gCOD/(L.d), an HRT of 0.1 d and a COD/N ratio of 12 gCOD/gN to achieve aggregation, based on previous research of Driessens et al. (1987). First, the influence of OLR, coupled to HRT (section 3.1.1), was examined by operating the reactor in four subsequent phases: (i) HRT 0.1 d (OLR 6.1 gCOD/(L.d)), (ii) HRT 0.04 d (OLR 14.6 gCOD/(L.d)), (iii) HRT 0.1 d (OLR 6.1 gCOD/(L.d)), and (iv) HRT 0.3 d (OLR 2.0 gCOD/(L.d)). The sedimentation flux was determined at HRT 0.04 and 0.1 d (no aggregation was observed at HRT of 0.3 d) for different dilutions of biomass harvested from the reactor.

To study the effect of metabolites accumulation at long HRT (1 d), aggregation was first established at an HRT of 0.1 d in two separate photobioreactors. The HRT was then increased to 1 d. When disaggregation occurred, tannic acid, an organic flocculant used in wastewater treatment, was added to the feed at 10 mg/(L.d) to one photobioreactor. In the photobioreactor without flocculant dosing, the medium in the recirculation loop was pasteurized in flasks for 15 minutes at 60 °C, 60-65 °C, and 70-80 °C.

To investigate the effect of accumulated metabolites, *Rb. capsulatus* was axenically cultivated in batch (no aggregation was observed) for 2 to 4 d. The broth was centrifuged at a relative g force of 20000g to separate the biomass and the supernatant. Before feeding the supernatant to the reactor, it was enriched with carbon and nitrogen to achieve an OLR of 12.2 gCOD/(L.d) and COD/N ratio of 12 gCOD/gN. Half of the minerals (see section 2.1) were also added. To test the hypothesis of heat-labile disaggregation metabolites, the supernatant was first autoclaved (section 3.1.2). The reactor was operated at five subsequential phases, at an HRT of 0.1 d: (i) start-up phase without the addition of supernatant, (ii) addition of supernatant of non-aggregated axenic batch cultures grown for 3 to 4 d to study the effect of disaggregation metabolites on PNSB aggregation, (iii) re-aggregation by stopping the addition of supernatant, (iv) addition of autoclaved supernatant and (v) addition of non-autoclaved supernatant of batch cultures grown for 2 to 3 d to examine the effect of lower concentrations of the disaggregation metabolites.

To explore the influence of the influent COD/N ratio (section 3.2.1), two upflow anaerobic photobioreactors were set up in parallel, both initially operated at a COD/N ratio of 12 gCOD/gN and HRT of 0.1 d, based on Driessens et al. (1987). After 15 days, the COD/N ratio was doubled to 24 gCOD/gN in one reactor and 6 gCOD/gN in the other. The last experiment (section 3.2.2) had the objective to investigate the effect of the nitrogen source, the nitrogen source was changed to glutamate, as a proxy for organic nitrogen, after achieving aggregation in a startup phase with ammonium at a COD/N ratio of 12 gCOD/gN. The reactor was operated in two phases with glutamate as the nitrogen source: (i) at a COD/N ratio of 35 gCOD/gN and (ii) at 22 gCOD/gN. The COD/N ratios for the experiment were based on the COD/N uptake ratios of PNSB (16-20 gCOD/gN) (Hülsen et al., 2014). These COD/N uptake ratios are lower compared to anaerobic digestion (57-140 gCOD/gN) because the fraction of incoming COD that is converted to biomass is higher (0.5-1.0 vs. 0.01 $gCOD_{biomass}/gCOD_{removed}$ for anaerobic digestion), resulting in a higher N-need (Mata-Alvarez, 2003; Metcalf et al., 1991). An overview of the different test conditions is presented in Table 1.

2.3 Analytical procedures

COD and NH_4^+-N were determined according to standard procedures NBN T91-201 and NBN T91-252, respectively. The biomass content, harvested directly from the reactor (the biomass content in the recirculation vessels was negligible), was measured after drying the sample at 105 °C (total suspended solids, TSS) and incineration at 650 °C for two hours (volatile suspended solids, VSS). Four biomass concentrations (100, 80, 60 and 40 %v/v) were tested in sedimentation cylinders of 50 mL to determine the sedimentation flux according to Verstraete et al. (1984). This parameter was selected because it is typically used to determine sedimentation for hindered settling (i.e., settling of intermediate concentrations of aggregates) (Verstraete and van Vaerenbergh, 1986). The sludge retention time (SRT) was not controlled and calculated based on the ratio of total biomass in the reactor to the biomass flow in the effluent (Equation 1), with Q representing the volumetric flow of the effluent, V the volume of the reactor and C the biomass concentration in the reactor and the effluent. The SRT to HRT ratio (SRT/HRT, unitless) was used as an indicator for biomass retention in the system. Higher SRT to HRT ratios or improved biomass retention implies less sludge washout due to better biomass aggregation and sedimentation.

$$SRT = \frac{C_{biomass,in\ reactor} * V_{reactor}}{Q * C_{biomass,effluent}} \qquad Equation\ 1$$

3 Results and discussion

3.1 Operational strategies to enhance aggregation

To analyze the influence of operational strategies on aggregation in PNSB, different OLR and HRT were tested. First, a range of OLR (2-14.6 gCOD/(L.d)) and HRT (0.3-0.04 d) was examined (section 3.1.1), as well as aggregation strategies at a relative long HRT of 1 d (section 3.1.2).

3.1.1 Organic loading rate and hydraulic retention time improve aggregation

Both the OLR and the HRT affected PNSB aggregation in the upflow reactor (Figure 2). Aggregation was highest at an OLR of 6.1 gCOD/(L.d) (HRT 0.1 d), resulting in an average aggregation indicator (SRT/HRT ratio) of 90 ± 40 and a sedimentation flux of 5.9 kgTSS/(m^2.h). Increasing the OLR to 14.6 gCOD/(L.d) (HRT 0.04 d), decreased the aggregation indicator to 62 ± 31. Lowering the OLR to 2.0

gCOD/(L.d) (HRT 0.3 d), on the other hand, drastically decreased the aggregation indicator to 10 ± 1. Similar trends were observed by Driessens et al. (1987), where an increase in OLR from 6.1 gCOD/(L.d) to 24.4 gCOD/(L.d) caused a decrease of the aggregation indicator from 26 to 16, using a similar reactor setup and feed. The sedimentation flux obtained by Driessens et al. (1987) at 6.1 gCOD/L was, however, substantially lower (2 kgTSS/(m^2.h)) than what was achieved in this research (5.9 kgTSS/(m^2.h)).

Overall, COD removal efficiencies of 90 ± 1% were obtained. Only at the highest OLR of 14.6 gCOD/(L.d) (HRT of 0.04 d), the COD removal efficiency decreased to 82 ± 6%, probably due to overloading of the system at shorter HRT (Alloul et al., 2019).

The effect of limited process parameters on PNSB aggregation has been studied before. Apart from the research of Driessens et al. (1987), the influence of OLR and HRT on aggregation of PNSB have not been studied. Stegman et al. (2021), testing different upflow rates, reached an SRT/HRT ratio (aggregation indicator) of 15 in an upflow reactor, lower than what was achieved in this research. The lower biomass retention may be the result of higher hydrodynamic shear due to the higher upflow velocity applied in the reactor (up to 9 m/h) (Tiwari et al., 2006). A sequencing batch reactor with increasing OLR and decreasing HRT has also been used to aggregate enriched PNSB, resulting in biomass with a sedimentation flux of 4.7 kgVSS/(m^2.h) (Cerruti et al., 2020). In the studies of Cerruti et al. (2020) and Stegman et al. (2021) however, both the OLR and HRT changed throughout the experiment, and the biomass aggregation was only quantified at the end of the experiment, not at different OLR of HRT.

For UASB reactors, it has been shown that HRT impacts the performance, yet the organics to nitrogen ratio, micronutrients content, shear, upflow velocity, and type of microorganisms are also crucial for aggregation (Tiwari et al., 2006). Typical HRT used in UASB systems varies from 0.13 to 3 d with upflow rates ranging between 0.1 and 2 h/m (Khan et al., 2011; Latif et al., 2011). The OLR has also been shown to be an important factor for aggregation in UASB reactors, as overloading the system can lead to the accumulation of volatile fatty acids, due to activity of acidogenic fermentative microorganisms, which can lower the reactor pH and negatively impact aggregation. UASB systems are therefore typically operated at an OLR between 2 to 4.5 gCOD/(L.d) (Tiwari et al., 2006). In this research, a high OLR did not impact the pH, but concomitant shorter HRT may play a role in the reduced aggregation. Aerobic granular sludge favors OLR between 0.5-10 gCOD/(L.d) and COD/N ratios between 2-30 gCOD/gN to maintain stable aggregates. Lower OLR or higher COD/N ratios can cause disaggregation due to filamentous overgrowth. The HRT in these systems varies between 0.17-1.0 d, as a consequence of the slow-growing microorganisms associated with aerobic granular sludge (de Sousa Rollemberg et al., 2018).

3.1.2 Improving aggregation at long hydraulic retention time in presence of growth metabolites

The previous section (3.1.1) showed that a relatively longer HRT (0.3 d) coupled to a high OLR (14.6 gCOD/(L.d)) decreased aggregation of *Rb. capsulatus* biomass in an anaerobic upflow reactor (Figure 2). In this section, only the effect of a long HRT was examined by decoupling the OLR and HRT. The results in Figure 3A show that a relatively longer HRT (while maintaining a constant OLR), results in a decline of the aggregation indicator (SRT/HRT ratio) from 12 to 3 and COD removal efficiency from

97% to 87 %, indicating a decrease in biomass aggregation in the reactor. To avoid disaggregation and washout of the biomass, tannic acid, a biological flocculant (Ge et al., 2019; Wu et al., 2020), was added to the influent, which lead to a gradual recovery and an increase of the aggregation indicator to 23 and COD removal efficiency to 91%. These insights indicate that HRT control is essential for optimal aggregation. Similar trends have been reported for aerobic flocs and aerobic granules, where a prolonged HRT negatively affected the production of extracellular polymeric substances and aggregation (Pan et al., 2004; Rosman et al., 2014; Trebuch et al., 2020).

The disaggregation at relatively long HRT was probably caused by the accumulation of metabolites that hinder aggregation. This phenomenon is also observed in other systems, where microalgae excrete organic matter, consisting out of a wide range of polysaccharides, proteins, nucleic acids and more, which causes disaggregation of the sludge bed (Pivokonsky et al., 2016). To explore whether accumulation of growth metabolites caused disaggregation at high HRT, a pasteurization step was included in the recirculation, since it was postulated that proportion of metabolites may be heat-labile.

Pasteurization at 60 °C, 60-65 °C and 70-80 °C improved the aggregation indicator from 3 to 6, 13 and 19 respectively, suggesting that the presence and accumulation of heat-labile metabolites in the medium at higher HRT negatively impact aggregation. The nature of the metabolites requires to be further determined, but these observations indicate the importance of short HRT for aggregation of PNSB.

To study the effect of PNSB metabolites furthermore, supernatant from batch cultures was fed in the reactor. After addition of supernatant, the aggregation indicator

(SRT/HRT ratio) abruptly decreased in the reactor (Figure 3B). Supernatant from 3-4 day old batch cultures showed a higher decrease in the aggregation indicator (from 21 to 5) than supernatant from 2-3 day old batch culture (aggregation indicator of 12). Aggregation was reestablished when the reactor was switched to the original feed (data not shown). Autoclaving the supernatant reversed this negative effect, improving aggregation and increasing the aggregation indicator to 115. These observations indicate the presence of a heat-labile metabolite in batch *Rb. capsulatus* cultures with the potential to cause disaggregation.

3.2 Wastewater characteristics influence aggregation

To study the influence of wastewater composition on aggregation, different COD/N ratios were tested (section 3.2.1) and the nitrogen source was changed from ammonium to glutamate (section 3.2.2).

3.2.1 Influence of the chemical oxygen demand to nitrogen ratio

The composition of wastewater can vary depending on the source (Muys et al., 2020) These differences create an imbalance in nutrient availability, which can cause a shift in the metabolism and biomass composition (e.g. polyhydroxyalkanoate production at high COD/N and polyphosphate accumulation at low COD/N) (Capson-Tojo et al., 2020; Hiraishi and Kitamura, 1985). The impact of the COD/N ratio on PNSB aggregation is, however, unknown. Therefore, investigating this factor creates a first step in the transition to real wastewater. Both decreasing as increasing the COD/N ratio showed improvement in the aggregation indicator (SRT/HRT ratio, 65 at 6 gCOD/gN, 47 at 12 gCOD/gN, 75 at 24 gCOD/gN). These findings were also confirmed with the sedimentation fluxes (Figure 4A), making the COD/N ratio an important factor in PNSB aggregation as well. For growth, an optimal influent COD/N around 16 gCOD/gN has been found to prevent carbon or nitrogen limitations (Hülsen et al., 2014). Further research should, however, clear out how this relates to the aggregation of PNSB. Wastewater streams from plant-based food processing have overall a high COD/N ratio (Verstraete et al., 2016), however, a pre-fermentation step can lower the COD content (Alloul et al., 2018).

3.2.2 Aggregation with organic nitrogen

Apart from the COD/N ratio, the type of nitrogen source can also vary in wastewater, yet the effect of nitrogen source has not been studied for PNSB aggregation. Only ammonium has, thus far, been used as a nitrogen source (Cerruti et al., 2020; Driessens et al., 1987; Stegman et al., 2021). PNSB metabolize both inorganic and organic nitrogen sources, such as glutamate and yeast extract (Imhoff, 2006). The nitrogen source was, therefore, changed to glutamate as an initial attempt to mimic the complexity of real wastewater. At first, the aggregation index decreased at a COD/N ratio of 35 gCOD/gN, indicating a disaggregation, however, the aggregation index increased when the COD/N ratio was decreased to 22 gCOD/gN, restoring the aggregation (Figure 4B). These trends were contrary to the results with ammonium (section 3.2.1), where aggregation improved at a COD/N ratio of 24 gCOD/gN (Figure 4A). Aggregation with the organic nitrogen source was, consistently, inferior to aggregation with inorganic nitrogen, as the sedimentation flux was 5.6 kgTSS/(m^2.h) for ammonium at 24 gCOD/gN, and only 1.2 kgTSS/(m^2.h) for glutamate at 22 gCOD/gN. The effect of organic nitrogen demands further investigation by selecting different N sources (e.g., urea) and mixing inorganic and organic nitrogen, to mimic real wastewater.

3.3 Future perspectives

Although certain operational approaches to affect PNSB aggregation have been explored, some parameters have not been studied. The temperature of the incoming wastewater, for example, can vary depending on the type and treatment stage. Furthermore, for UASB systems, the pH has an impact on aggregation (Tiwari et al., 2006), yet only a constant temperature was used in this experiment, as well as a constant influent pH. Both temperature and pH, however, can affect flocculation of photosynthetic bacteria (Lu et al., 2019) and could influence aggregation in the upflow reactor. Apart from operational tools, more research needs to be done on scale-up of the upflow photobioreactors, as sufficient illumination is necessary for phototrophic growth. Granulation of the biomass enables more light penetration into the reactor, compared to non-aggregated cells in suspension (Fradinho et al., 2021), however, only the biomass on the surface of the aggregates is illuminated. The research of Cerruti et al. (2020) shows that the microbial community is homogenous throughout the granules due to the fast growth of PNSB, yet, nothing is known on the impact on the distribution of the microbial community in the granules over a longer period.

4 Conclusions

The PNSB model organism *Rb. capsulatus* showed the best aggregation in an upflow photobioreactor at OLR of 6.1 gCOD/(L.d) and HRT of 0.1 d, reaching a sedimentation rate of 5.9 kgTSS/(m^2.h). Increasing the HRT did not improve aggregation, possibly due to the accumulation of heat-labile metabolites interfering with aggregation. Results indicate that wastewater streams with inorganic nitrogen and either high or low COD/N ratios are better suited for aggregation. In addition, wastewater with a low COD content is preferred, as this allows to maintain a short HRT without overloading the system.

E-supplementary data for this work can be found in e-version of this paper online

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38.

Figure captions

Table 1. Objectives and operational conditions for aggregation in a non-axenic upflow photobioreactor inoculated with a pure *Rhodobacter capsulatus* ATCC 23782 culture. HRT: hydraulic retention time, OLR: organic loading rate, COD: chemical oxygen demand.

Figure 1. Configuration of the anaerobic upflow photobioreactor for aggregation of purple non-sulfur bacteria, with an illuminated volume of 440 mL, and a decanter on top. The reactor was inoculated with a pure culture of *Rhodobacter capsulatus* ATCC 23782 and operated non-axenically.

Figure 2. Conversion, aggregation and sedimentation features in an anaerobic upflow photobioreactor inoculated with *Rhodobacter capsulatus* ATCC 23782. Panel A. Influence of organic loading rate (OLR) on the aggregation indicator (sludge to hydraulic retention time ratio SRT/HRT) and chemical oxygen demand (COD) removal efficiency. OLR was controlled by varying the HRT. The decrease of the aggregation index at day 5 is due to a measurement error. Panel B. The sedimentation fluxes of aggregated biomass at HRT 0.1 d and HRT 0.04 d, determined for different dilutions of aggregated biomass (no settling observed at HRT of 0.3 d).

Figure 3. Conversion and aggregation features in an anaerobic upflow photobioreactor inoculated with *Rhodobacter capsulatus* ATCC 23782. Panel A. Influence of a long hydraulic retention time (HRT 1 d) and addition of tannic acid on the aggregation indicator (sludge retention time to hydraulic retention time ratio, SRT/HRT) and chemical oxygen demand (COD) removal efficiency of aggregated biomass. Panel B.

Influence of addition of supernatant on the aggregation indicator and chemical oxygen demand (COD) removal efficiency of aggregated biomass.

Figure 4. Sedimentation, conversion and aggregation features in an anaerobic upflow photobioreactor inoculated with *Rhodobacter capsulatus* ATCC 23782. Panel A. The sedimentation fluxes of aggregated biomass at COD/N ratio of 6, 12 and 24 gCOD/gN with ammonium as nitrogen source, determined for four different dilutions of aggregated biomass. Panel B. Influence of an organic nitrogen (glutamate) on the aggregation indicator (sludge retention time to hydraulic retention time ratio SRT/HRT) and chemical oxygen demand (COD) removal efficiency grown at COD/N ratios of 35 and 22 gCOD/gNwith glutamate as nitrogen (and partial COD) source.

Tables and figures

Table 1.

Objective	*HRT (d)*	*OLR (gCOD/(L.d))*	*COD/N (gCOD/gN)*	*Nitrogen source*	*Rem*
Influence of OLR and HRT	0.04 0.1 0.3	14.6 6.1 2.0	12	Ammonium	
Aggregation at long HRT	1	12.2 6.1	12	Ammonium	Addi Paste
Influence of COD/N ratio	0.1	3.0 6.1 12.2	6 12 24	Ammonium	

Influence of N source	0.1	8.6 11.1	22 35	Glutamate	
Influence of metabolites	0.1	12.2	12	Ammonium	Addi batc

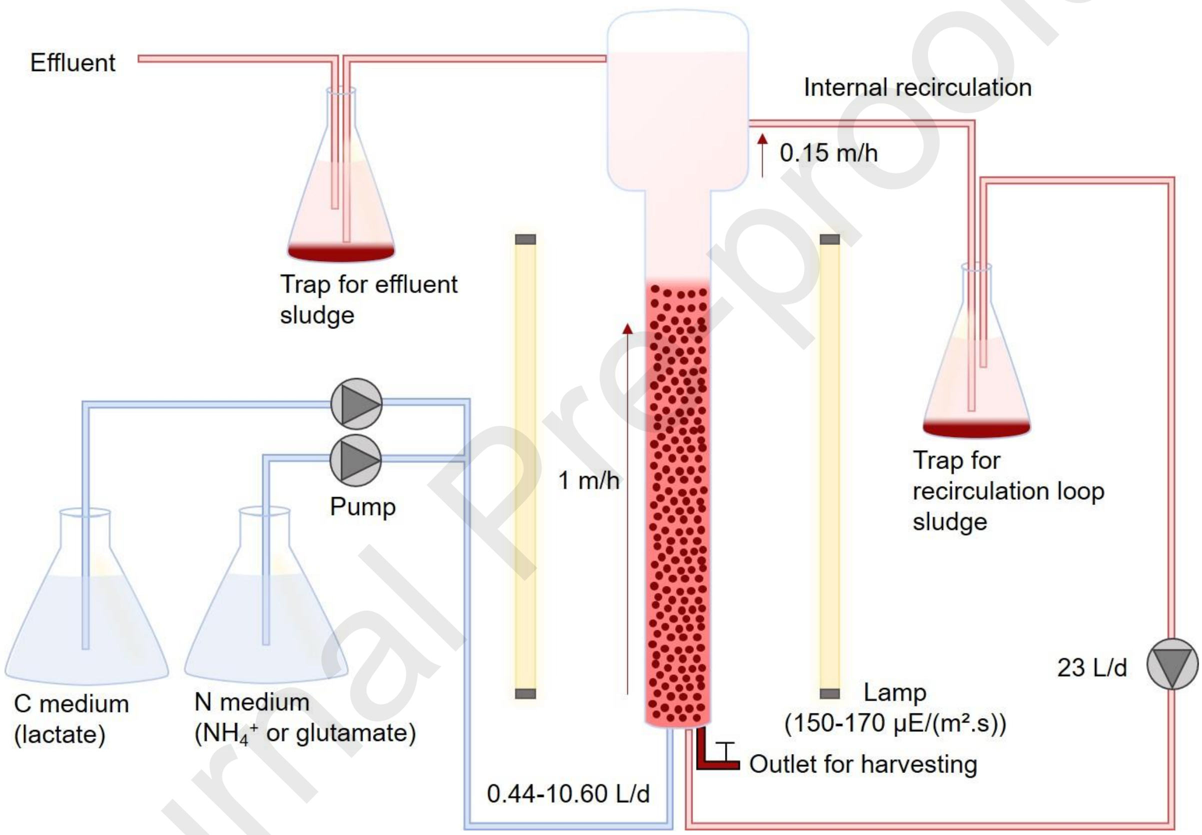


Figure 1

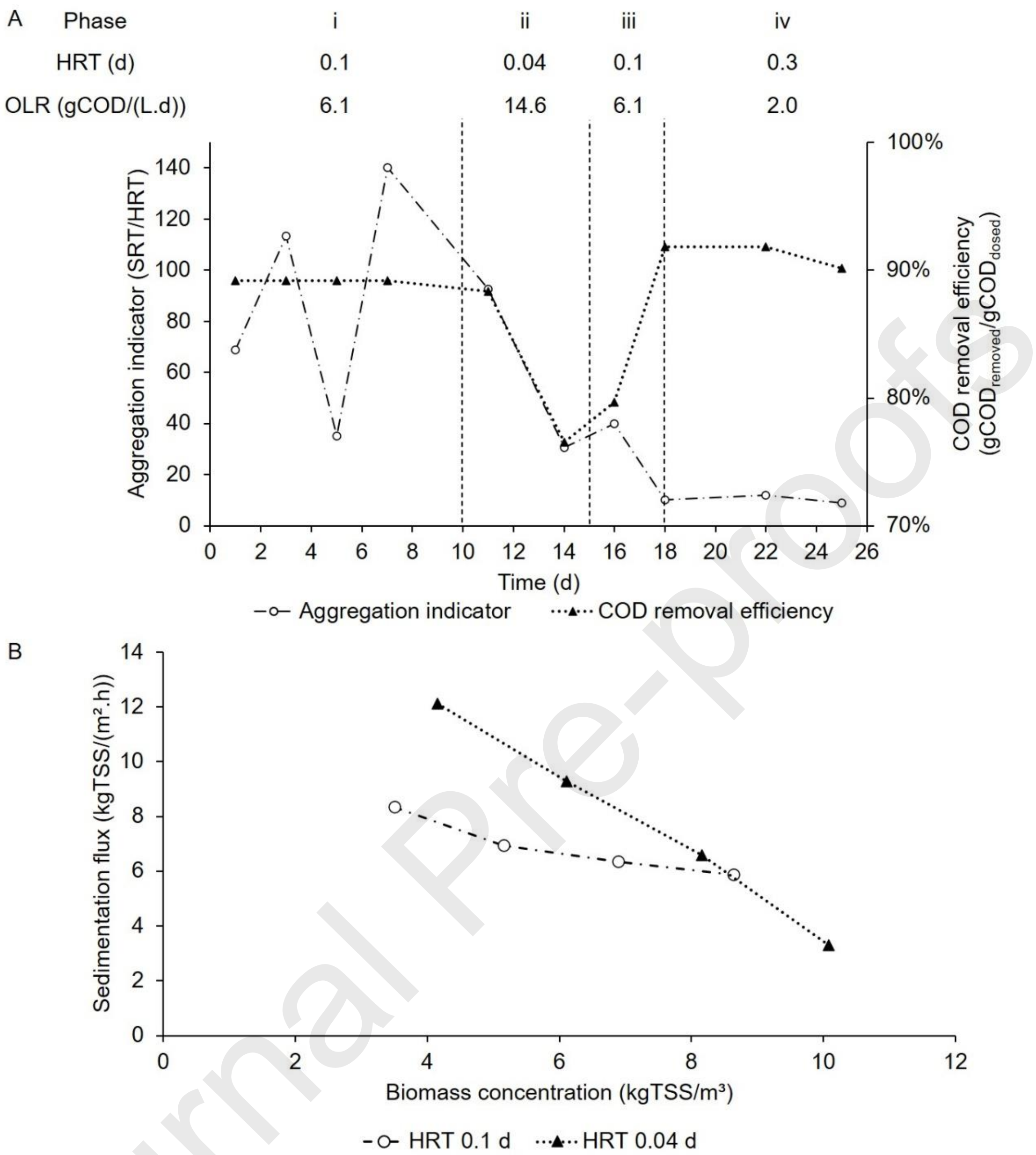

A
Phase
i
ii
iii
iv
HRT (d)
0.1
0.04
0.1
0.3
OLR (gCOD/(L.d))
6.1
14.6
6.1
2.0
Aggregation indicator (SRT/HRT)
COD removal efficiency (gCOD$_{removed}$/gCOD$_{dosed}$)
Time (d)
Aggregation indicator
COD removal efficiency
B
Sedimentation flux (kgTSS/(m².h))
Biomass concentration (kgTSS/m³)
HRT 0.1 d
HRT 0.04 d

Figure 2

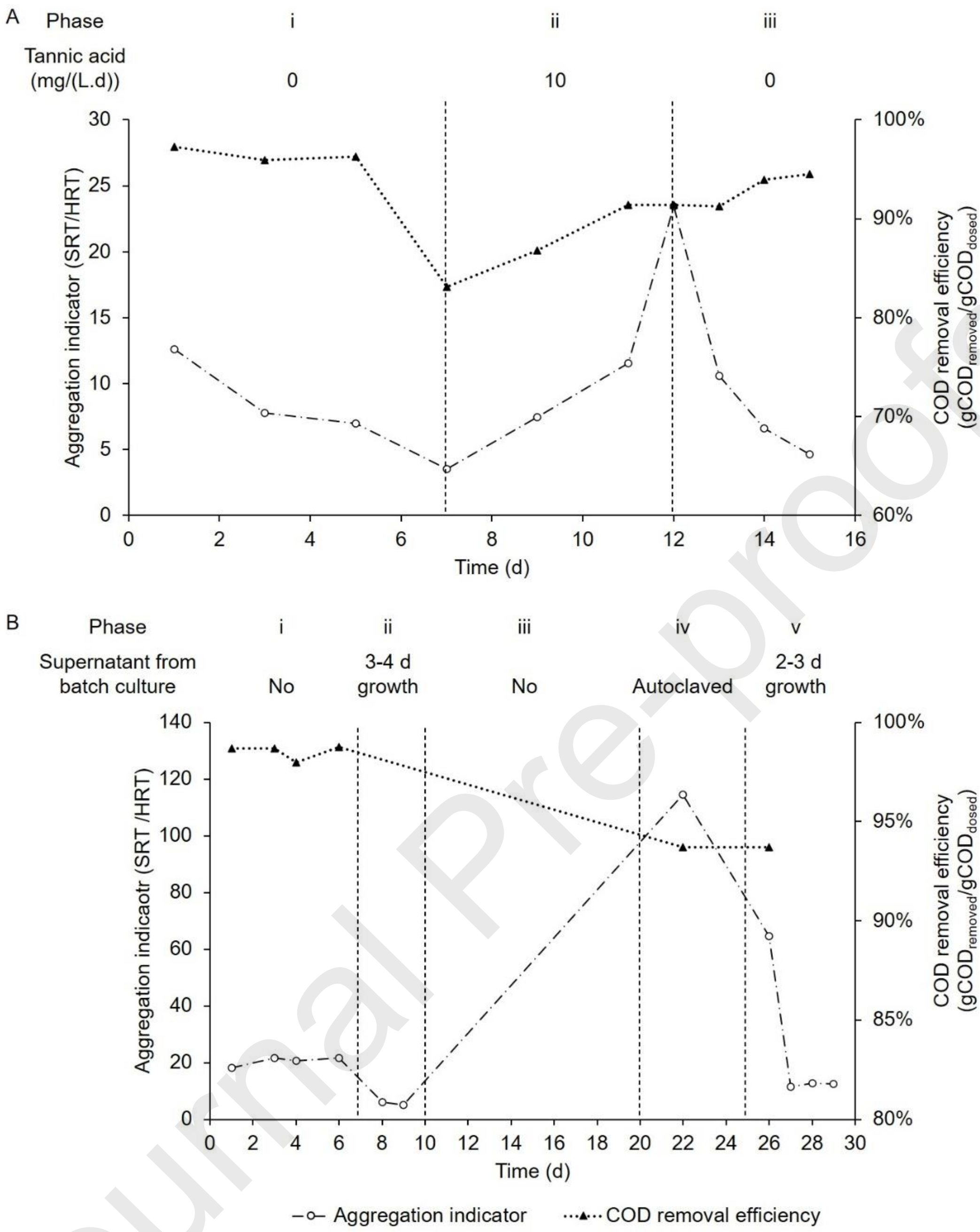

A
Phase
i
ii
iii
Tannic acid
(mg/(L.d))
0
10
0
Aggregation indicator (SRT/HRT)
COD removal efficiency
(gCODremoved/gCODdosed)
Time (d)
B
Phase
i
ii
iii
iv
v
Supernatant from
batch culture
No
3-4 d
growth
No
Autoclaved
2-3 d
growth
Aggregation indicaotr (SRT /HRT)
COD removal efficiency
(gCODremoved/gCODdosed)
Time (d)
Aggregation indicator
COD removal efficiency

Figure 3

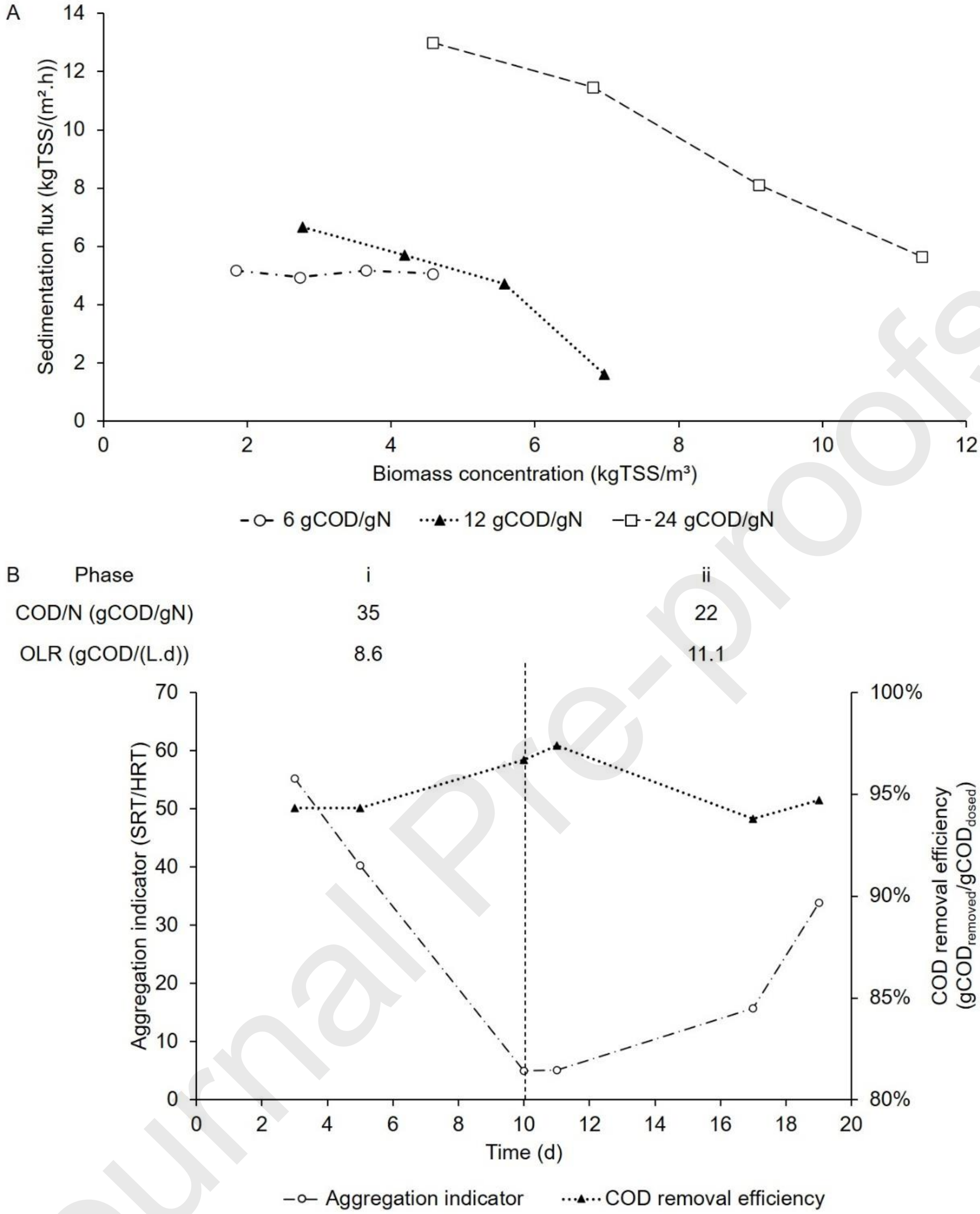


Figure 4

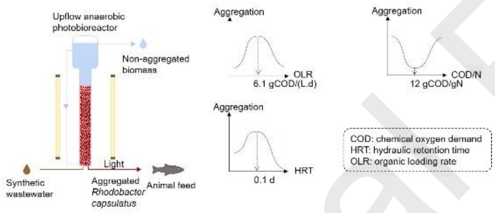
Naïm Blansaer: Writing – Original draft, Writing – Review & editing, Visualization, Project administration **Abbas Alloul:** Writing – Original draft, Writing – Review &

editing **Willy Verstraete:** Conceptualization, Supervision **Siegfried E. Vlaeminck:** Writing – Review & editing **Barth F. Smets:** Conceptualization, Investigation

Declaration of interests

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



- *Rhodobacter capsulatus* grew in aggregates in an upflow photobioreactor
- Optimal loading rate and hydraulic retention time were 6.1 gCOD/(L.d) and 0.1 d
- The fastest settling aggregates had a sedimentation flux of 5.9 kgTSS/(m^2.h)
- The ratio COD/nitrogen ratio and the nitrogen source impacted aggregation
- *Rb. capsulatus* may produce metabolites inducing disaggregation