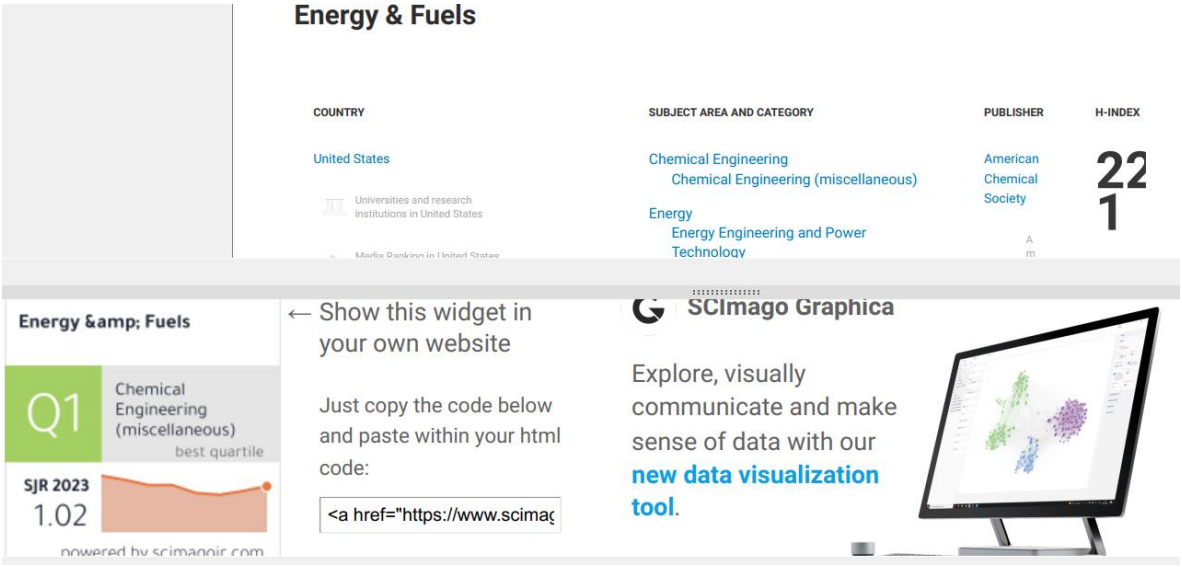

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Author(s): Abimbola, Tobi; Christodoulatos, Christos; Lawal, Adeniyi

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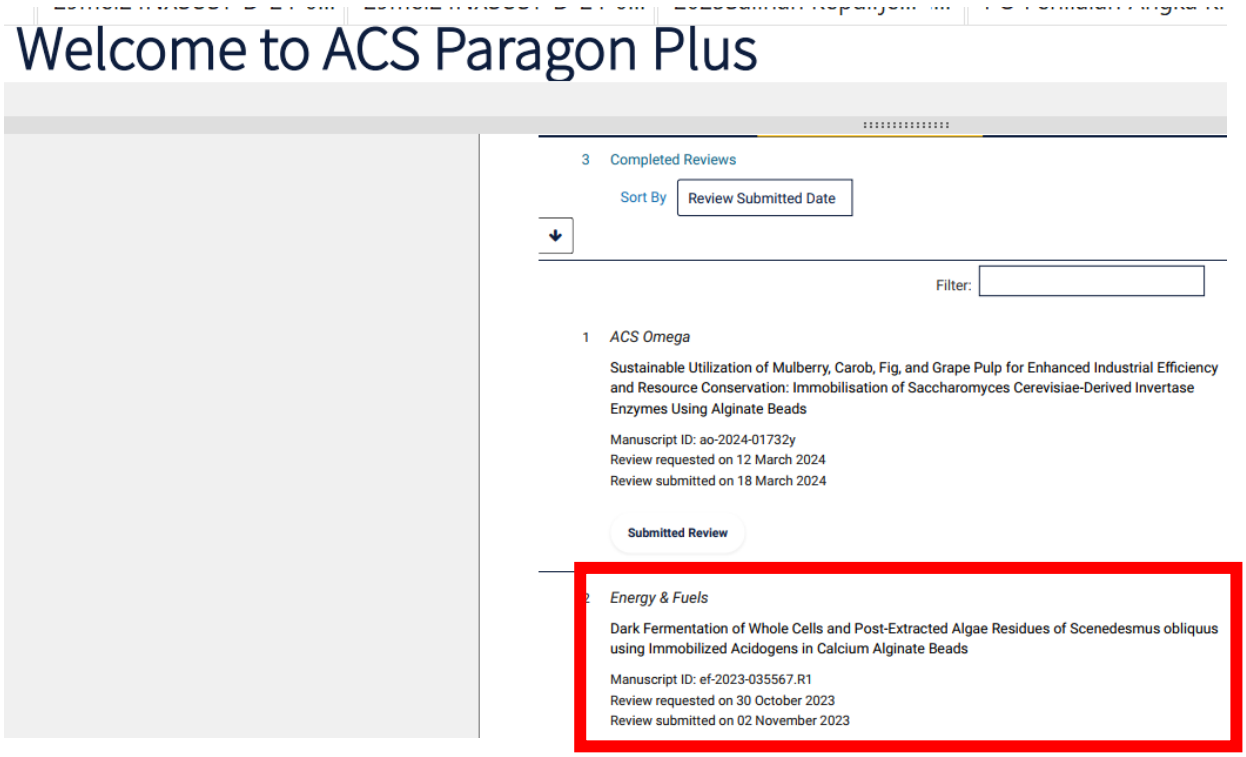

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Manuscript Type:	Article
Date Submitted by the Author:	22-Sep-2023
Complete List of Authors:	Abimbola, Tobi; Stevens Institute of Technology, Chemical Engineering Christodoulatos, Christos; Stevens Institute of Technology, Civil and Environmental Engineering Lawal, Adeniyi; Stevens Institute of Technology, Chemical, Biomedical, and Materials Engineering

Dark Fermentation of Whole Cells and Post-Extracted Algae Residues of *Scenedesmus obliquus* using Immobilized Acidogens in Calcium Alginate Beads

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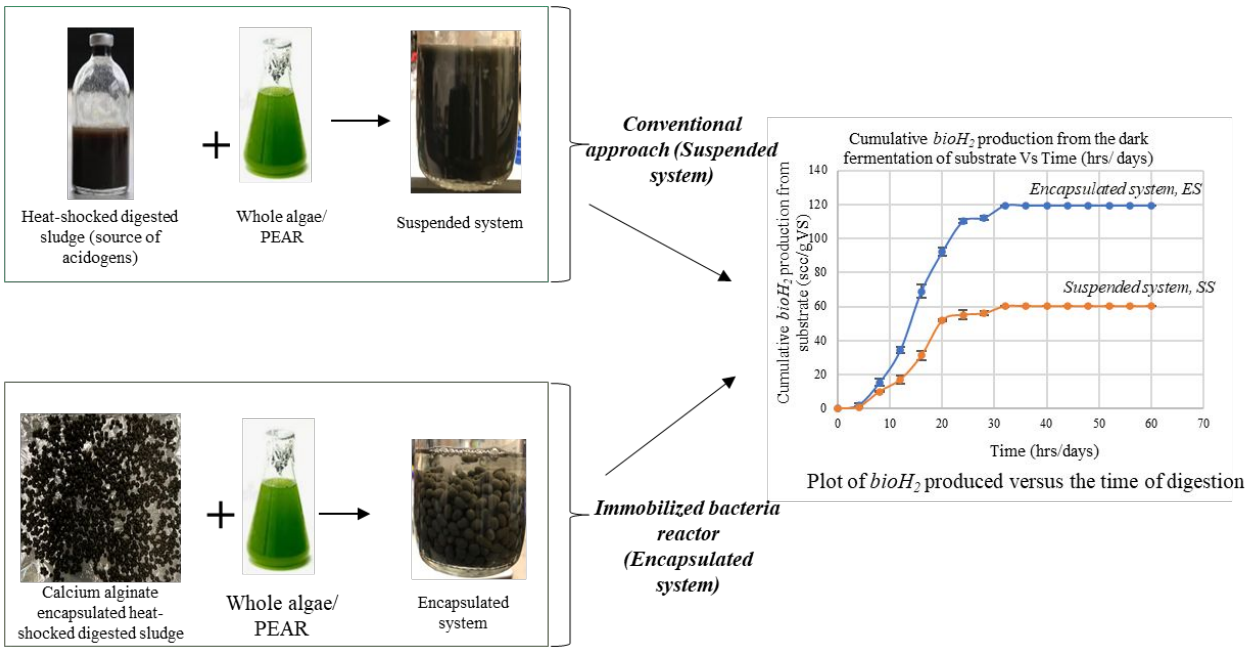
Abstract

In this work, dark fermentation (DF) of alga and post-extracted algae residue (PEAR) of *Scenedesmus obliquus* is performed in an immobilized batch fermenter using heat-shocked-digested sludge (DS) encapsulated in calcium alginate, for the first time. Encapsulation parameters at which stable beads are formed were optimized using glucose as a model compound. Microbes encapsulated in beads formed at 2% sodium alginate produced biohydrogen with over 25% more than the yield from conventional fermenters, digesting alga and its PEAR. Reuse of immobilized microbes was demonstrated with 60% efficiency compared to the performance of fresh microbes while digestate after DF was recovered for further processing into other bioenergy forms such as biomethane (a downstream process impossible with the conventional suspended fermenters). This study demonstrates a clean bioenergy approach in which the biohydrogen produced can be used as a sustainable H_2 source for green-diesel production while higher bioH_2 yields make alga-to-biofuel production more economically attractive.

Keywords: Dark fermentation, Microalgae, Post-extracted algae residue, Calcium alginate encapsulation, Heat-shocked digested sludge.

TOC Graphic


Heat-shocked digested sludge (source of acidogens)
Whole algae/ PEAR
Suspended system
Conventional approach (Suspended system)
Calcium alginate encapsulated heat-shocked digested sludge
Whole algae/ PEAR
Encapsulated system
Immobilized bacteria reactor (Encapsulated system)
Cumulative $bioH_2$ production from the dark fermentation of substrate Vs Time (hrs/ days)
Cumulative $bioH_2$ production from substrate (scc/g VS)
Encapsulated system, ES
Suspended system, SS
Time (hrs/days)
Plot of $bioH_2$ produced versus the time of digestion

1. Introduction

Microalgae assimilate CO_2 during photosynthesis for growth while producing biomass that can be converted to bioenergy. They uptake nutrients such as nitrogen and phosphorus, enabling their application in wastewater bioremediation (Carvalho Júnior et al., 2011). They are characterized by a short generation time, multiplying exponentially under favorable environmental conditions (Buono et al., 2016). Microalgal biomass can be used for several applications ranging from bioenergy to pharmaceutical applications. They accumulate high amounts of lipids that can be extracted, recovered, and processed into biodiesel via transesterification or hydrotreated into hydrocarbons. The biomass residue after extracting oil can be utilized for bio-oil, ethanol or methane production (Wang et al., 2013). These attributes made microalgae favorable for carbon capture technologies and bioenergy production. Thus, as part of the efforts to provide more research insights on the diverse applicability of algae to sustainable energy, the whole alga of *Scenedesmus obliquus* and its post-extracted residue (PEAR) after oil extraction are explored as biomass or substrates for biohydrogen ($bioH_2$) generation in this current study.

Hydrogen (H_2) is considered one of the most favorable alternatives for sustainable energy because of its high conversion efficiency to usable power, non-polluting oxidative products, and high gravimetric energy (Elsharnouby et al., 2013). Hydrogen has a caloric content of about 120MJ/Kg, which is more than twice the energetic value of either natural gas or gasoline. In addition to that, the only product of combustion generated from H_2 is water hence, no greenhouse gas is released into the atmosphere using H_2 as fuel (Cheng & Liu, 2011). At present, H_2 is produced from non-renewable sources, such as oil, natural gas and coal. Predominantly, H_2 is synthesized commercially from autothermal reforming (ATR), partial oxidation or steam reforming of methane (SRM) (Chen et al., 2023). Although it was predicted that H_2 could be synthesized from steam reforming at $7/GJ, the

emission of CO_2 from this process is detrimental to the environment, making it less attractive for industrial application (El-Emam & Özcan, 2019). Therefore, utilizing algae (that require CO_2 to thrive) and implementing dark fermentation is more eco-friendly and if the process parameters are optimized, can be more economically feasible.

Dark fermentation (DF) is a method of biohydrogen ($bioH_2$) production that involves digesting suitable biomass such as algae in an environmentally friendly fashion. Biologically, $bioH_2$ can also be produced from photo-fermentation but $bioH_2$ yields adopting dark fermentation are more promising and its operation is simpler (Elsharnouby et al., 2013). The first three stages of anaerobic digestion (AD) are similar to the stages involved in the DF process (namely, hydrolysis, acidogenesis, and acetogenesis). However, for anaerobic digestion, acetogenic bacteria transform the products from the acidogenesis stage into either acetate or a mixture of CO_2 and H_2. The hydrogen produced from the acetogenesis is consumed by the methanogenic bacteria. According to the literature, acetogenesis reactions are not thermodynamically spontaneous if the partial pressure of H_2 is higher than 10^{-4} atm (Uddin & Wright, 2022). This is the reason why it is better to perform dark fermentation in a semi-continuous or continuous fashion to remove the biohydrogen as it is produced to avoid too much accumulation. Pure hydrogen-forming bacteria such as *Clostridium beijerinckii* or a mixed culture of digested sludge collected from a wastewater processing facility containing a consortium of microbes can be used as a source of acidogenic microbes to implement DF. To make the digested sludge rich in hydrogen-producing bacteria, methanogenic activity is suppressed by adopting pretreatment strategies such as pH and heat shock (Wang et al., 2011). Most studies focused on the utilization of pretreated sludge to produce $bioH_2$ using the conventional suspended system approach (Singh et al., 2013b). However, in the continuous operation of suspended microbes, challenges associated with biomass washout at high dilution rates and inseparability of the digestate from the spent bacteria for

further processing have been identified. Cell immobilization has been reported to be a suitable remedy to these challenges (Singh et al., 2013a). Hence, in this study, bioH_2 synthesis from the DF of alga and its PEAR was performed in a batch reactor filled with immobilized acidogens to recover the digestate after DF for further processing into bioCH_4 via 2-stage AD as proposed by (Abimbola et al., 2023)

The use of immobilized bacteria cells for DF can lead to a reduction in the cost of production as a result of repeated use of the immobilized cells, increased cells-to-substrate ratio, protection of the cells in the immobilized environment, and improved productivity (Gotovtsev et al., 2015). Encapsulation is one of the methods of cell immobilization and it involves the enclosure of biocatalysts in an aqueous medium inside a semi-permeable membrane (Blandino et al., 1999). Calcium alginate gel is an example of a hydrogel widely applied. Of all of the different biopolymers utilizable in the formation of semipermeable membrane gels/capsules, alginate is the most frequently used because calcium alginate gels can be formed under mild conditions (Jen et al., 1996). The gelling properties of alginate depend on its monomeric composition, block structure, molecular size and concentration of polymer and calcium ions used (Ouwerx et al., 1998). Therefore, to deploy encapsulated acidogens for DF, it is imperative to establish the best conditions for encapsulation and how they impact the bioH_2 yield from digesting substrates.

This study is aimed at comparing the performance of the conventional DF fermenters with the immobilized bacteria reactor system for digesting alga and its PEAR, adopting the calcium encapsulation strategy. The use of encapsulated acidogens to disintegrate either alga or its PEAR to generate bioH_2 has not been reported anywhere else in the literature. Specifically, the approach in this study considers the use of PEAR generated after stripping algae of oil using an optimized hexane extraction method reported recently by Abimbola et al, 2021, where it was anticipated that digesting

the PEAR can improve the performance of the alga-to-biofuel pathway proposed. Also, reports have shown that the immobilization of microbes leads to an increase in biogas productivity, but challenges associated with mass transfer resistance are often faced (Sigurdardóttir et al., 2018). Thus, in this work, the conditions (concentration of sodium alginate, mixing speed and digestion time) that may influence the bioH_2 yield from the digestion of substrates while retaining stable encapsulated microbes were investigated. Preliminary tests were performed using glucose as a model substrate to observe the stability of the encapsulated microbes. Subsequently, encapsulation and digestion parameters were implemented to digest whole alga and PEAR of *S.obliqqus* while establishing the best conditions for bioH_2 yield. This study demonstrates a circular approach of bioenergy utilization whereby bioH_2 produced from DF contributes to the H_2 requirement for hydrotreatment of algal oil, generated CO_2 is used to grow algae, recovered digestate can be processed further to generate biomethane via 2-stage AD (Abimbola et al., 2023), encapsulated bacteria can be re-used and the experimental data from this study can be used in the techno-economic analysis (TEA) of alga-biofuel technology.

2. Materials and Methods

2.1 Algae Cultivation and Harvesting

Abraham et al., 2018 recently performed an extensive study to explore different freshwater algal strains for cultivation in energetics-laden wastewater (with high nitrogen content) from energetics processing. Of all the algal strains investigated, *Scenedesmus obliquus* had the highest growth rate and was able to withstand the toxicity of the energetics-laden wastewater (Abraham et al., 2018). Based on the promising attributes of *S.obliquus* reported in the work by Abraham et al., 2018, it was selected for this study and cultivated in 100L raceways containing fresh water. Detailed growth conditions for *S.obliquus* can be found in Abraham et al., 2018. After the growth period,

harvested algal slurry at a very low concentration was centrifuged for 15 minutes at 3700 rpm using a Marathon 8k centrifuge purchased from Fisher Scientific. At this rpm, the potential stress that can be imposed on the algal cells is reduced, thus, keeping the cellular components that may be released into the supernatant during centrifugation within the algal cells until they are ready to be processed (Molina Grima et al., 2003).

2.2 Total solids and volatile solids determination, algal oil extraction and generation of post-extracted algae residue

After harvest, the total solids content of algae is estimated while the amount of bioH_2 produced from either whole algae or PEAR after AD is reported per gram of volatile solids. Thus, both total solids (TS) and volatile solids (VS) content were determined using the procedure described in (Abimbola et al., 2021). Hexane extraction was performed on acid-hydrolyzed algal slurry and the downstream separation of the lipids was performed in a separating funnel also as described in (Abimbola et al., 2021). The PEAR was recovered as a by-product and preserved for digestion.

2.3 Collection, degassing and pretreatment of digested sludge.

Digested sludge (DS) containing a consortium of anaerobes was collected from a secondary anaerobic digester operated at 35℃ located in Bergen County Utility Authority (BCUA), Little Ferry, New Jersey. Degassing is performed each time before the DF experiment to ensure that the aqueous carrier for the microbes in the DS does not contain an organic load that can be digested. To degas the digested sludge, residual solids are removed by filtering the DS with a sieve of 2mm opening. If these solids are not removed, they may impede the DF process by decreasing the rate of mass transfer between the substrates and the microbes. At a temperature of 35℃ and a mixing speed of 150 rpm, the filtered DS is allowed to acclimatize under anaerobic conditions to release biogas from the digestion of the residual solids remaining after filtering. The biogas released is quantified using a

water displacement method by monitoring the level of displaced acidic water as a result of the accumulation of biogas. In most experiments, degassing took about 5-7 days. Before using the degassed DS as a bacteria source, the TS and VS of the DS are determined following the procedure in section 2.2.

To pretreat the DS to suppress methanogens, either heat shock or pH control was adopted in this study. To perform heat shock, a Bottom Stirred Autoclave Reactor operated at 150rpm was used to induce a temperature shock at 90℃ for 15mins while for pH control, a handheld mixer was used to thoroughly homogenize digested sludge in a beaker while adding sulfuric acid dropwise until a pH of 5.5 is reached. Other methods explored are briefly explained in the discussion section.

2.4 Encapsulation of bacteria cells in calcium alginate beads

A known mass of sodium alginate (Bio grade 90-106%, product #: JK603390) is first mixed with pretreated digested sludge to give a homogeneous suspension with concentration varying from 2 to 6% w/v. The mixture of sodium alginate and microbes is then withdrawn into a 50mL syringe and with the aid of a syringe pump, the mixture is extruded at 5mL/min into a solution of 0.15M calcium chloride. Beads (of about 3mm in size) encapsulating the bacteria are generated instantaneously. Afterwards, the beads encapsulating the microbes are filtered, washed with DI water, and stored in DI water until they are ready for use. To examine the surface morphology of the beads, a good approach is employing scanning electron microscopy (SEM). However, this method requires that the beads be pretreated using either lyophilization, critical point drying or cryo-preparation. Applying any of these pretreatments can potentially shrink the beads by about 50% (Walker et al., 2003). Thus, for this study, SEM imaging of the gels was not performed because the images obtained would not be an accurate representation of the soft calcium alginate gels.

2.5 Anaerobic fermenter for dark fermentation of substrates

The batch fermenter is a 245mL capacity microcosm bottle equipped with a rubber stopper to prevent leakage of produced biogas under the maximum proof pressure of 50psig. The working volume of the microcosm bottle is 90mL while the remaining 155mL serves as the headspace to trap produced biogas. In each fermenter, the substrate and sieved digested sludge (immobilized or suspended) are mixed such that the volatile solid (VS) ratio of the DS to the substrate is 2:1. Several authors reported 2:1 as the optimal ratio at which bioH_2 was maximized (Pellera & Gidarakos, 2016). Argon gas is used to purge each fermenter for at least 5 minutes to create an anaerobic environment and then the reactors are tightly covered and arranged in a shaker controlled at a temperature of 37°C and a shaker speed of 150rpm. In all experimental runs, a control sample containing only the bacteria source was prepared to estimate the actual biogas produced from digesting the substrate by subtracting the biogas produced from the control sample from the tested samples. The loading rate of either the whole alga or PEAR was 75g/L while the ratio of the TS to VS ranged between 1.1-1.5.

2.6 HPLC analysis for glucose

For experimental studies that involve the digestion of glucose, it is projected that there might be less than 100% conversion of glucose at the end of the DF process. Thus, a Shimadzu HPLC equipped with a refractory index (RI) detector and an Aminex HPX-87C column is used for glucose detection and quantification. The mobile phase is 0.005M sulfuric acid, while the mobile feed rate is set at 0.6 mL/min for good peak resolution to detect glucose.

2.7 GC analysis and biohydrogen quantification

The biogas produced in the fermenter is quantified following Equation 1 (all terms in Equation 1 are defined in Table 1). The calculated volume of biogas has a unit of standard cubic centimeters (scc)/g glucose or scc/gVS of alga or PEAR. The composition of biogas produced from all fermenters

is determined by injecting gas samples into a Shimadzu GC-14B-TCD equipped with Plot Q and Mole sieve columns in series. Argon is used as a carrier gas and the GC oven is operated isothermally at 35℃ for 15 minutes. Gas samples were manually introduced into the GC using a 10mL capacity airtight analytical syringe. The detector temperature was set at 150℃ while the injector temperature was set at 220℃.

$$V_{std} = \frac{\Delta P * V_{int} * T_{std}}{P_{std} * T_i} \quad (1)$$

Table 1: Definition of terms in Equation 1

Symbol	Definition (units)
ΔP	Gauge pressure of the headspace read on the manometer (psig)
T_i	Temperature at which the AD fermenters are operated, 308K (35°C)
V_{int}	The capacity of the headspace of the microcosm (~155mL)
P_{std}	Standard pressure (14.696 psia)
T_{std}	Standard temperature, 273K (0 °C standard temperature)
V_{std}	Volume of biogas produced at standard temperature and pressure (mL)

3. Results and Discussion

3.1 Preliminary study on the fermentation of glucose to establish stable encapsulation parameters.

3.1.1 Pretreatment condition for hydrogen-producing-rich digested sludge.

Although the use of pure microbes for biohydrogen has been reported by various authors in the literature as a good way to improve hydrogen yield and have better biokinetic control of the microbes, from a technical standpoint, a mixed culture is desirable because it can be deployed under non-sterile conditions. A mixed culture also encourages synergies between microbes (Elsharnouby et

al., 2013). However, there are disagreements on the optimal pretreatment conditions required for enriching hydrogen-producing bacteria in mixed cultures (see Table 3).

For this study, the use of chemical pretreatment was not explored to avoid incurring costs on the purchase of materials. As an initial test, the digestion of glucose using digested sludge pretreated with heat shock, pH load, and vacuum imposition was tested (Rajhi et al., 2015). Table 2 shows that all three pretreatment methods resulted in methanogenic suppression (based on the $bioH_2$ yield presented) while the *CIG-No pretreatment* gave a higher yield of biomethane because of the presence of active methanogens. Also, a combination of heat shock and pH load was used to pretreat the digested sludge but the amount of biogas produced was significantly low such that sampling was impossible (reduced bioactivity and consequently low biogas yield). Imposition of vacuum (*CIG vacuumed*) gave a substantial amount of $bioH_2$ (0.93 mol $bioH_2$/mol glucose consumed) from digesting glucose but resulted in sucking out the samples to be tested (a sign that implosion of the fermenter may occur) therefore, the method was abandoned and not used in subsequent experiments. Only heat shock and pH load were considered for further examination.

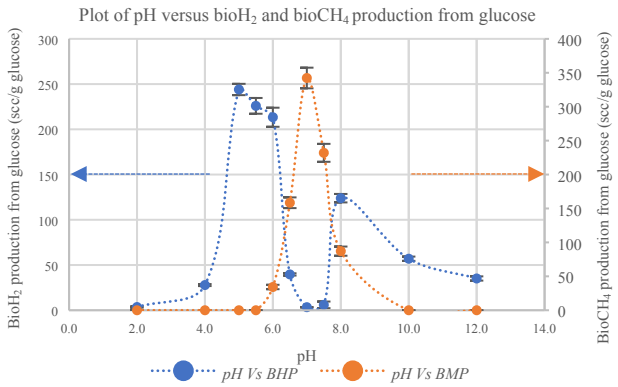
Table 2: *$BioH_2$* and *$bioCH_4$* generated from glucose to establish pretreatment methods that can suppress methanogens in digested sludge.

Sample #	Range of gas composition			Cum. total biogas (scc)	Cum. $bioCH_4$ (scc/g glucose)	Cum. $bioH_2$ (scc/g glucose)	mols of CH_4 produced	mols of CH_4/mol of glucose consumed	mols of H_2 produced	mols of H_2/mol of glucose consumed
	H_2 (%)	CH_4 (%)	CO_2 (%)							
CI (no pretreatment)	0	32-55	45-68	17.55	3.21	2.25	0.0001	0.026	0.0001	0.018
CI (Heat Shock-HS)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CI (low pH 5.5)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CIG (no pretreatment)	5-16	40-67	17-45	434.64	197.87	70.79	0.0088	1.592	0.0032	0.569
CIG (Heat Shock-HS)	27-40	5-35	25-68	407.02	10.11	177.92	0.0005	0.081	0.0079	1.431
CIG (low pH 5.5)	40-45	0-6	49-60	224.06	11.66	99.00	0.0005	0.094	0.0044	0.796
CIG (vacuumed)	12-40	10-56	14-78	312.87	60.00	115.47	0.0027	0.483	0.0052	0.929

**CI- Control sample containing only digested sludge without any added substrate at different pretreatment conditions in the parenthesis*

**CIG- Test sample containing 1g of glucose for digestion at different pretreatment conditions in the parenthesis*

To establish the optimum conditions specifically for the digested sludge used for this study, pH of the digested sludge was varied from 2.0 to 12.0 while the heat shock temperature varied from 80℃ to 100℃ with a step of 10℃. An initial study on pH control suggests that the maximum yield of $bioH_2$ can be produced from digesting glucose using digested sludge pretreated with a pH between 4.0 and 6.5. Thus, the step change in the pH between 4.0 and 6.5 was minimized to identify the local maximum for $bioH_2$ production. From Figure 1a, it can be observed that the maximum $bioH_2$ production from glucose is at a pH of 5.0 (244 scc $bioH_2$/g glucose; 1.96 mol H_2/mol glucose). At pH of 2.0 to 5.5, the methanogens were completely inhibited, and no methane was produced. Interestingly, at pH values where $bioH_2$ production diminish, the amount of $bioCH_4$ produced increased, and a maximum $bioCH_4$ production was attained at a pH of 7.0 (342 scc $bioCH_4$/g glucose; 2.75mol CH_4/mol glucose). On the basic side of the pH range, $bioH_2$ was produced at pH between 8.0-12.0 and $bioCH_4$ was reduced at these points indicating that $bioH_2$-producing bacteria outnumber the methanogens in the digested sludge.



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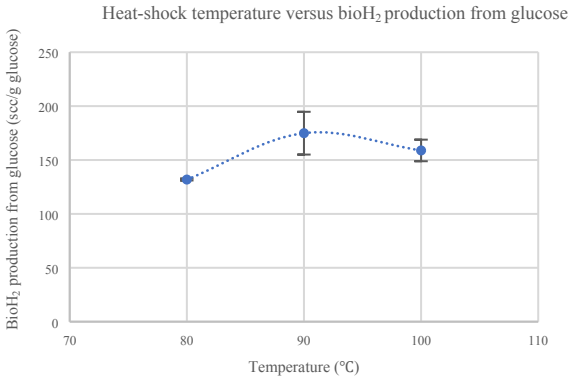


(b.)

Figure 1: Plot of (a.) bioH_2 and bioCH_4 production from glucose at different values of pH and (b.) bioH_2 production from glucose at different temperatures for heat shock for the pretreatment of the digested sludge.

Heat shock pretreatment parameters reported in the literature vary depending on the source of the bacteria and the kind of substrates digested with temperatures ranging from 80℃ to 121℃ and exposure times varying from 15 to 20 mins (Zhu & Béland, 2006). In this case, heat shock was used to treat the digested sludge at temperatures varied from 80℃ to 100℃ with a step of 10℃. Figure 1b shows the bioH_2 production from glucose at these temperatures and the maximum bioH_2 was produced at a temperature of 90℃ (174.89 scc bioH_2/g glucose; 1.41 mol H_2/mol glucose) whereas no bioCH_4 was produced at any of the temperatures tested.

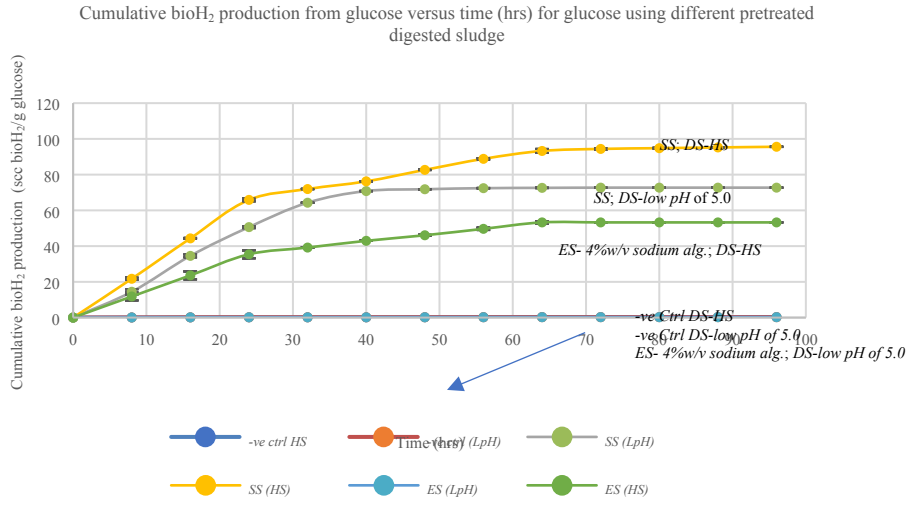
In conclusion, this study shows that the best pretreatment conditions to efficiently suppress methanogens in the digested sludge received for this study and maximize bioH_2 production from glucose are a pH of 5.0 for pH control and a temperature of 90℃ for heat shock pretreatment. These bioH_2 maxima in this study are obtained at pretreatment conditions within the range reported in the literature for heat shock and pH control (Kan, 2013) (see Table 3).

3.1.2 Pretreatment condition for digested sludge before encapsulation in calcium alginate for bioH_2 production

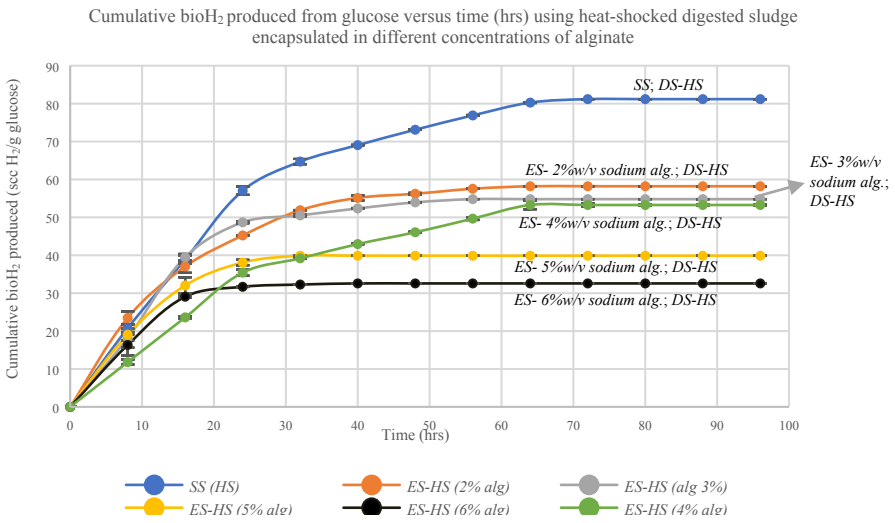
An initial study performed showed that uniform gels can be formed when the concentration of sodium alginate varied from 2%w/v to 6%w/v with a step of 1%w/v. The concentration of sodium alginate is the most important factor that affects the size, shape and encapsulation efficiency of calcium alginate beads (Lotfipour et al., 2012). For this preliminary study, 4% w/v of sodium alginate (which is the medium of the range of concentrations where feasible encapsulation can be performed) was selected. Here, the pretreatment method applicable without interfering with the gelation of the

beads encapsulating the digested sludge is investigated. Based on optimum conditions reported for bioH_2 production from the DF of glucose in suspended pure anaerobes in literature, 10g/L of glucose was digested (Yin & Wang, 2017), a shaker speed of 100rpm was used to induce mixing (Beckers et al., 2015), while the temperature was controlled at 37℃. The encapsulated system is represented by *ES* while suspended system is described as *SS*.

Assuming *ES- 4% w/v sodium alg.*, DS pretreated with heat shock at 90℃ and pH adjustment to 5.0 was encapsulated and used to digest 10g/L glucose. From Figure 2a, glucose digested in the suspended system released 95.59 scc bioH_2/g glucose (0.77mol H_2/mol glucose) and 72.71 scc bioH_2/g glucose (0.58mol H_2/mol glucose) using heat-shocked (*SS*; *DS-HS*) and pH control to 5.0 (*SS*; *DS-low pH*) digested sludge as the source of acidogens, respectively. The encapsulated heat-shocked digested sludge (*ES-4%w/v sodium alg.*; *DS-HS*) processed glucose to produce a cumulative bioH_2 of 53.26scc bioH_2/g glucose (0.43mol H_2/mol glucose) after 96 hours of dark fermentation (DF). The bioH_2 yield from the encapsulated heat-shocked sample (*ES-4%w/v sodium alg.*; *DS-HS*) is about 55.7% of the bioH_2 generated from the suspended system (*SS*; *DS-HS*).



(a.)



(b.)

Figure 2: Plot of cumulative bioH_2 production from glucose versus time (a.) using both the suspended and encapsulated digested sludge pretreated at different pretreatment conditions (b.) using both the suspended and digested sludge encapsulated at different concentrations of sodium alginate.

The encapsulated digested sludge at an adjusted pH of 5.0, on the other hand, did not produce any bioH_2. Instead, rod-like shaped beads were observed under this condition compared to the spherical shape seen in encapsulated heat-shocked digested sludge beads. This observation is consistent with what was reported by Chuang et al, 2017 where the aspect ratio of calcium alginate beads increased with a decrease in pH. The aspect ratio in this case is the ratio of the longer radius to the shorter radius of the calcium alginate bead formed (Chuang et al., 2017).

A recent report on the use of encapsulated anaerobic sludge from organic solid wastes in calcium alginate suggests a pH of 7.9, a temperature of 30.3℃ and 90 hours of digestion time as the optimum conditions for bioH_2 production using digested sludge heat-shocked in an autoclave at 121℃ for suppression of methanogens (Sekoai et al., 2016). Also, heat shock was identified as a method to enrich digested sludge with spore-forming bacteria before encapsulation in calcium alginate to degrade glucose while reporting a good buffering pH regulation capacity in the immobilized system

compared to the suspended system (Penniston & Gueguim Kana, 2018). In addition, in the review performed by Ghimire et al., 2015 on dark fermentation of organic biomass for bioH_2 production, more than 50% of the research work reported applied heat shock on mixed sludge for digestion of organic biomass to generate hydrogen (Ghimire et al., 2015). These reviews suggest that heat shock is a favorable choice for most researchers for encapsulation. It can be concluded from this experiment that the best pretreatment to condition the digested sludge for bioH_2 production before encapsulation in calcium alginate is heat shock. However, the performance of the encapsulated system (55.7% of the suspended system) measured relative to the suspended system suggests that the production of bioH_2 using encapsulated heat-shocked digested sludge should be optimized.

3.1.3 Effect of sodium alginate concentration on bioH_2 production from glucose using encapsulated heat-shocked digested sludge

The outcome of the experiment performed in section 3.1.2 shows that encapsulation of heat-shocked digested sludge is feasible. Thus, the concentration of sodium alginate used to encapsulate heat-shocked digested sludge varied from 2 to 6 w/v% with a step of 1, based on the range of concentration of sodium alginate from preliminary tests. The concentration of glucose remained at 10g/L, and all conditions used for anaerobic digestion in previous tests are maintained.

Figure 2b shows the progression of bioH_2 produced over a period of 96hrs. Interestingly, the heat-shocked digested sludge encapsulated at the lowest concentration of sodium alginate (*ES- 2%w/v sodium alg.; DS-HS*) gave the highest yield of bioH_2 from digesting glucose (58.21 scc bioH_2/g glucose; 0.47mol H_2/mol glucose) and this yield is 72% of the bioH_2 produced from the suspended system (*SS; DS-HS*). The amount of bioH_2 produced decreased with an increase in the concentration of sodium alginate. The lowest bioH_2 is produced at *ES- 6%w/v sodium alg.; DS-HS* with a bioH_2 yield of 33 scc bioH_2/g glucose (0.27mol H_2/mol glucose). Thakur et al., 2014 immobilized *Klebsiella*

oxytoca ATCC 13182 in calcium alginate at different concentrations of sodium alginate from 1-5% w/v to digest used rice mill effluent and 2% w/v sodium alginate gave the highest bioH_2 while a similar trend (decrease in bioH_2 production with increase in the concentration of sodium alginate used for bead formation) observed in this study was reported (Thakur et al., 2014). According to the work by Damayanti et al., 2018, a mixed culture of bacteria pretreated with acid and subsequently regulated to a pH of 7.0 before encapsulating in beads formed from 1, 2 and 3%w/v sodium alginate was used to digest 10g/L of glucose. At 1% w/v sodium alginate, the beads formed were non-spherical and brittle compared to 2 and 3% w/v sodium alginate beads (Damayanti et al., 2018). This is similar to the observation made at 1% w/v sodium alginate encapsulation in an initial study. Based on the bioH_2 yield reported, the best encapsulation concentration is at 2% w/v sodium alginate while lower yields of bioH_2 were reported at higher concentrations of sodium alginate (Damayanti et al., 2018).

According to the number average sizes of beads formed here, the size of the beads increased with an increase in sodium alginate concentration due to increased cross-linking by the Ca^{2+} ions in the $CaCl_2$ at higher concentrations of sodium alginate (Kaur et al., 2018). Beads formed at 5 and 6w/v% sodium alginate were non-spherical and encapsulated microbes at these conditions gave the least bioH_2. Thus, the use of 5 and 6w/v% sodium alginate concentration was eliminated for future experiments with heat-shocked digested sludge. Although *ES- 2%w/v sodium alg.; DS-HS* gave the highest bioH_2 using encapsulated heat-shocked digested sludge to process glucose, further examination on how the varying concentration of sodium alginate affects bioH_2 digesting whole alga and PEAR should be confirmed.

Table 3: Comparison of bioH_2 yields from the dark formation of glucose using different pretreatment conditions to suppress methanogens in mixed bacteria sources in the suspended system with the current work.

S.N	Bacteria source/ Seed	Pretreatment method; Suspended system (SS)	Substrate	Conc. of substrate	Mode of operation of the fermenter	Fermenter Temp. (℃)	BioH$_2$ yield (mol H$_2$/mol glucose)	Reference
1	Digested sludge	Heat shock at 100℃ for 15mins	Glucose	1g of glucose	Batch mode	35℃	1.78 mol H$_2$/mol glucose	(Wang & Wan, 2008)
2	Anaerobic sludge	Heat shock by baking at 104℃ for 2h	Glucose	3g-COD/L	Batch mode	25℃	0.968 mol H$_2$/mol glucose	(Oh et al., 2003)
3	Anaerobic sludge	Heat pretreatment at 100℃ for 30mins	Glucose	18.75 g/L	Batch mode	35℃	1.08 mol H$_2$/mol glucose	(Hu & Chen, 2007)
4	Anaerobic digested sludge	Heat pretreatment at 100℃ for 2hrs	Glucose	20g/L	Batch mode	35℃	1.40 mol H$_2$/mol glucose	(Kawagoshi et al., 2005)
5	Digested sludge	Heat pretreatment at 90℃ for 15mins	Glucose	1g of glucose	Batch mode	37℃	1.41 mol H$_2$/mol glucose	**This work**
6	Anaerobic sludge	pH control to 3.0 for 24h	Glucose	18.75 g/L	Batch mode	35℃	0.72 mol H$_2$/mol glucose	(Hu & Chen, 2007)
7	Sewage sludge	Pretreatment with a pH of 3 for 24hrs	Glucose	20g-COD/L	Batch mode	35℃	1.0 mol H$_2$/mol glucose	(Chen et al., 2002)
8	Anaerobic digested sludge	Pretreatment with a pH of 3 for 18hrs	Glucose	20g/L	Batch mode	35℃	1.35 mol H$_2$/mol glucose	(Kawagoshi et al., 2005)
9	Digested sludge	Pretreatment with a pH of 3 for 24hrs	Glucose	1g of glucose	Batch mode	35℃	0.80 mol H$_2$/mol glucose	(Wang & Wan, 2008)
10	Digested sludge	Pretreatment with a pH of 5	Glucose	1g of glucose	Batch mode	37℃	1.96 mol H$_2$/mol glucose	**This work**
11	Anaerobic sludge	Pretreatment with 5% chloroform	Glucose	18.75 g/L	Batch mode	35℃	1.17 mol H$_2$/mol glucose	(Hu & Chen, 2007)
12	Digested sludge	Aeration with air for 24hrs	Glucose	1g glucose	Batch mode	35℃	0.86 mol H$_2$/mol glucose	(Wang & Wan, 2008)
13	Sludge compost	Aerobic conditioning at 60℃ for 3 days	Glucose	1% glucose in 500mL medium	Batch mode	60℃	2.1 mol H$_2$/mol glucose	(Morimoto et al., 2004)

3.2 Dark fermentation of whole alga and post-extracted algae residue of *S.obliquus* using the immobilized reactor.

3.2.1 Effect of sodium alginate concentration on bioH$_2$ production from the whole alga of *S.obliquus* using encapsulated heat-shocked digested sludge.

Adopting the parameters obtained from digesting glucose, the performance of the encapsulated microbes is tested here to digest the whole alga of *S.obliquus* by alternating the concentration of sodium alginate required for encapsulation. Alga is a more complex substrate compared to glucose; thus, the hydraulic time of the fermenter takes about 5-10 days. Based on an initial study performed, subjecting the encapsulated beads to consistent mixing may introduce mechanical attrition and consequently bead breakage. Therefore, a digestion time of 5 days was suggested for complete bioH$_2$ production but to allow for confirmation of completion of all dark

fermentation reactions, a digestion time of 10 days was recommended as the optimum number of days for DF and more than 90% recovery of encapsulated biocatalysts (heat-shocked digested sludge) was achieved. Figure 3a shows that after 9 days of DF, whole algae yielded bioH_2 that decreased with an increase in sodium alginate concentration. The maximum bioH_2 was realized at *ES- 2%w/v sodium alg.; DS-HS* (56 scc bioH_2/g VS of algae) while the suspended system performed least of all the samples tested (25 scc bioH_2/g VS of algae). *This trend was consistently obtained under these experimental conditions, and it is proposed that these parameters can be implemented on a bench-scale bio fermenter*. The bioH_2 production from digesting whole algae here is comparable to the bioH_2 reported in the work by Batista et al., 2015. About 56.8 scc H_2/g VS. was reported in this work from digesting *S.obliquus* using the suspended *Enterobacter aerogenes* (Batista et al., 2015). Pure bacteria under strictly anaerobic conditions perform better than mixed-digested sludge. Hence, the likely reason why the suspended system in this case produced 50% less than the yield reported by Batista et al., 2015 where pure bacteria was utilized.

3.2.2 Effect of sodium alginate concentration on bioH_2 production from PEAR using encapsulated heat-shocked digested sludge.

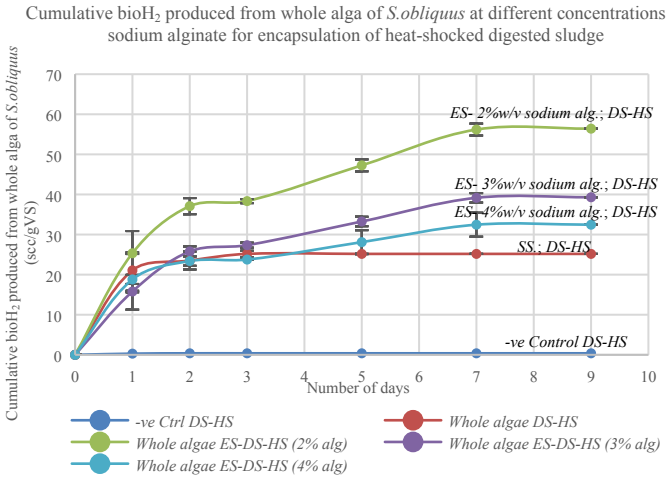
Post-extracted algae residue (PEAR) of *S.obliquus* generated after oil extraction using hexane extraction method was digested with encapsulated heat-shocked digested sludge at different concentrations of sodium alginate. For this experiment, the extracted oil from the whole algae of *S.obliquus* was 12% of the dry weight of the algal biomass. The concentration of sodium alginate used for bead formation varied from 2% w/v to 4% w/v with a step of 1. According to Figure 3b, after 9 days of digestion, the sample with 2% w/v sodium alginate beads gave the highest cumulative bioH_2 production (32.11scc bioH_2/gVS of PEAR). The suspended system gave a cumulative bioH_2 production (25.17 scc/gVS) 19% higher than the cumulative bioH_2 production recovered from the

least performing sample (4% w/v sodium alginate sample). However, it is observed from Figure 3b that the suspended system reached the plateau faster (after 2 days) than any of the encapsulated systems. This may be attributed to the improved mass transfer in the suspended system for the first 48hrs of the digestion. Subsequently, the release of organic acids into the suspended system truncates the process of further production of bioH_2 (pH decreased from 6.5 to 5.5). However, in the case of the encapsulated system, the encapsulation network protects the microbes from the effect of the released volatile acids giving the microbes a longer lifetime for digestion to take place.

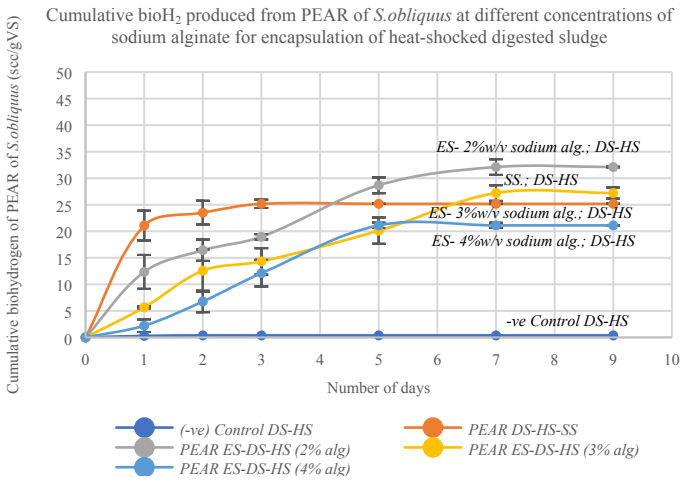
3.2.3 Reuse of encapsulated heat-shocked digested sludge for bioH_2 production from the dark fermentation of whole alga of *S.obliquus*

One of the advantages of adopting the encapsulation of microbes in calcium alginate is the recovery of the beads after dark fermentation (DF). At the optimum encapsulation parameters in section 3.2.1, the whole alga of *S.obliquus* is digested at *ES-2%w/v sodium alg.; DS-HS* while the reuse of the encapsulated microbes after DF is evaluated. The alga was fed into the fermenter at a volatile solid (*VS*) content of 1.55wt.%. From Figure 3c, bioH_2 produced from digesting the whole algae is 56 scc bioH_2/gVS using fresh encapsulated heat-shocked digested sludge (*ES-2%w/v sodium alg.; DS-HS, 1st use*). After the first use, the beads were recovered with about 7% mass reduction compared to the initial mass of the beads fed into the fermenter. For the second cycle, fresh algae from the same sample used for the first run were fed at the same volatile solids (*VS*) content. About 60% (34 scc bioH_2/gVS) of the yield from the first cycle is produced when whole algae are digested with the recycled beads after the first use (*ES-2%w/v sodium alg.; DS-HS, 2nd use*). As can be observed in Figure 3c, both *ES-2%w/v sodium alg.; DS-HS, 1st use* and *ES-2%w/v sodium alg.; DS-HS, 2nd use* produced a cumulative bioH_2 higher than the yield from digesting the whole algae using the suspended heat-shocked digested sludge (*SS; DS-HS*) (25 scc bioH_2/gVS).

The decline in the bioactivity of *ES-2%w/v sodium alg.; DS-HS, 2nd use* apparent in the lower amount of bioH_2 released compared to 1st use is most likely due to the exposure or release of a fraction of the encapsulated cells during fermentation. Another possible explanation for this decline is that in the 1st use, the pH of the local environment of the encapsulated microbes might have been altered due to the absorption of volatile fatty acids (VFAs). In subsequent batches using recycled encapsulated bacteria, the microbes need to adjust before fermentation can take place, thus, a decline in bioactivity (Hutchinson et al., 2020).



(a.)



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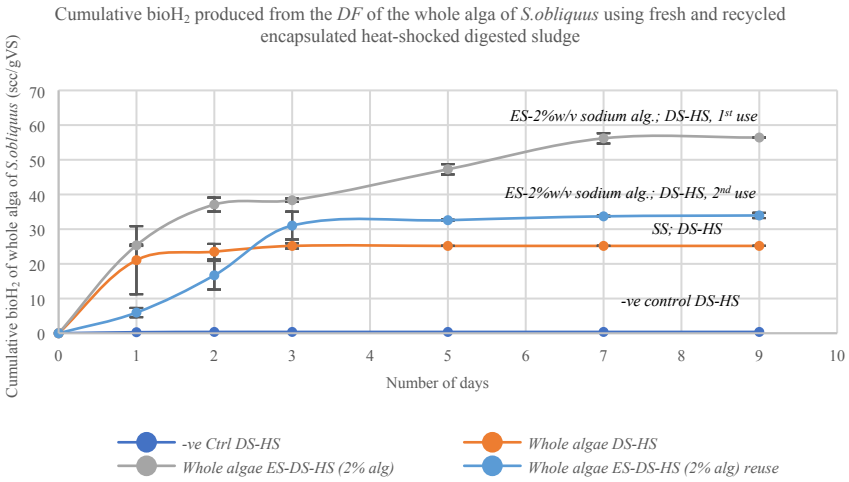

(c.)

Figure 3: Plot of cumulative bioH_2 produced from (a.) the whole alga of *S.obliquus* (b.) PEAR of *S.obliquus* using both the suspended and digested sludge encapsulated at different concentrations of sodium alginate and (c.) the dark fermentation of the whole alga of *S.obliquus* using encapsulated heat-shocked digested sludge at 1st and 2nd use.

The same effect was observed in the study by Hutchinson et al., 2020 where recycled cells entrapped in 3%w/v calcium alginate used to ferment balsamic-styled vinegar showed a 7% decline in bioactivity in the second cycle of fermentation, based on sugar consumption rates reported (Hutchinson et al., 2020). Similarly, after Shyamkumar et al., 2014 discovered that encapsulated *C.glutamicum* using *2* %w/v sodium alginate gave the highest yield of glutamic acid, a reusability test was performed, and results showed that the productivity of glutamic acid decreased by 25% after 5 cycles (Shyamkumar et al., 2014). These reviewed studies showed that the observation made here has been experienced in other applications that do not involve the fermentation of microalgae. Thus, it was concluded that the encapsulated heat-shocked digested sludge recovered after AD can be reused. Also, it was reported that the cost of making 250g of calcium alginate beads is about $64 (Hutchinson et al., 2020). Thus, the reuse of beads is encouraged to avoid recurrent expenses from making beads for every batch of fermentation.

4. Conclusions

Dark fermentation (DF) of the whole alga of *S.obliquus* and its post-extracted algae residue (PEAR) using immobilized heat-shocked digested sludge is first reported in this work. Immobilized fermenter using 2% w/v sodium alginate to form beads encapsulating heat-shocked-digested sludge (DS) digested either of the substrates producing more than 25% of the bioH_2 from the conventional fermenter (operated in the suspended fashion). The reuse of immobilized microbes recovered after 10 days of fermentation was 60% efficient compared to fresh encapsulated microbes. Separation and

further processing of digestate to other bioenergy forms which the conventional fermenter disallows was feasible by adopting the immobilized reactor. It is anticipated that the recovered digestate can be further processed to produce biomethane by adopting a 2-stage anaerobic digestion strategy. This will improve the economic viability of alga-to-biofuel. A typical pathway applicable to this work is the extraction of lipids using hexane extraction (for subsequent transformation to green diesel) followed by fermenting the post-extracted algae residue using the strategy presented here while the digestate can be processed to produce methane using untreated digested sludge. BioH_2 generated can be used in the hydrotreatment of algal oil to green diesel instead of using fossil hydrogen available in the market. Overall, this work provides data that can be utilized for initial scale-up parameters for immobilized bio fermenters.

Acknowledgements

This work was supported under the Net Zero Technologies for the Army's Industrial Munitions Base project sponsored by LEIDOS Inc. Contract number: Prime Contract 2009-372. Authors would like to acknowledge Dr. Juliana Abraham for assisting with culturing *S.obliquus* used for this study.

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