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Research review paper

Progress in biohythane production from microalgae-wastewater sludge co-digestion: An integrated biorefinery approach

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ABSTRACT

Recent advances in microalgae to biohythane (bio-H_2 and bio-CH_4) conversion have achieved growing attention due to their eco-friendly and energy-efficient nature. Although microalgae are considered a potential 3rd – 4th generation biomass, their low C/N ratio and cell-wall biopolymers are challenging for biohythane production. This study emphasizes the solutions to mitigate the adverse effects of microalgae-based biohythane production using co-digestion with wastewater sludge. Wastewater sludge, an emerging environmental concern, is reviewed to be an effective co-substrate with microalgae to establish a biorefinery approach. The future trends and prospects of this biorefinery approach is critically reviewed to attain a profitable process. This study also reviewed the advantages of microalgae-wastewater co-cultivation and the application of activated sludge for bio-flocculation as a cost-effective solution for microalgae cultivation and harvesting. Microalgae-wastewater co-cultivation is also recommended to be effective for biohythane purification. The liquid digestate is suggested to be used as a culture media to enhance microalgal growth; whereas, the solid digestate could be transformed into resources through hydrothermal processes as a solution of digestate management. A practical biorefinery approach combining the synergistic benefits of microalgae-wastewater sludge and its biological conversion to biohythane would be an adjoining link to the beginning of a sustainable future.

1. Introduction

Generation of carbon-neutral H_2 fuel and one carbon-involved CH_4 fuel can potentially enhance the decarbonization of certain carbon-emitting activities (Bălănescu and Homutescu, 2021). These two gaseous energy carriers could be considered clean fuels for transport engines (Li et al., 2021). Powerful rocket and jet engines already use H_2 fuel as an ideal exhaust. Still, current researches propose a mixture of H_2 and CH_4 (hythane) fuel for lower-powered engines like wheeled vehicles (Sandalcı et al., 2019). Biohythane (10–25% bio-H_2 and 75–90% bio-CH_4) application in transport engine was initially introduced in 1995, where biohythane combustion showed a tremendous environment-friendly nature as it emitted nearly 45% less NO_x gas than compressed natural gases (Hora and Agarwal, 2018). Other advantages of biohythane over CH_4 based fuel are as follows, i) a reduction of CO_2 emission by improved H/C ratio; ii) the improvement of shorter range of CH_4 flammability, ultimately reducing the combustion period; iii) blaze speed is enhanced by the introduction of H_2, improving fuel heating efficiency; and iv) refining the fuel ignition by shortening the extinguishing distance (Shanmugam et al., 2021a). The synergistic benefits of biohythane mixture could transition pollutant-rich fossil fuel to hydrogen-based renewable fuel. Ekins and Huges (2010) predicted that hydrogen-based fuel would revolutionize the market and political institutions like coal (19th century) and petroleum (20th century), and so extensive research is currently directed to biohythane processing.

Biohythane can be processed using either electrochemical or

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biological processes; however, electrochemical processes are energy-intensive and expensive (Huang et al., 2017). The profitable solution of biohythane synthesis is biological processes such as dark fermentation and anaerobic digestion. While operating the biological conversion processes, various difficulties like the formation of inhibitory metabolites and fluctuation of operating parameters slowed down the synthesis of biohythane. This could be solved by establishing a two-staged operation of the anaerobic digestion process. Hans and Kumar (2019) stated that two-stage anaerobic digestion could optimize operating conditions for anaerobes, which is the energy-efficient way of converting organic biomass to biohythane. One of the potential 3rd - 4th generation biomass for biohythane conversion is microalgae (Alam et al., 2020). The high carbohydrate and micronutrient content (phosphorous, sulfur, iron, cobalt, and zinc) of microalgae makes it one of the promising feedstocks for biohythane productivity (Ghimire et al., 2017). Even so, the mono-digestion of microalgae having a lower C/N ratio (<6.5) and cell-wall biopolymers showed a slow rate of degradation and formation of ammonia, inhibiting the biohythane conversion efficiency (Ghimire et al., 2017). Although several studies found using pretreatment methods (Kendir and Ugurlu, 2018) and nanoparticle inclusion (Rana et al., 2020) effective in solving the issue, the operating cost increased significantly. Alternatively, co-digestion of microalgae with a mixture of waste feedstocks having high C/N ratio might work as a double-edge solution in terms of providing optimum condition for biohythane production and waste management. Co-digestion provides numerous benefits such as, system optimization, dilution of inhibitory components, C/N/P nutrient balance, high organic loading, higher anaerobic productivity and energy neutrality(Solé-Bundó et al., 2019b). However, microalgae co-digestion with Agri-industrial waste feedstocks resulted in solids accumulation, reduced biodegradability and nitrogen backload (Carminati et al., 2018). The most sustainable and economical approach is the co-digestion of microalgae with wastewater sludge, which has effectively weakened the inhibitory effects of ammonia generation and enhance biodegradability, facilitating biohythane production.

Wastewater sludge (WWS) is potential biomass for anaerobic digestion, a growing environmental concern worldwide. Based on the last five years' Scopus database, more research was conducted on anaerobic digestion of wastewater sludge than microalgal biomass (Fig. 1). Since the production of WWS (250-300 million metric tons/year) is rising at a high rate, biorefinery approaches of microalgae-wastewater sludge co-digestion could be a promising prospect to achieve sustainable development goals (Strande et al., 2014). Tena et al., (2020) observed 14 times higher hydrogen yield for food waste fermentation while co-fermented with wastewater sludge. Lu and Zhang, (2016) found 84 % improvement of microalgal biomethane production rate while co-digestion with wastewater sludge. Even so, there is a research gap regarding the integrated approach of biohythane generation from microalgae-wastewater sludge.

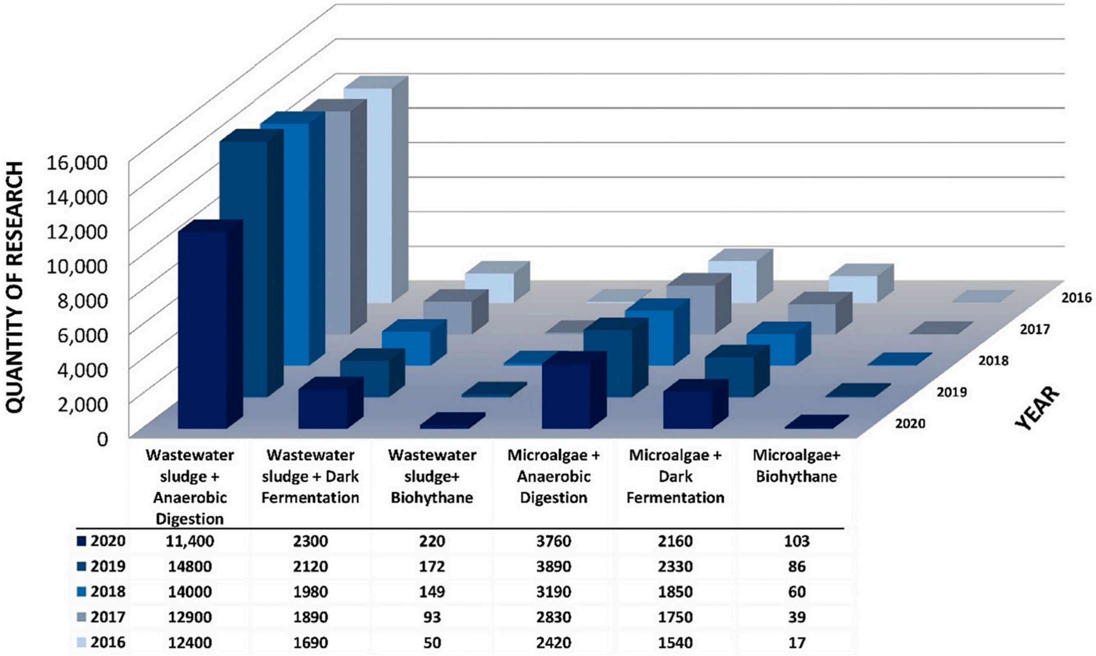
This study took the opportunity to review the aspects of microalgal biomass co-digestion with wastewater sludge and its techno-economic assessment to produce biohythane. Following the production of biohythane, microalgae-bacteria culture is recommended for purifying biohythane as a cost-effective approach. The elevated photosynthetic ability of microalgae would help to cleanse CO_2 from the biohythane mixture and utilize it as a carbon source for biomass production (Angelidaki et al., 2018). This article also summarized future trends on the biorefinery approach and recommended microalgae-wastewater co-cultivation and bio-flocculation as an economical solution for biomass production. The hydrothermal technology is also reviewed to be effective in terms of digestate management. The revolutionary approach to formulating different bioenergy sources using the biorefinery ability of microalgae-wastewater sludge would be an environment-friendly pathway to a cleaner future.

2. Biohythane production through single/two-stage anaerobic digestion

Anaerobic digestion (AD) is a biological process that combines numerous anaerobes to synthesize biohythane from biomass. AD process is a combination of four phases (i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis). These phases could be run through either single-stage anaerobic digestion (SSAD) or two-stage anaerobic digestion (TSAD).

2.1. Single-stage anaerobic digestion

Conventionally, SSAD leads to the production of about 60-65% bio-CH_4 as a major product, CO_2 and H_2 as minor products, and trace



	Wastewater sludge + Anaerobic Digestion	Wastewater sludge + Dark Fermentation	Wastewater sludge+ Biohythane	Microalgae + Anaerobic Digestion	Microalgae + Dark Fermentation	Microalgae+ Biohythane
■ 2020	11,400	2300	220	3760	2160	103
■ 2019	14800	2120	172	3890	2330	86
■ 2018	14000	1980	149	3190	1850	60
■ 2017	12900	1890	93	2830	1750	39
■ 2016	12400	1690	50	2420	1540	17

Fig. 1. Quantitative analysis of researches conducted on anaerobic conversion of microalgae and wastewater sludge.

amounts of H_2S and NH_3. The key advantages associated with SSAD are the simple design and low operating costs. Ta et al. (2020) introduced a novel approach to produce biohythane in SSAD using gel-entrapped hydrogenic and methanogenic bacteria. The entrapped hydrogenic/methanogenic bacteria ratio of 2/3 resulted in the highest biohythane productivity (64.6 mL bio-H_2/L-d and 395 mL bio-CH_4/L-d) using the SSAD system (Ta et al., 2020). Still, Ta et al. (2020) faced several drawbacks such as lack of pH control, the high hydraulic retention time (HRT), and low COD removal rate while operating the SSAD system. Kumar et al. (2017) also faced several drawbacks such as high retention time, inconsistent CH_4 production, and the lack of parameters control while operating an SSAD system. Remarkably, the pH fluctuations during SSAD reduce the microbial growth rate and its efficiency to convert biomass; thus, the energy conversion efficiency is not up to the mark (Croce et al., 2016).

2.2. Two-stage anaerobic digestion

The integrated TSAD system generates biohythane where H_2 is produced through dark/photo-fermentation in the first stage, and CH_4 is produced through methanogenesis in the later stage, as shown in Fig. 2. During dark fermentation (DF), the hydrolytic anaerobes break biomass metabolites into monomers. Then the fermentative anaerobes utilize monomers to produce volatile fatty acids (VFA), alcohol, and H_2. Subsequently, the acetogens convert the VFA to acetic acid, H_2, and CO_2 gas. The fermentative broth is transferred to the methanogenic reactor, where methanogenesis occurs. The methanogens consume acetic acid and VFA to produce CH_4 and CO_2 gas. The TSAD process has the potentiality to reduce inhibitory metabolites and provide an optimum condition for biomass digestion. As a result, the TSAD of biomass has achieved a higher energy conversion rate than SSAD (Kumar et al., 2019). So, the process optimization of the TSAD is the crucial factor to recover the maximum energy from biomass.

2.3. Impact of process optimization

The energy conversion efficiency of biomass depends on several factors such as biomass quality, pretreatment efficiency, and growth environment for anaerobes. Suitable biomass is considered to have positive digestive characteristics: nutrient balance (C/N ratio = 20-30 and N/P ratio = 6.5-7), high volatile solids to total solids ratio (VS/TS> 0.7), and simple cell-wall structure. Biomass having these characteristics would increase the microbial activities and biohythane yield. Due to these reasons, AD of food waste (FW) showed better energy yield than other biomass types (Table 1). Otherwise, co-digestion would optimize these factors, which positively impacted the energy yield (Jehlee et al., 2019). The two-stage co-digestion of *Chlorella* sp. and glycerol waste generated 50% more biohythane than two-stage mono-digestion of *Chlorella* sp. (Jehlee et al., 2019). The synergistic approach of *Chlorella* sp. and glycerol waste co-digestion improved the nutrient balance (C/N ratio=30.1), which increased the microbial activities and biohythane conversion rate (Jehlee et al., 2019). So, the co-digestion approach should be considered by ensuring proper nutrient balance to increase microbial metabolism.

The sustainable anaerobic growth environment could be ensured by optimizing pH, temperature, HRT parameters and reducing toxic elements. The anaerobes are sensitive to both of the organic (e.g., chlorophenols, halogenated aliphatic and long chain fatty acids) and inorganic (e.g., ammonia, sulfide and heavy metals) toxicants (Chen et al., 2014). Chen et al., (2014) recommended that, toxicity sensors with rapid response time need to be implemented in the digesters to monitor and control toxicity. The presence of heavy metal ions (Zn++, Cd++, Cu+, or Cu++) in the substrate could create inhibitory conditions whereas, the presence of light metal ions Mg+, Na+, and Ca++ ions would be required for enhancing the methyl coenzyme M reductases and monoxide dehydrogenases mechanisms for bio-CH_4 productivity. However, excess concentration of the metal ions would cause toxicity for anaerobes and so concentration of these metals should be monitored to keep them below the threshold level to enhance biohythane production (Shanmugam et al., 2021b).

Between mesophilic (30° - 40 °C) and thermophilic (50° - 60 °C) thermal conditions, Ward et al. (2008) observed 95% of biomass conversion within 11 days under thermophilic conditions in SSAD, which is 2.5 times faster than mesophilic digestion. One of the major benefits of thermophilic digestion over mesophilic digestion is the enhancement of bacterial performance and so, shorter HRT is required. Although thermophilic digestion has several benefits like higher biomass biodegradability, higher volatile fatty acid formation rate, and enhanced biohythane productivity, it has drawbacks like higher operating costs and difficulty in maintaining biomass digestion with a high organic

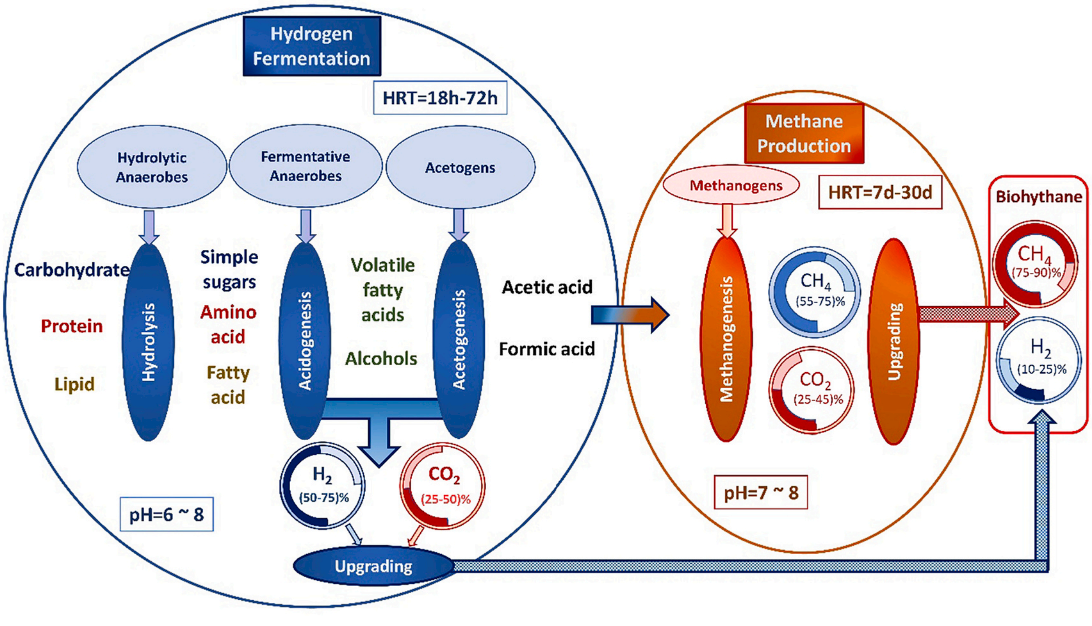


Fig. 2. bio-H_2 fermentation and bio-CH_4 production through two-stage anaerobic digestion.

Table 1
Benefits of TSAD over SSAD regarding energy yield efficiency.

Substrates	Methods						Highlights	Bio-fuel yield		Energy yield (MJ/kg VS) [a]	References
	Pretreatment	Reactor Type	HRT (day)	pH	Temperature (°C)	Inoculum		Bio-hydrogen (mL H_2/gVS)	Bio-methane (mL CH_4/gVS)		
Mono-digestion											
Dark Fermentation (DF)											
Microalgae (Chlorella sp.)	Enzymatic Saccharification (4g/g VS)	Batch- 60 mL serum bottles	-	5.5	35	Anaerobic granules collected from ICWTF Pretreated (100°C for two hours)	Pretreatment yielded 82.46% higher biohydrogen	42.24	-	0.45	(Sriyod et al., 2021)
Sewage Sludge (SS)	-	Batch- 250 mL glass bottle	14	5.5	55	Anaerobic digester sludge	Hydrogen production was low due to the low C/N ratio (5.1) of SS	4.45	-	0.05	(Tena et al., 2020)
Anaerobic Sludge	Freezing and thawing (-17°C for 24h)	Batch- 125 mL serum bottles	4.16	6.0	35	Wastewater sludge *(Clostridium bifermentans)* thermal pretreatment (121°C for 30 mins)	Pretreatment increased biohydrogen productivity by 1.5-2.5 times	60	-	0.65	(Wang et al., 2003)
Starch Processing wastewater	-	Batch- 120mL bioreactor	2	4-7	35	UASB sludge- Base (NaOH) treated (pH 11 for 24h)	COD degradation was increased by 20% by base treated bacteria	138	-	1.5	(Sinbuathong and Sillapacharoenkul, 2020)
Waste Activated Sludge (WAS)	Freezing (-5°C for 4 h) and Nitrite Pretreatment	Batch- serum bottles	14	6.4	35	Pretreated WAS acts both as inoculum and substrate	Pretreatment increased biohydrogen productivity by 13.4 times	19.4	-	0.21	(Liu et al., 2020)
Food Waste (FW)	Potassium ferrate pretreatment (0.4 g/g TS)	Batch- Working Volume= 1L	4	6.8	35	MWWTP	Pretreatment increased biohydrogen productivity by 3 times	173.5	-	1.86	(Kuang et al., 2020)
Single-stage anaerobic digestion (SSAD)											
Microalgae (Scenedesmus obliques)	Drying at 105°C for 24h	Batch- 250mL reactor	32	-	38	-	Drying as a pretreatment decreased the biogas production by 80%	-	177.94	6.36	(Mussgnug et al., 2010)
Microalgae (Chlorella pyrenoidosa)	Nanoparticle supplementation	Batch	30	6.8-7.6	37	Anaerobic digestate	Nanoparticle application increased biogas yield by 25%	-	605	21.66	(Rana et al., 2020)
Microalgae (Chlorella sp.)	Hydrothermal pretreatment at 130°C	Batch-Automatic methane potential test system-II	24	7	37	Anaerobic sludge collected from the sewage treatment plant	Hydrothermal pretreatment increased the protein recovery by 69.81%	-	194.63	6.97	(Wu et al., 2020b)
Chlorella sp.	Hydrothermal pretreatment at 150°C	Batch-Automatic methane potential test system-II	24	7	37	Anaerobic sludge collected from the sewage treatment plant	Hydrothermal pretreatment increased the protein recovery by 69.81%	-	125	4.47	(Wu et al., 2020b)
Sewage Sludge	Alkali thermal pretreatment	Continuous anaerobic digester	15	7.0	37	MWWTP sludge thermally treated (134°C for 30m)	Pretreatment reduced HRT from 25d to 15d.	-	247	8.84	(Liu et al., 2020)
Anaerobic Sludge	-	Semi-continuous batch reactor	30 (5[b])	6.8 -8.8	55	UASB sludge	Single-staged fermentation provides less solid reduction and biomethane generation	-	400.7	14.34	(Erden and Filibeli, 2010)
Starch Processing wastewater	-	Continuous (OLR-10 g/L. d) in Upward flow anaerobic reactor	0.77	8.0	25	Anaerobic digestate	Biomethane production was increased by increasing the organic loading rate up to 10 g/L. d	-	311	11.13	(Araujo et al., 2018)
Anaerobic Sludge	Fenton pretreatment	Semi-continuous batch reactor	30 (5[b])	6.8 -8.8	55	UASB sludge		-	547.3	19.59	(Erden and Filibeli, 2010)

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Table 1 (*continued*)

Substrates	Methods						Highlights	Bio-fuel yield		Energy yield (MJ/kg VS) [a]	References
	Pretreatment	Reactor Type	HRT (day)	pH	Temperature (°C)	Inoculum		Bio-hydrogen (mL H_2/gVS)	Bio-methane (mL CH_4/gVS)		
							Fenton pretreatment increased the biomethane production by 36.5%				
FW	-	Batch: Box type reactor (25x50x25) - 304 stainless steel	Recirculation	7-8	37	Screened liquid fraction of dairy manure	VS removal efficiency was 91.4%	-	477	17.07	(Rico et al., 2020)
Food Solid Waste	Milled pretreatment	Semi-continuous reactor (0.8 L)	21	7.0-7.3	35	CSTR digestate	Increased methane productivity by 13.6% in a dual solid-liquid (ADSL) system	-	643	23.01	(Zhang et al., 2013)
Food Liquid Waste	Milled pretreatment	Semi-continuous reactor (0.8 L)	13.9	7.4-7.5	35	CSTR digestate	Increased methane productivity by 13.6% in a dual solid-liquid (ADSL) system	-	659	23.59	(Zhang et al., 2013)
Two-stage anaerobic digestion (TSAD)											
Microalgae (Chlorella sp.)	Hydrothermal pretreatment at 150°C	Batch-Automatic methane potential test system-II	1^{DF} 22^{AD}	6^{DF} 7^{AD}	37	DF=Anaerobic sludge (100°C for 15 mins); AD=Anaerobic sludge	Increased the energy recovery by 22.23–146.78% than SSD	5.15	434.38	15.61	(Wu et al., 2020a)
Microalgae (Chlorella vulgaris)	Enzyme pretreatment	Batch- 500mL glass bottle	5^{DF} 20^{AD}	7.5	60^{DF} 37^{AD}	Anaerobic sludge from municipal WWTP	MSD improved the biohydrogen and biomethane productivity	138	416	16.38	(Wieczorek et al., 2014)
SS	Fe_3O_4 nanoparticle	CSTR	1^{DF} 12^{AD}	7.0	36	MWWTP sludge	Nanoparticle application increased 15.1% of hydrogen yield and 58.7% of methane yield	11.9	109.8	4.06	(Zhang et al., 2020)
Petro-chemical wastewater	-	Batch- 500mL serum bottle	3^{DF} 62^{AD}	4.97	55	Thermophilic anaerobically digested sludge	MSD could efficiently remove organic content from the wastewater	88	321	12.44	(Jariyaboon et al., 2015)
MEG contaminated wastewater	Thermal pretreatment (105°C for 30 mins)	Batch- 300mL bottle	36	7	55	Baking yeast company's WWTP ISR=5.29^{DF}, 3.78^{AD} g VSS/g COD	The highest biomethane yield was observed for ISR 5.29 g VSS/g COD	22.7	151.83	5.68	(Elreedy et al., 2017)
SS	Alkali pretreatment	MEC	9	-	30	DF=Fermentative clostridium sp., AD=Hydrogenotrophic methanobacterium	MEC and alkali pretreatment enhanced the biomethane productivity of sewage sludge	75	187	7.50	(Liu et al., 2016)
Starch Processing wastewater	-	Batch-500mL serum bottle	4^{DF} 45^{AD}	6.5-5.8	55	Starch Processing wastewater	MSD increased energy conversion efficiency	81.5	310	13.98	(Khongkliang et al., 2015)
Anaerobic Sludge	-	Semi-batch reactors	30 (5[b])	6.8 -8.8	37^{DF} 55^{AD}	UASB sludge	MSD increased biomethane production by 36%	-	544.6	19.48	(Erden and Filibeli, 2010)
Anaerobic Sludge	Fenton pretreatment	Semi-batch reactors	30 (5[b])	6.8 -8.8	37^{DF} 55^{AD}	UASB sludge	Fenton pretreatment increased the biomethane production by 3%	-	561.1	20.08	(Erden and Filibeli, 2010)
FW	-	CSTR	$8h^{DF}$ 20^{AD}	5.5^{DF} 7.5^{AD}	37	Brown water	OLR of 1.24 g VS/L. d was found optimum in terms of methane production and organic removal	99.9	728	27.14	(Paudel et al., 2017)
FW	-	DF=batch reactor AD=continuous	$160h^{DF}$ 26.6^{AD}	Not adjusted	40	UASB tank sludge	The optimal OLR for MSD was considered to be 22.65 kg VS/m3 d for Hydrogen production and 4.61 kg VS/m3 d for methane production	65	546	20.25	(Wang and Zhao, 2009)
FW	-	Mixing tank, batch H_2 production	1.3^{DF} 5^{AD}	5.5^{DF} 7.2^{AD}	55^{DF} 35^{AD}	WWTP anaerobic sludge	Recirculation technology effectively maintained optimum pH for hydrogen production, and	205	464	18.82	(Chu et al., 2008)

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Table 1 (*continued*)

Substrates	Methods						Highlights	Bio-fuel yield		Energy yield (MJ/kg VS) [a]	References
	Pretreatment	Reactor Type	HRT (day)	pH	Temperature (°C)	Inoculum		Bio-hydrogen (mL H_2/gVS)	Bio-methane (mL CH_4/gVS)		
		reactor, filter CH_4 production reactor					methanogen-rich suspended media added to the methanogenesis reactor effectively shortened the HRT.				
FW	-	Batch reactor integrated with recirculation technology	7	5.2^{DF} 8.1^{AD}	55	WWTP anaerobic sludge	Recirculation technology effectively maintained the stability of the pilot-scale operation.	220	710	27.79	(Micolucci et al., 2014)
FW	-	CSTR	Recirculation	5.4^{DF} 7.8^{AD}	55^{DF} 37^{AD}	AD sludge	Digestate recirculation in comparison with a no-recirculation system reduced the need for alkali addition to maintaining pH in the H_2-reactor by 54%	135	529.5	20.41	(Algapani et al., 2019)
Co-digestion Dark Fermentation (DF)											
FW+SS= 75:25 (VS)	-	Batch- 250 mL glass bottle	14	5.5	55	Anaerobic digester sludge	Co-fermentation yielded 14 times more biohydrogen than mono-digestion of SS	43.25	-	0.46	(Tena et al., 2020)
FW+SS= 10:1 (COD)	Thermal (90°C for 20 m)	Batch-Working Volume=3L	1.5	5.4	35	Sewage Sludge	Co-fermentation yielded 13% higher biohydrogen than mono-digestion of FW	165	-	1.78	(Kim et al., 2011)
Single-stage anaerobic digestion (SSAD)											
MA+FW= 0.2:0.8	-	Batch- BMP	40	7.2	35	CSTR digestate	Co-digestion of microalgae with FW showed a 4.99-fold increase in biomethane yield.	-	639.8	22.90	(Zhen et al., 2016)
Sewage Sludge + WAS	-	Semi-continuous batch reactor	44[b]	8.1	37	WWTP mesophilic digestate	Co-digestion yielded 1.15 times more biomethane than SS mono-digestion	-	181	6.48	(Villamil et al., 2020)
FW+SS= 50:50 (VS)	-	Batch	12	-	55	Anaerobic chemostats	Thermophilic digestion increased biomethane yield by 30% than mesophilic digestion	-	280	10.02	(Kim et al., 2003)
Two-stage anaerobic digestion (TSAD)											
Chlorella pyrenoidosa and cassava starch	Steam heating + dil. H_2SO_4	Batch- 300mL glass fermenter	3^{DF} 3.5^{PF} 21^{AD}	6^{DF} 7^{PF} 8^{AD}	35^{DF} 30^{PF} 35^{AD}	AD sludge obtained from a methane plant	Co-generation of H_2 and CH_4 improved the energy production efficiency to 67.2%	920.2	126	14.45	(Xia et al., 2014)
Chlorella sp. and 2% glycerol wastes	-	Batch-250mL serum bottle	7^{DF} 30^{AD}	-	55	DF=Heat-shock treatment of anaerobic sludge (100°C for 60 min); AD= Methanogenic sludge from United Palm Oil Industry	The addition of glycerol waste optimized the C/N ratio to 30.1 that enhanced the biohythane productivity	39.8	577.3	21.09	(Jehlee et al., 2019)
FW and microalgae	Thermochemical pretreatment (140°C	Cylinder shaped reactor	6^{DF} 10^{AD}	7	37			141.5	275	11.37	(Sun et al., 2019)

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Table 1 (*continued*)

Substrates	Methods						Highlights	Bio-fuel yield		Energy yield (MJ/kg VS) [a]	References
	Pretreatment	Reactor Type	HRT (day)	pH	Temperature (°C)	Inoculum		Bio-hydrogen (mL H_2/gVS)	Bio-methane (mL CH_4/gVS)		
	for 10 mins with the addition of 1% H_2SO_4)					WWTP sludge thermally pretreated for DF and untreated sludge for AD	The entire system showed the ratio of net energy input to net energy output was 0.24.				
Co-digestion of aMSW and bWAS	-	CSTR	3^{DF} 17^{AD}	5.3^{DF} 8.2^{AD}	55	WWTP sludge	Co-digestion increased biomethane production by 17%	24	570	20.66	(Bolzonella et al., 2020)
FW + SS + 1% GL	-	Batch- 250 mL glass bottle	1.5^{DF} 40^{AD}	5.5^{DF} 7^{AD}	35	AD sludge	Glycerol increased the H_2 and CH_4 production efficiency in an MSD process	140.2	342	13.75	(Silva et al., 2018)
FW+SS= 85:15 (VS)	-	Batch-serum bottle (300mL)	2^{DF} 42^{AD}	5.5^{DF} 7^{AD}	37	WWTP activated sludge	Co-digestion had higher VS removal efficiency than SSD	106.4	353.5	13.8	(Liu et al., 2013)
FW+SS= 3:1 (VS)	-	Batch- 300 mL glass reactor	2^{DF} 30^{AD}	6^{DF} 8^{AD}	35	AD sludge	The biohydrogen yield of 174.6 mL/gVS was 49.9% higher than the mono-fermentation of FW	174.6	264.1	11.34	(Cheng et al., 2016)

DF=dark fermentation, PF=photo fermentation, AD= anaerobic digestion, FW=food wastes, SS= sewage sludge, WAS=waste activated sludge, COD=chemical oxygen demand, VS=volatile solids, GL= glycerol waste, CSTR=continuous stirred tank reactor, UASB=up-flow anaerobic sludge blanket, MWWTP= municipal wastewater treatment plant, WWTP=wastewater treatment plant, ICWTF= internal circulation (IC) wastewater treatment facility, ISR= inoculum to substrate ratio, MEG= mono-ethylene-glycol, MEC= microbial electrolytic cell, OLR= organic loading rate.

[a] Energy yield is demonstrated as the summation of bio-CH_4 and bio-H_2 total heating values of the produced biofuels.

[b] Sludge retention time.

loading rate (OLR) (Wang et al., 2018). Otherwise, mesophilic digestion could maintain a high OLR of biomass, but requires longer HRT (Paudel et al., 2017). To make the process economical, thermophilic fermentation and mesophilic methanogenesis are recommended in the TSAD process to produce biohythane.

The pH range should be between 6 and 8 for enhancing the hydrogenase activity (H_2 producing enzyme) of acetogens, as illustrated in Table 1. Kawagoshi et al. (2005) also recommended maintaining pH 6 inside the fermentative reactor to maximize H_2 production and observed no H_2 production below pH 5. This phenomenon indicates that the hydrogenase activity of H_2-producing bacteria (HPB) is inhibited at pH$\leq$5, which results in ethanol and acetones formation instead of H_2 generation (Liu et al., 2013). As presented in Table 1, researchers recommended a pH range from 7 to 8 for methanogenesis since methanogens can't grow under pH 6.5 and over pH 9. Maintaining this parameter in SSAD is difficult, whereas TSAD could provide the optimum pH condition for anaerobic growth, resulting in maximum microbial efficiency and biohythane production.

Another influential factor in increasing microbial metabolism is solubilizing biomass metabolites before digestion. Pretreatment could increase the biomass solubilization rate and improve the total nitrogen content in the mixture, which would increase microbial activity. However, excessive pretreatment would negatively impact the digestion process. As illustrated in Table 1, hydrothermal pretreatment (130°C for 30 mins) of *Chlorella* sp. increased the bio-CH_4 productivity by 25%; however, the bio-CH_4 productivity was decreased by 27% while pretreated at 150°C for 30 mins (Wu et al., 2020b). In another study, thermal pretreatment (105°C for 12 h) of *Scenedesmus obliques* decreased the bio-CH_4 yield by 80% due to the long pretreatment period (Mussgnug et al., 2010). For this reason, the pretreatment method and its duration should be very carefully selected based on the biomass characteristics (Satlewal et al., 2018). As presented in Table 1, milled pretreatment is suitable for FW due to its lignin-based cellular structure (Ma and Liu, 2019). Whereas supercritical fluid extraction, enzymatic and thermochemical pretreatment is effective for microalgae and wastewater biomass because of their glycoprotein and peptidoglycan-based cellular structure (Kendir and Ugurlu, 2018). Nobre et al. (2013) investigated the biorefinery prospects of *Nannochloropsis* sp. and recommended supercritical CO_2 extracted biomass to be suitable for bio-H_2 production. The lipid extracted biomass exhibited 26 % higher bio-H_2 productivity (60.6 mL/gVS) compared to non-extracted biomass (40 mL/gVS). Lunprom et al. (2019) observed that the combined pretreatment of acid-thermal hydrolysate (1.5% HCl at 100°C) improved the biohythane productivity by 5.5-fold than the control condition. On the contrary, the enzymatic pretreatment increases biomass hydrolysis, which spikes the bacterial decomposition rate. Enzymatic hydrolysis before TSAD of *Chlorella vulgaris* increased the bio-H_2 productivity by 7-folds and bio-CH_4 productivity by 70% (Wieczorek et al., 2014). So, proper pretreatment based on biomass characteristics could enhance the AD performance of both SSAD and TSAD.

As presented in Table 1, the TSAD has achieved a better energy yield than SSAD due to providing optimum growth media for acetogenic and methanogenic anaerobes. The SSAD could not ensure a consistent growth environment for the fermentative and methanogenic bacteria, making it complicated and time-consuming. Rana et al. (2020) applied Iron-oxide nanoparticles (NP) into the microalgae growth medium to solve this issue, which positively impacted biomass growth and bio-CH_4 yield. Iron-oxide NP has a high surface-to-volume ratio that stimulates bacterial metabolism and reduces inhibitory components during SSAD (Rana et al., 2020). Again, the electrically conductive material NP excites the acetogen and methanogens by direct interspecies electron transfer, which ultimately increases the bio-CH_4 productivity. However, nano-Au, nano-CuO and nano-CeO_2 showed toxicity during mesophilic and thermophilic digestion (Chen et al., 2014). So, further evaluation of the NP economic feasibility and environmental impact on a large scale is required before practical implementation. On the contrary, the best result was observed for the TSAD of *Chlorella* sp., which was 1.5 times more than the SSAD process (Wu et al., 2020a, 2020b). So, the synergic production of bio-CH_4 and bio-H_2 (biohythane) in TSAD is recommended to achieve maximum energy at the lowest cost.

3. Microalgae for biohythane production

Microalgae are autotrophic microorganisms that photosynthetically convert both inorganic and organic carbon into superior organic compounds (Bohutskyi and Bouwer, 2013). Due to having a lignin-free cell wall and high organic fractions, numerous researches have been conducted on microalgal biomass to produce bio-H_2 and bio-CH_4 since the 1950s (Golueke et al., 1957). However, biohythane generation from microalgal biomass is a novel concept that has an emerging perspective. Some challenging factors of microalgal biohythane productivity are the low C/N ratio, low biodegradability, biochemical composition, and cell wall properties. The challenges could be overcome using proper microalgal strain selection, co-digestion and pretreatment method. In this context, Jehlee et al., (2019) used carbohydrate rich *Chlorella* biomass for TSAD and optimized the nutrient balance of the TSAD reactor (C/N ratio of 19–41) by introducing organic co-substrate (molasses, POME, and glycerol waste) with microalgae which improved 8–100% bio-H_2 and 80–264% bio-CH_4 yield. The governing factors that influence microalgal biohythane productivity like enzymatic efficiency, cellular composition and biochemical composition of microalgae, pretreatment methods and the operating parameters are demonstrated in this section.

3.1. Microalgal strain

Microalgae show distinctive ratios of organic compounds (proteins, lipids, and carbohydrates) (Fig. 3), which directly influence biohythane production. As presented in Table 2, microalgae with high lipid and low carbohydrate content show less bio-H_2 productivity. For instance, *Chlorella* sp. possesses a high proportion of lipid (28.8%) than carbohydrates (19.5%) which produces less H_2 (5.2 mL/g VS) (Phukan et al., 2011). The H_2 production rate was hampered because of the slow degradation of microalgal lipid (i.e., saturated fatty acid). On the contrary, *Chlorella pyrenoidosa* has shallow lipid content (2%) and higher carbohydrate content (26%) that showed high production of bio-H_2 in DF (75.6 mL/g VS) and Photo-fermentation (PF) (122.7 mL/g VS) (Xia et al., 2013a). This happened due to the higher bacterial metabolism to break down the carbohydrate into simple sugar rather than protein or lipid (Stack and Gerlt, 2021). Therefore, investigations are ongoing on lipid extracted biomass to produce biohythane (Tibbetts et al., 2016). While comparing the lipid extracted and whole *Scenedesmus* sp. biomass, the lipid extracted biomass showed a higher amount of biohythane (440 ±19.5 mL/g VS) production during TSAD under mesophilic conditions (Tibbetts et al., 2016). Another reason for the biohythane increment might be the cell wall disruption during the lipid extraction process, which improves the biomass solubilization rate. Again, comparing the Table 2 data of micro- and macro-algal biohythane generation, *Laminaria japonica* (carbohydrate of 56.4%) macro-algae generated comparatively more biohythane (551.3 mL/g VS) than other microalgal species (Shi et al., 2011). To conclude, microalgal strain should be selected based on high carbohydrate content to maximize the biohythane production rate.

3.1.1. Enzymatic effects

Microalgae produce bio-H_2 in dark anaerobic condition through hydrogenases and nitrogenases mechanism. There are four major types of hydrogenases, such as [FeFe]-hydrogenase, [NiFe]-hydrogenase, [Fe]-hydrogenase, and hox-hydrogenase responsible for hydrogen generation (Vignais and Billoud, 2007). Among the hydrogenases, [FeFe]-hydrogenase in microalgal species (*Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas*) and hox-hydrogenase in cyanobacteria catalyses the algae-derived-hydrogen evaluation. Whereas, [NiFe]-hydrogenase is present in anaerobic bacteria and archaea that catalyses both hydrogen evaluation and uptake (Wang et al., 2020). On the contrary, the nitrogenases fixes nitrogens in the atmosphere through ferritin cycle and produces hydrogen as a by-product. Based on the metal cofactors, three types of nitrogenases such as Mo-nitrogenase, Fe-nitrogenase, and V-nitrogenase are responsible for algae-based-hydrogen generation (Wang et al., 2020). After hydrogen fermentation, the microalgal broth is converted to bio-CH_4 through monoxide dehydrogenases enzyme and methyl co-enzymatic metabolism (M-reductases, Coenzyme F420 (F420-0: EC:1.12.98.1; F420-1: 6.3.2.31.6)) of methanogens (Jiang et al., 2019). The enzymatic efficiency would be enhanced through providing optimum operating conditions of anaerobic reactor as well as proper microalgal strain for the evaluation of biohythane.

3.1.2. C/N ratio

Microalgae have a diversified C/N ratio (4.6 - 10.8) among species that significantly impact biohythane productivity (Table 2). The low C/N ratio (<15) negatively affects the balance of carbon and nitrogen inside the reactor, which leads to ammonia inhibition. As a consequence, the digestion process becomes unstable, and biohythane production decreases. To overcome this limitation, co-digestion with carbonaceous materials is suggested to reduce the inhibitory effects. As an example, *Chlorella pyrenoidosa* exhibited higher biohythane productivity (increment of 3.7 folds) when co-digested with *Cassava* starch (C/N ratio of mixed biomass = 25) (Xia et al., 2014). As presented in Table 2, the co-digestion of *Laminaria digitata and Nannochloropsis oceanica* ((C/N ratio of mixed biomass = 20) also doubled the biohythane productivity than mono-digestion of *Nannochloropsis oceanica* (Ding et al., 2016). To conclude, the C/N ratio of microalgae needs to be optimized (C/N ratio= 20-30) using carbonaceous co-substrates for getting the maximum biohythane yield.

3.1.3. Cell wall properties

The microalgal cell wall consists of various types of carbohydrates and proteins (Takeda, 1988). Takeda (1988) reported two types of cell walls of *Chlorella* sp. One comprises glucose-mannose (e.g., *Chlorella luteoviridis, Chlorella fusca, Chlorella minutissima, Chlorella saccharophila, Chlorella protothecoides,* and *Chlorella zofingiensis*), and another contains amino sugar and glucosamine (e.g., *Chlorella vulgaris, Chlorella sorokiniana,* and *Chlorella kessleri*) (Takeda, 1991; Takeda, 1988). While cultivating in wastewater, *Chlorella vulgaris* formed a rigid biopolymer (algaenans) in the cell wall to protect against bacterial extracellular enzymes (Chiu et al., 2015). The microalgae genomes mutation helps to fight against the parasites. Bohutskyi et al. (2014) observed rigid cell walls (polysaccharides) in *Chlorella* sp., *Scenedesmus* sp., and *Nannochloropsis* sp., which resulted in less biohythane productivity. The rigid cell walls hinder hydrolytic enzymes' mechanism during the AD process and lower the production rate. Because of the inhibitory nature of these biopolymers (e.g., sporopollenin and algaenan), proper pretreatment is required to break down biopolymers and increase biohythane productivity.

3.2. Pretreatment methods

The slow degradation of microalgal biopolymers and lipid fractions makes hydrolysis a rate-limiting stage step (Rasit et al., 2015). In order to overcome this limitation, the separation of lipids from biomass before AD is suggested to increase the lipid decomposition rate by breaking the biopolymer cell wall. In the late nineties, Chen and Oswald (1998) stated that the pretreatment of microalgal biomass is an essential step to synthesize bio-CH_4. During the TSAD process, the first stage acts as the pretreatment step for bio-CH_4 production. However, bio-H_2 production remains at low levels due to the slow decomposition of the microalgal rigid cell wall (Nagarajan et al., 2020). Microalgal biomass that contains carbohydrate-rich cell walls is one of the sources of bio-H_2 synthesis.

Therefore, proper pretreatment of biomass would enhance the hydrolysis of cell wall carbohydrates, ultimately increasing bio-H_2 production (Nagarajan et al., 2020). Various pretreatment methods: chemical, mechanical, thermal, and biological, have been introduced for enhancing biohythane production (Shanmugam et al., 2021b). However, if the energy consumption for the pretreatment is more than the net energy generation from biohythane production, the system becomes both economically unfeasible and less sustainable for large-scale operations. Currently, investigations are ongoing to determine a cost-effective solution of biomass pretreatment for enhancing biohythane generation.

3.2.1. *Mechanical pretreatment*

Mechanical pretreatment methods like ultrasonication and microwave radiation (2.5 GHz) have been applied widely to pretreat algal biomass (de Farias Silva et al., 2020). These processes use physical impacts or electromagnetic energy to disrupt microalgal cell wall structures. Thus, close connections between organic substrates and anaerobes are developed. Lately, Kumar et al. (2017) combined two types of mechanical pretreatment (e.g., electrolytic and ultrasonication) on microalgal mixed culture, which showed a 2-fold increase in bio-CH_4 yield than the untreated biomass. The electrolytic pretreatment significantly increased the intracellular organics; whereas, the ultrasonication treatment enhanced the solubilization rate of the complex metabolites (Kumar et al., 2017). However, research has found that microwave pretreatment on microalgae digestion has a positive energy balance (Passos et al., 2013). For example, microwave pretreatment on microalgae-wastewater biomass has increased the bio-CH_4 yield to 60% (Passos et al., 2014). Recently, an innovative approach of microwave pretreatment with nanoparticles has increased pretreatment efficiency. Zaidi et al. (2019) implemented a combination of mechanical (microwave) pretreatment and nanoparticle (iron-oxide) supplementation on microalgal biomass (*Enteromorpha*). They observed a significant increase in the energy yield (20.28 MJ/kg-VS). The microwave pretreatment increased the solubilization rate of metabolites; whereas, the nanoparticles acted as a catalyst to enhance bacterial performance throughout the digestion process. So, the combination of microwave pretreatment with nanoparticles is suggested, which would effectively dissolve the algae cell wall and increase biomass degradation rate to achieve a positive energy balance.

3.2.2. *Thermo-chemical pretreatment*

Over the last few decades, researchers used thermal pretreatment methods (55°-170°C) to solubilize microalgal biomass (Wu et al., 2020a). Currently, advanced technologies such as hydrothermal (110°-150°C) and steam explosion (160°-170°C) are implemented to enhance the hydrolysis process of biomass (Wu et al., 2020a). Wu et al. (2020a) applied hydrothermal pretreatment (150°C) on *Chlorella* sp. for generating biohythane and observed a 12.78% increase in energy recovery. The pressurized heating degrades and oxidizes the biomass, which makes it easy for bacteria to digest. On the other hand, chemical pretreatment uses acid, alkali, Fenton, and oxidizing agents to disrupt the microalgal cell wall, which negatively impacts the environment (Sankaran et al., 2020). Moreover, chemicals require a comparatively long exposure time for hydrolysis, contaminating the biomass (Kendir and Ugurlu, 2018). A novel concept of combining chemical and thermal pretreatment has been introduced to solubilize the biomass faster (Arun et al., 2020). Bohutskyi et al. (2014) investigated a thermo-alkaline pretreatment on *Chlorella* and *Nannochloropsis*, which increased bio-CH_4 yield up to 30-40%. Again, thermo-acidic (e.g., H_2SO_4) pretreatment on *Chlorella vulgaris* showed a 65% increment of bio-CH_4 production (Mendez et al., 2013). Lunprom et al., (2019) reported an enhanced hydrogen and methane yields of 12.5 and 81 mL/g VS from acid-thermal pretreated *Chlorella* sp. The acid thermal pretreatment improved the biodegradability of microalgal cell wall which resulted in the maximum biohythane productivity. The thermo-chemical pretreatment can damage the amorphous structure of algae cells, which increases biohythane productivity. So, thermo-chemical pretreatment is suggested to get the benefits of both thermal and chemical treatments and increase energy conversion efficiency.

3.2.3. *Biological pretreatment*

Biological pretreatment is considered an eco-friendly method, which uses microorganism-derived hydrolytic enzymes to biodegrade microalgal cell-wall. The hydrolytic enzymes break the complex formation of

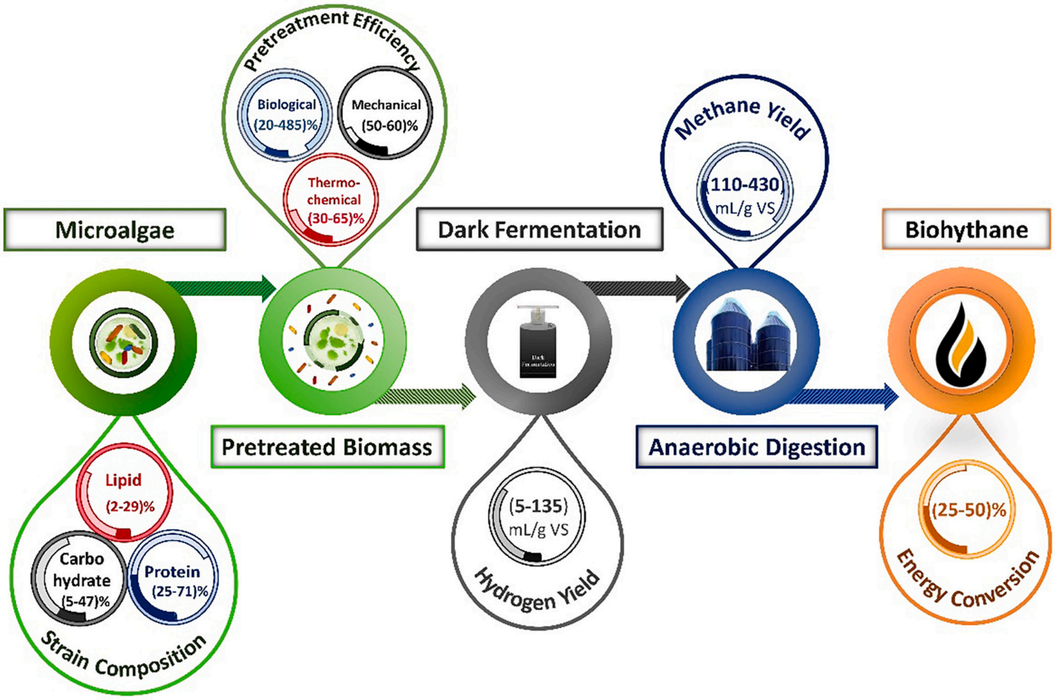

Fig. 3. Potentiality of microalgae for biohythane production.

Table 2
Biohythane generation potential from different algal biomass.

Microalgae species	Microalgal phyco-remediation	Growth Rate $mgL^{-1}d^{-1}$	C/N ratio	Carbohydrates (%)	Proteins (%)	Lipids (%)	Experimental Conditions	Highlights	Biohythane Production Potential		References
									H_2 mL/gVS	CH_4 mL/gVS	
Single strain microalgae											
Arthrospira maxima	Textile wastewater	18.97	4.6	13-16	60-71	6-7	PT: Enzyme hydrolysis and bacterial domestication; DF: Mesophilic Batch (35°C), HRT:35h; AD: Mesophilic Batch (35°C); HRT:16d	The cogeneration of H_2 and CH_4 increased energy conversion efficiency to 27.7%.	78.7	109.5	(Cheng et al., 2011; García-López et al., 2020)
Chlorella sp.	MWW	50	7.06	19.5	43.2	28.8	PT: Hydrothermal (150°C), 0 minute; DF: Mesophilic Batch (37°C), HRT- 48h; AD: Mesophilic Batch (37°C); HRT: 24d	TSAD and hydrothermal pretreatment enhanced energy recovery to 64.32%.	5.15	434.38	(Wu et al., 2020a)
Chlorella pyrenoidosa	Starch processing wastewater	630	4.6-8.2	26	57	2	PT: Steam heating with dilute H_2SO_4; DF: Mesophilic Batch (35°C), HRT: 48h; PF: Zeolite treatment of supernatant+ solid residue of DF–> Mesophilic Batch (30°C), HRT: 96h; AD: Mesophilic Batch (35°C), HRT: 30d	Three-stage digestion obtained an energy conversion efficiency of 34.0%.	75.6^{DF} 122.7^{PF}	186.2	(Tan et al., 2019; Xia et al., 2013a)
Chlorella Sorokiniana	MWW	2.8[a]	5.3	23.3	39.3	29	PHP: Continuous white light at 35–40 μ-mol photons m-2 s-1 for 72h; PT: Sonication (200 Watts at 80% amplitude for 10 min), HRT: 96h; AD: Mesophilic Bench-scale (30°C), HRT: 42d	Sonication pretreatment improved the bio-CH_4 generation by 25.7% than untreated biomass.	41.34[d]	388	(Beltrán et al., 2016; Pongpadung et al., 2018)
Scenedesmus sp.	Brewery effluent	2.6[a]	6	37.7	30	12.6	PT: 8 g/L NaOH at 100°C; DF: Mesophilic Batch (37°C), HRT: 70h; AD: Mesophilic Batch (37°C); HRT: 50d	Alkali-thermal pretreatment enhanced the biohythane yield.	46	354	(Yang et al., 2011)
Lipid extracted Scenedesmus sp. *AMDD.*	-	-	6.1	47	40	0.7	PT: 4% (w/w) pre-treated with 8 g/L NaOH at 100°C for 8h; DF: Mesophilic Batch (37°C), HRT: 65h; AD: Mesophilic Batch (37°C) HRT: 37.5d	The TSAD increased the CH_4 yield by 22% more than the SSAD	46 ± 2.4	393.6 ± 19.5	(Yang et al., 2011)
Scenedesmus obtusiusculus	Meat processing wastewater	500	7	28	25	20	PT: Dilute acid-thermal pretreatment (100 °C, 1.7 h, 3% HCl, 30 g TS/L); DF: Mesophilic Batch (37°C), pH-7-7.5; AD: Mesophilic Batch (37°C), pH-8.5	Acid-thermal pretreatment increased the bio-H_2 yield by 1.7-fold and bio-CH_4 yield by 1.3-fold.	48	296	(Rincón-Pérez et al., 2020)
Nannochloropsis oceanica sp.	Palm oil mill effluent	1.27[a]	7.3	33.3[g]	15.2[g]	51.5[g]	PT: Microwave (80°–180°C for 5–25 min) with dilute H_2SO_4 (0–2.0% v/v); DF: Mesophilic Batch (35°C), pH=6; PF: Zeolite treatment of supernatant for removal of NH4++ solid residue of DF–> Mesophilic Batch (30°C), pH=7; AD: Mesophilic Batch (35°C), pH=8	The TSAD increased the energy yield by 1.3 times than SSAD.	39^{DF} 150^{PF}	65[j]	(Xia et al., 2013b)
Two-strain microalgae co-digestion											
Scenedesmus, Keratococcus, and Oscillatoria	Anaerobic digestate	-	6.5[i]	20	50	19	PT: Thermal (90°C) acidic (1% H_2SO_4) hydrolysis for two h; DF: Mesophilic (36°C) horizontal shaking (150 rpm) Batch reactor, HRT: until H_2 production stopped; AD: Mesophilic	Co-digestion of microalgal biomass showed energy recovery of up to 15.9 kJ/g-VS.	45	432	(Carrillo-Reyes and Buitrón, 2016)

(continued on next page)

Table 2 (*continued*)

Microalgae species	Microalgal phyco-remediation	Growth Rate $mgL^{-1}d^{-1}$	C/N ratio	Carbohydrates (%)	Proteins (%)	Lipids (%)	Experimental Conditions	Highlights	Biohythane Production Potential		References
									H_2 mL/gVS	CH_4 mL/gVS	
							(36°C) horizontal shaking (150 rpm) Batch reactor, HRT: until CH_4 production stopped				
Macro- and microalgal co-digestion											
Laminaria digitata and Chlorella pyrenoidosa	Petroleum wastewater	-	20^{i}	93.0^{e} 33.7^{f}	7.8^{e} 45.3^{f}	1^{e} 21^{f}	DF: Mesophilic Batch (37°C), HRT: 65h; AD: Mesophilic Batch (37°C)	The co-digestion increased energy conversion efficiency up to 57.1%.	97	224.3	(Ding et al., 2016)
Laminaria digitata and Nannochloropsis oceanica	POME and Heavy metal removal	-	20^{i}	93.0^{e} 33.3^{g}	7.8^{e} 15.2^{g}	1^{e} 51.5^{g}	DF: Mesophilic Batch (37°C), HRT: 65d; AD: Mesophilic Batch (37°C), HRT: 26d	The co-digestion increased energy conversion efficiency up to 70.9%.	94.5	295.9	(Ding et al., 2016)
Single strain macroalgae											
Laminaria japonica (sea-weed)	Crude-oil contaminated wetlands	-	16	56.4	8.4	1.6	DF: Mesophilic (35°C) condition in ASBR, HRT: 6d; AD: Mesophilic (35°C) condition in UASBr OLR: 3.5g COD/L/d, Operating period- 90d	TSAD showed an enhanced energy yield of 17.5 kJ/g-VS.	87.8	463.5	(Shi et al., 2011; Yin and Wang, 2018)
Cyanobacteria											
Microcystis	Wastewater (Heavy metal removal)	2191^{b}	8	15.4^{c}	24.3^{c}	-	PT: Steam heating with dilute H_2SO4 (1% v/v); DF: Mesophilic Batch (35°C), HRT: 48h; PF: Zeolite treatment of supernatant+ solid residue of DF—> Mesophilic Batch (30°C), HRT: 96h; AD: Mesophilic Batch (35°C), HRT: 18d	Cogeneration of biohythane from steam-heated algae biomass increased the energy conversion efficiency by 47%	256.7	253.5	(Cheng et al., 2014)

PT=pretreatment, DF= dark fermentation, PF=photo fermentation, AD=anaerobic digestion, PHP= photosynthetic H_2 production, MWW= municipal wastewater, HRT= hydraulic retention time, h= hour, d= day, ASBR= anaerobic sequencing batch reactor, UASBr= up-flow anaerobic sludge blanket reactor, OLR= organic loading rate, POME= palm oil mill effluent.

[a] g/L.

[b] µg/g.

[c] mg/L.

[d] mL/L.

[e] % of VS for Laminaria digitate.

[f] % of VS for Chlorella pyrenoidosa.

[g] % of VS for Nannochloropsis oceanica.

[i] C/N value of the mixture.

[j] CH_4 mL/g TS.

organic compounds into simple compounds that could be easily consumed by H_2-producing bacteria. While applying the biological pretreatment (e.g., enzymolysis) on *C. vulgaris* before DF, the biohythane productivity of *C. vulgaris* was enhanced up to seven times (Wieczorek et al., 2014). The combined effect of cellulase (Onozuka R-10) and pectinase (Macerozyme R-10) enzyme could effectively degrade the cell wall of *C. vulgaris.* Again, hydrogenogens domestication and glucoamylase enzymolysis pretreatment (24h at 60°C) on *Arthrospira maxima* also exhibited a 1.5 times increment of biohythane productivity (Cheng et al., 2011). The glucoamylase effectively promoted the hydrolysis of cell-wall carbohydrates; whereas, genetically modified hydrogenogens (hydrogenogenic 16S rDNA) showed impressive H_2 producing ability (Cheng et al., 2011).

However, the effectiveness of enzyme hydrolysis depends on the microalgal cell wall characteristics, which vary significantly among species. The use of an enzyme cocktail (e.g., a mixture of cellulases, xylanases, α-amylases, proteases, and amyloglucosidases) is recommended (Hom-Diaz et al., 2016; Prajapati et al., 2015). This enzyme cocktail will increase biomass degradation more than other pretreatment methods (Prajapati et al., 2015). Biological pretreatment has achieved a maximum bioenergy conversion rate at the lowest operating cost among the pretreatment methods. In this regard, biological pretreatment on microalgal biomass needs more investigations to optimize biohythane productivity.

3.3. *Operating parameters*

Due to the low C/N ratio (4-10) in microalgae, proper maintenance of operation parameters is necessary to reduce inhibitors (Fig. 4). pH should be maintained around 5-6 in fermentative reactors and 7-8 in methanogenic reactors to minimize the formation of the inhibitor (e.g., ammonia, sulfide, and toxicants). The pH optimization would also increase the inoculum (anaerobes) performance. At the same time, the ratio of inoculum supplementation has a significant impact on reducing inhibitor formation. Sun et al. (2011) reported that biohythane productivity of *Chlorella* sp. was maximum while the inoculum to substrate (ISR) ratio was 0.3 for DF and 2 for methanogenesis. The *Enterobacter* sp., *Nitrobacter* sp. and *Clostridium* sp. inoculum are reviewed to be effective for microalgal bio-H_2 production and *Methanobacterium/Methanosarcina* inoculum are suitable for microalgal bio-CH_4 production (Sun et al., 2019). The continuous stirred tank reactor (CSTR) configuration is suitable for microalgal TSAD because it improves the anaerobes' performance and helps to maintain optimum pH (Zabed et al., 2020). Temperature is also an essential factor in improving microbial performance, which may be in mesophilic conditions (20 -42°C) or thermophilic conditions (42 -75°C). However, research has shown that thermophilic condition increases bacterial performance resulting in lower HRT (Gebreeyessus and Jenicek, 2016). The HRT values in fermentative and methanogenic reactors vary according to the substrate's characteristics (Solé-Bundó et al., 2018). Typically, the HRT of microalgal digestion requires approximately 18-72 hours for the fermentative reactor and 7-30 days for the methanogenic reactor (Solé-Bundó et al., 2018). To conclude, microalgal digestion requires an optimum set of parameters for each reactor in a TSAD system to achieve maximum biohythane productivity.

4. Wastewater sludge for biohythane production

Recently, wastewater has become a major environmental issue for most countries worldwide (Lüthi et al., 2020). Industrial effluent and domestic wastewater sludge/septage are often dumped into the river without proper treatment, which lowers the dissolved oxygen level of the waterbody (Zheng et al., 2019). On the other hand, this wastewater sludge could be an asset if it is used as a resource for bioenergy generation (Arun et al., 2020; Mahdy et al., 2015). Research on wastewater sludge suggested that sludge could be utilized either in cement industries as a solid waste fuel or converted into bioenergy by thermal and biological processes (Liu et al., 2019; Villamil et al., 2020). One of the prospects of managing wastewater sludge is to generate bioenergy via TSAD. Ting and Lee (2007) investigated on the wastewater sludge TSAD process and reported 500 % more biohythane production (132.44 g/kg dry solids) for TSAD compared to SSAD process. They recommended the positive energy feasibility of sludge TSAD process for large scale-up (Ting and Lee, 2007). In another study, Siddiqui et al. (2011) investigated sludge co-digestion with organic wastes, in which they recommended enhanced biohythane productivity (129.1 mL/g VS bio-H_2 and 617.6 mL/gVS bio-CH_4) and superior energy recovery (13.4 %) for TSAD process. The addition of wastewater sludge with food waste improved the C/N ratio (20) that enhanced the biohythane production and energy recovery process. In the context of wastewater sludge, this section critically analyses the characteristics of wastewater sludge for its applicability in the TSAD process. Finally, the challenges and prospects of wastewater sludge using proper pretreatment methods through TSAD are also reviewed.

4.1. Characteristics of wastewater sludge

Wastewater sludge has various pollutants that affect the anaerobes' performance during digestion (Table 3). Fecal sludge and domestic septage primarily contain organic pollutants suitable for microbial activities (Strande and Brdjanovic, 2014). The organics-rich sludge has a proper nutrient balance, which also improves AD performance. The introduction of sewage sludge in food waste co-digestion improved two-fold productivity of biohythane through TSAD (Zhu et al., 2011). The organic fraction of sewage sludge is dominated by proteinaceous substances and bacterial cells that could be hydrolysed and acidified in the hydrogen reactor and used for bio-CH_4 production in the following methane reactor (Zhu et al., 2011). On the other hand, industrial wastewater has additional concerns about heavy metals and toxicants, negatively affecting the AD process (Alhelou et al., 2019). In order to solve this issue, buffers and trace elements are used for maximizing bacterial growth (Elreedy et al., 2017). Gadow and Li (2020) reported that the TSAD of textile wastewater sludge using bacteria growth media constituted of buffer and trace elements could generate 2750 $L/m^3/d$ bio-H_2 and 1460 $L/m^3/d$ bio-CH_4 from OLR of 6.49 kg $COD/m^3/d$. Therefore, proper analysis of wastewater characteristics is required, and then the microbial consortia should be selected for AD. The major governing factors are the C/N ratio and total solids content in the sludge, which affects the AD performance.

4.1.1. C/N ratio

Carbon and nitrogen are the key elements that are essential for anaerobes' growth. As presented in Table 3, domestic septage (e.g., anaerobic sludge) contains a comparatively higher C/N ratio (20-30) than other types of wastewater sludge. Research has found that the anaerobes' performed better at a high C/N ratio (20-30) (Reyna-Gómez et al., 2019). In this context, domestic wastewater sludge/septage is suggested to be a suitable substrate for AD (Lu et al., 2019). On the other hand, sewage sludge has a comparatively low C/N ratio of 10 (Gao et al., 2020). The waste-activated sludge (WAS) contains low organic carbon (C/N around 5.5) since it is mainly consists of aerobic bacteria (Xia et al., 2018; Kavitha et al., 2016). Consequently, the low C/N value of WAS often generates inhibitory compounds like ammonia during the AD process. To overcome this limitation, Hallaji et al. (2019) increased the C/N ratio of WAS by adding fruit waste and cheese whey and reported better AD performance. Warrajareansri and Wongthanate (2021) optimized the C/N ratio of starch processing wastewater sludge to 30 which improved the biohythane productivity of 206.47 mL/g COD. So, it is suggested to maintain a proper nutrient balance (C/N ratio of 20-30) of wastewater sludge by adding co-substrates and improve AD efficiency.

4.1.2. Solids content

The feedstock's total solids content is the main design parameter that dominates the reactor type selection for the AD process. The wastewater sludge usually has a high moisture content (97-99.5%). So, most of the researchers used either an up-flow anaerobic sludge blanket (UASB) or a continuous stirred tank reactor (CSTR) to maximize anaerobes' performance (Araujo et al., 2018; Wang et al., 2013). UASB is suitable for domestic wastewater due to its low solid content (TS<2%). The integrated CSTR bio-H_2 reactor with UASB for bio-CH_4 is ideal for wastewater sludge because of its comparatively higher solid content (2<TS<12%) (Capela et al., 2008; Zhang et al., 2020). Gebreeyessus and Jenicek (2016) reviewed that the sewage sludge two-staged configurations of combined CSTR with UASB/ABR were more stable than a single-staged CSTR. Liu et al. (2013) reviewed that the stability of wastewater sludge TSAD process that was ensured by operating two-staged configurations of CSTR bio-H_2 with UASB or up flow packed reactor for bio-CH_4 production. Therefore, a two-stage configuration of wastewater sludge digestion is suggested to process biohythane.

4.2. Pretreatment

Wastewater sludge contains various pathogens and anaerobes, which inhibit the TSAD process. Pretreatment plays a significant role in destroying inhibitory organisms and increasing the solubility of volatile solids. A study on thermal pretreatment on fecal sludge showed improved production of bio-CH_4 and inactivation of pathogens (Yin et al., 2016). Recently, Mirmasoumi et al. (2018) compared the performance of ultrasonic and thermal pretreatment on sewage sludge, where the thermal pretreatment showed 13.2% more yield than ultrasonic pretreatment. For minimizing the system energy consumption, low-temperature pretreatment is recommended. The performance of low-temperature thermal pretreatment could be enhanced by merging with alkaline pretreatment. Xiao et al. (2020) compared the high-temperature thermal pretreatment (HTTP) with low-temperature thermo-alkaline pretreatment (LTTAP), where the LTTAP showed 20% higher energy efficiency than the HTTP. In another study, Liu et al. (2020) reported that alkali-thermal pretreatment of sewage sludge not only increased bio-CH_4 production by 33.5% but also reduced the HRT from 25 to 15 days than thermal pretreatment. LTTAP has also improved the bio-CH_4 production of WAS by 308.7% than untreated sludge (Zou et al., 2020). Therefore, LTTAP is recommended for wastewater sludge digestion, which would increase biohythane productivity at lower operating costs.

4.3. *Operating parameters*

The TSAD process of wastewater sludge requires proper maintenance of operating parameters: temperature, pH, OLR, and HRT. Research has shown that temperature significantly impacts the microbial performance of AD (Gao et al., 2020). Mirmasoumi et al. (2018) compared the sewage sludge digestion in both mesophilic and thermophilic ranges, where thermophilic temperature yielded 160.8% more bio-CH_4 than mesophilic temperature. The high temperature (thermophilic range) not only helps the microbes to grow faster but also requires less inoculum. Mirmasoumi et al. (2018) also investigated optimum ISR of 3 for mesophilic AD, whereas ISR of 1 was optimum for thermophilic AD. Besides, the wastewater feedstock possesses an optimum pH range (6-8) that excels in microbial activities (Table 3). DF within pH range of 5-6 and AD within pH range of 7-8 is suitable for bacterial metabolism. In this context, wastewater sludge could be considered as one of the potential substrates to enhance TSAD reactor stability and microbes' performance.

The optimum OLR and HRT of wastewater sludge depend on the inoculum growth rate, reactor type, and feedstock characteristics. Wang et al. (2013) introduced the TSAD process in the CSTR-UASB reactor, where sugary wastewater was converted to biohythane; they also optimized the HRT of H_2 production to 5h and CH_4 production to 15h. The two-phase system could effectively convert 92% of the substrate to biohythane (Wang et al., 2013). So, proper maintenance of these operating parameters is required for the efficient digestion of wastewater sludge.

5. Microalgae-wastewater sludge biorefinery

Over the past few decades, researchers reported wastewater feedstock as a resource based on proper utilization (Bhatia et al., 2020; Zhang et al., 2014). The wastewater sludge might be used as a cultivation medium to produce microalgal biomass and converted to bioenergy (Bhatia et al., 2020; Shahid et al., 2020). The advanced studies also suggested that microalgae consume micropollutants from wastewater and produce high-valued biomass (Ansari et al., 2019). The integrated microalgae-wastewater biomass could be further utilized as a feedstock for bioenergy generation (Fig. 5).

5.1. *Co-cultivation of microalgae and wastewater*

Due to the presence of micropollutants, trace minerals, and disease-creating microorganisms in the wastewater, the untreated disposal into water bodies would potentially contaminate the aqueous culture (Raheem et al., 2018). So, treatment is a must before exposing wastewater to the environment. Since wastewater contains various pollutants and trace minerals (K, Ca, Mg, Fe, Cu and Mn), treatment methods have become costly and economically unfeasible (Frascari et al., 2018). Research has dealt this problem using microalgae-bacteria symbiosis that would act as a double edge solution of pollutant removal and organic-rich biomass production (Ansari et al., 2019). Biofilms produced by microalgae-bacteria consortia could effectively remove ammonia, phosphate and acetate through volatilization (ammonia) and precipitation (phosphorus) in complex with metal ions (Ca, Mg and Fe) (Pacheco et al., 2015). The biofilm could also produce polypeptides called chelating agents which are capable of binding heavy metals (Hg, Cd, and Pb). Thus, microalgae-bacteria consortia could be an excellent alternative to municipal wastewater treatment in terms of toxic heavy metals and organic pollutants removal. In this context, microalgae-wastewater cultivation methods (i.e., high-rated algal ponds (HRAP), photobioreactors (PBRs), algal turf scrubber (ATS), and hybrid cultivation system) have been introduced (González-Fernández et al., 2012). Among these systems, the hybrid system (PBRs with HRAP/ATS system) can maximize the beneficial factors of each cultivating method and enhance wastewater treatment efficiency (Yun et al., 2018). A novel design (hybrid anaerobic baffled reactor (HABR) + PBRs) was introduced by Khalekuzzaman et al. (2020), where HABR effluent (>92% removal of COD and TSS) was used to cultivate microalgae-bacteria biomass (lipid content of 38%). Again, Yun et al. (2018) used PBR and open raceway pond (ORP) hybrid cultivation process that resulted in 40-60% more microalgal productivity and enhanced lipid content.

Moreover, the wastewater sludge also enhances the microalgal flocculation process (bio-flocculation) that could significantly reduce the cost of microalgae harvesting processes (Choi et al., 2020). Leong et al. (2018) used co-cultivation of microalgae and activated sludge (MA: AS=0.75:1) where the co-culture effectively reduced nitrogen concentration in wastewater by 96 % and improved bio-flocculation efficiency of 42 %. Recently, Choi et al. (2020) introduced activated sludge-derived extracellular polymeric substance (ADS-EPS) to bio-flocculate microalgal biomass where ADS-EPS showed up to 87.24% of *Chlorella vulgaris* recovery. Therefore, the hybrid cultivation of microalgae-wastewater sludge is suggested to get an economic benefit over biomass production and wastewater treatment.

5.2. *Co-digestion of microalgae and wastewater sludge*

The microalgae-wastewater sludge could be converted to bioenergy via different physicochemical and biological processes (Arun et al., 2020). Most conversion processes are economically unfeasible due to the high operating costs and low energy recovery rate (Choudhary et al., 2020; Gao et al., 2020). Based on microalgae-wastewater sludge, AD is the eco-friendly and energy-positive method for biofuel conversion (Mahdy et al., 2015). Several technical advancements on AD processes have been investigated over past decades using microalgae-wastewater integrated biomass for bio-CH_4 production (Solé-Bundó et al., 2020). However, the production of either bio-H_2 or biohythane from microalgae-wastewater sludge has not been implemented yet. As presented in Table 4, the co-digestion of microalgae-wastewater sludge significantly increases the bio-CH_4 conversion rate. The optimum mixing ratio of primary sludge (PS) to microalgae is reported to be 75% of the total volume of biomass (Solé-Bundó et al., 2018). As an example, among different mixtures of primary sludges (PS) and microalgae (MA), the mixing ratio of MA: PS=25:75 showed the best result (460 mLCH_4/g VS) (Solé-Bundó et al., 2019a). However, while adding fat, oil, grease (FOG) in the MA: PS (50-50 VS) mixture, bio-CH_4 productivity increased by 42% because of the C/N ratio (18) increment (Solé-Bundó et al., 2020). Therefore, the C/N ratio should be optimized using microalgae-wastewater sludge co-digestion to improve biohythane production.

5.3. *Solid digestate to biofuels*

The economic management of the AD system requires the proper utilization of residue digestates. As AD reduces about 40-80% of volatile solids, some portion of the organics still remains in the digestate. The organics in digestates could efficiently be converted into biofuels using either microbial electrochemical technologies (MET) or thermochemical conversion methods (TCC) (Rezaee et al., 2020; Sciarria et al., 2019). The MET requires a continuous supply of electricity to operate, whereas

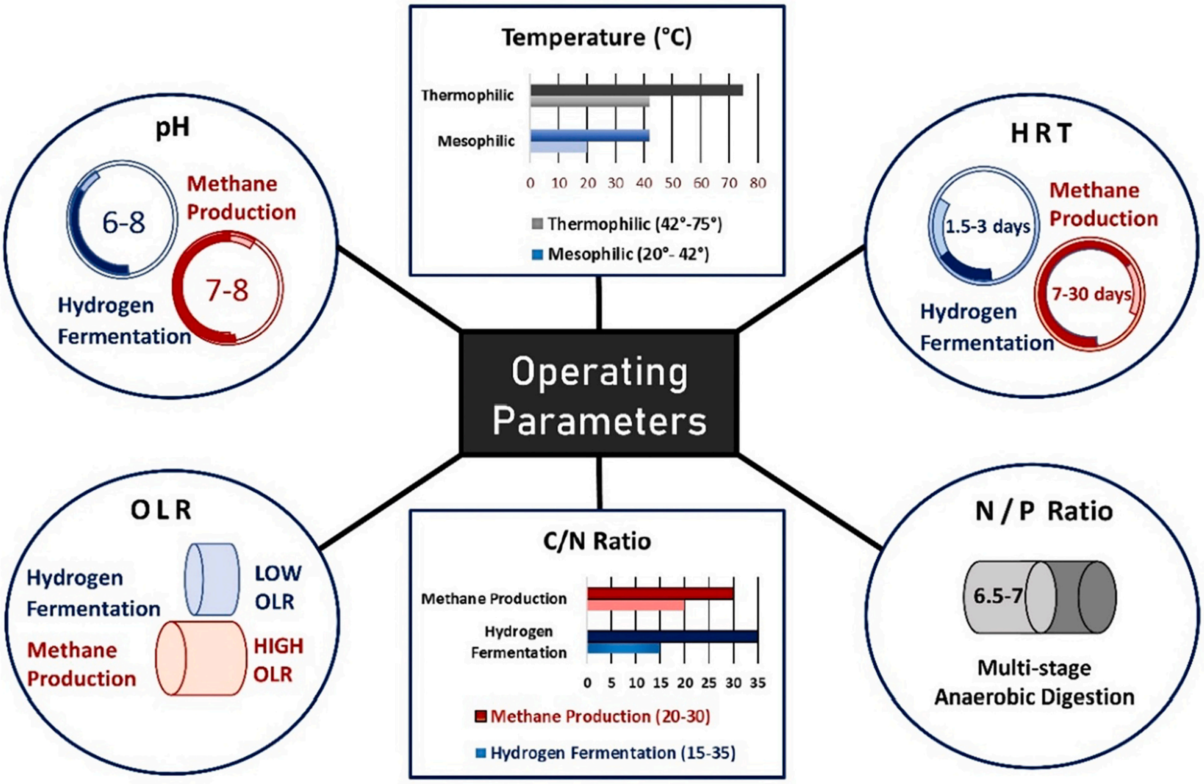

Fig. 4. Operating parameters during two stages of the microalgal AD process.

TCC requires high temperature and high pressurized conditions inside the reactor to facilitate the chemical reactions (Choudhary et al., 2020; Rezaee et al., 2020). Conventionally, researchers used gasification, pyrolysis, and hydrothermal carbonization on solid digestate to produce high-value biofuels (Chang et al., 2020; Miliotti et al., 2020). These processes are effective for anaerobic digestate of lignocellulosic and earth biomass due to their low moisture content (Feng and Lin, 2017). In the case of microalgae-wastewater sludge-derived digestate, the moisture content remains over 90%; thus, most of the TCC processes become economically unfeasible (Naqi et al., 2019). However, one of the thermochemical approaches, named hydrothermal liquefaction (HTL), utilizes moisture as a reaction media (i.e., solvent) at high temperature

Table 3
Characterization of wastewater biomass.

Type of sludge	Collection Zone	Total solids (%)	Volatile solids (% TS)	COD g/L	N/P ratio	C/N ratio	pH	Pollutants	References
Fecal Sludge	Warangal, USA	11.8	63.7		5.34	9.42	7±0.2	Coliform, bacteria, and pathogen	(Krueger et al., 2020)
	Narasapur, USA	10.7	47.4		1.65	11.14	7±0.1		(Krueger et al., 2020)
Domestic Septage	Khulna, Bangladesh	0.06 (TSS)	41 (VSS)	0.6 ±0.2	2.52	27.7	8.1 ±0.2	Pathogen and coliform, Ascaris eggs	(Khalekuzzaman et al., 2018)
	Lowell, USA	8-10	55-58	23.7 ± 1.3		36.2	6.59		(Lu et al., 2019)
	USA	11	32-81			20	6-8.8		(Schneiter et al., 1984)
	Cameron	3.7		31		30	7.9 ±0.4		(Nikiema et al., 2014)
Industrial sludge	Portugal	20.2	46	105 ±1.9			7.46 ±0.1	Heavy metal	(Capela et al., 2008)
Primary Sludge (PS)	Taiwan	5-9	60-80		5	10	5-8	Organic micro pollutants	(Tyagi and Lo, 2013)
	Jharkhand, India	8.7	60-80						(Bora et al., 2020)
	Québec City, Canada	9.5	18.8		1		5.61		(Zhang et al., 2014)
Secondary Sludge	Taiwan	0.8-1.2	59-68		4.8		6.5-8	Organotins, phenyltins	(Tyagi and Lo, 2013)
	India	0.83-12	30.88		1.875		5-8		(Pathak et al., 2009)
Waste Activated Sludge (WAS)	Nanjing, China	2.7	59	13.5 ±0.4		5.5	6.8 ±0.1	Bacteria, Heavy metal contaminations	(Huang et al., 2016)
	Bangkok, Thailand	1±0.1	80±1	11.7 ±0.4		5.5	6.7 ±0.2		(Seng et al., 2010)
A mixture of PS and WAS	Shenzhen City, China	19.22	64.6			7.3	7.7	Organotins, phenyltins, Coliforms	(Du et al., 2020)
AD Sludge	Loire, France	22.3±0.2	70.2±0.1			10.2	6.9 ±0.1	Organic Pollutants	(Maynaud et al., 2017)

TSS= total suspended solids, VSS= volatile suspended solids.

(280°-370°C) and pressure (10-25 MPa) conditions (Gollakota et al., 2018). Moreover, the presence of calcium and potassium compounds in the digestate would act as a catalyst to enhance the HTL process (Hossain et al., 2022a; Islam et al., 2022). As an output of HTL, biocrude/oil, biochar, and syngas fuel are formed from the metabolites (Hossain et al., 2022b). Okoro and Sun (2020) used HTL on anaerobic digestate and observed enhanced biocrude production performance (HHV of 36.7 MJ/kg). However, the major drawback of this process is the presence of hazardous compounds (i.e., heavy metals, nitrogen, and phosphate) in the HTL effluent aqueous products (Tommaso et al., 2015). Rao et al. (2018) introduced integrated HTL and membrane filtration technology to produce biofuels and treat HTL aqueous products efficiently. Therefore, the residual solid digestate produced in the TSAD could be converted into energy-dense biofuels using the HTL process, meanwhile ensuring the proper energy balance throughout the system.

6. Techno-economic perspective

Biohythane for wheeled vehicles was introduced in the 90s and still has not been commercialized fully. Therefore, offering further insight into the economic value of this green fuel and possible policy implementation can be deemed a revolutionary addition to the biofuel market worldwide (Bolzonella et al., 2018; Kongjan et al., 2018). In contrast, bio-H_2 is already commercialized as jet fuel. Other high-value product generations, such as solid oxide fuel cell (SOFC) materials and bio-CH4, were industrialized for large-scale biogas productions (Chowdhury et al., 2020). However, a recent study demonstrated that biohythane is a higher efficient SOFC material than syngas and bio-H_2 (Veluswamy et al., 2019). This study also presented that a 120 kW SOFC stack has been optimized using biohythane (58% CH_4 + 35 % CO_2 + 7 % H_2) with better performance, minor effects on water-gas shift reactions, and 6% less CO emission compared to bio-H_2 and syngas (Veluswamy et al., 2019). Since biohythane contains a higher amount of CH_4 gas integrated with H_2, the process of biohythane generation automatically gets beneficial for microalgae-wastewater sludge biorefinery compared to mere bio-CH_4 based on total energy recovery and the additional cost savings for bio-H_2 separation (Krishnan et al., 2018). Another techno-economic advantage of biohythane production is that it can be optimized with the existing processing techniques in different industries with very little modification of the process. The bio-H_2 production company, Hydrogen Component Inc., initiated the biohythane in the biofuel market and presented no additional requirement of new infrastructure, storage system upgrade, and engine modification of storing and implementing biohythane as a substitute for compressed natural gas (CNG) for wheeled engines (Bolzonella et al., 2018). Compared to CNG, biohythane displayed better performance for flammability in-vehicle engines due to the presence of H_2 and much lower greenhouse gas emissions. Besides, the calorific value of biohythane is competitive with CNG (Bolzonella et al., 2018). Therefore, for the microalgae-wastewater sludge biorefinery system, biohythane can be considered one of the high-value fuels.

Microalgae cultivation in different types of wastewater (such as a sugar mill, brewery, pulp, and paper mill, slaughterhouse, pharmaceutical, textile, mining, municipal sewage, and others) to accumulate organic and inorganic waste, dye removal as well CO_2 absorption is being introduced in various sectors recently to minimize the waste and greenhouse gases (Hossain et al., 2019; Hossain and Morni, 2020). Wastewater-based microalgae are utilized chiefly for biofuel generations, fish feed, or biofertilizers. Among these applications, biofuel has been presented with higher economic value. Biohythane production is one of the most profitable fuel forms than other biofuels such as biodiesel, bio-oil, biochar, bioethanol, and others due to microalgae's high moisture content. For microalgal TSAD or HTL, the drying process is not required, and bioenergy can be generated efficiently. The addition of potential microbial species can quickly produce biohythane from the mixture and store the biohythane for commercial applications for power and transportation system. A practical study presented that algal biomass grown in 1 kg of BOD from brewery wastewater could generate 1kWh electricity power by the mixture of bio-CH_4 and bio-H_2, and 1kWh of electrical power required to eliminate 1kg of BOD in the activated sludge process in this system. Therefore, the removal process has been obtained with a net cost of zero, and absorption of CO_2 by algal biomass is an additional environmental incentive (Amenorfenyo et al., 2019).

Microalgal biohythane has already been introduced for laboratory-scale research (Bolzonella et al., 2018). Optimization and enhancement of the biohythane efficiency from wastewater-borne algal biomass can be further obtained by comprehensive life cycle cost analysis and life cycle assessment. Then microalgal biohythane can only be ready for the practical implementation of the power and transport sector and commercialization. In addition, co-digestion of algal biomass with other waste biomass, especially the well-established industries with their regular batches of feedstock, is also recommended to be studied further to optimize algal feasibility biohythane economically. For instance, a previous study presented that co-digestion of skim latex serum and palm oil mill effluent can produce a large amount of biohythane, which generated total energy of $1.76x10^6$ GJ/year equivalent to $51x10^6$L gasoline (Kongjan et al., 2018).

7. Challenges and prospects

7.1. *Future of biofuels: biohythane*

The world is in a transitional state where the rise in fossil fuel consumption puts a question on fuels' future. In this context, researches are ongoing for finding out the eco-friendly solutions (Fig. 6) (Nguyen and Khanal, 2018; Vitova et al., 2015). Bio-H_2 is one of the environment-friendly fuels that does not emit any greenhouse gases (Singh and Das, 2018). One of the prospects of bio-H_2 synthesis would be using microalgae, which produces high-quality biomass in a short period (Singh and Das, 2018). A new concept regarding implementing several microalgal fermentative stages (DF before photo-fermentation) and even co-digestion with organic wastes are suggested to generate bio-H_2 (Ding et al., 2016; Xia et al., 2013a). Still, the energy conversion rate of standalone bio-H_2 generation could not achieve economic feasibility (Abbasi and Abbasi, 2011).

Nevertheless, the fermentative broth is an excellent substrate for CH_4 generation (Wang and Zhao, 2009). Therefore, producing CH_4 after the H_2 fermentation process would enhance the energy recovery rate. So, the integrated approach of producing biohythane by combining H_2 fermentation with CH_4 production is one solution to optimize energy conversion efficiency (Schievano et al., 2014). In addition, an effective selection of microalgal consortium, which ensures a better conversion mechanism during the TSAD process, is also essential for the robust production of biohythane. However, more investigations are required to improve the biohythane production potential as follows:

i) Genetically modified microalgae (GMM) for biohythane production would have significant impact on large-scale application. Recent advances in GMM processing (oxygen tolerating mutants) improved the photosynthetic mechanism of microalgae which enhanced biomass quality and productivity (Show et al., 2019). Anwar et al., (2019) investigated that the hydrogenase enzyme uptaking capability of eukaryotic algae could be enhanced through genetic modification which acts as biocatalyst to enhance biohydrogen production. Thus, the genetic and metabolic engineered microalgae would have high prospects of cost-effective and sustainable biohythane production (Ng et al., 2017).

ii) Optimization of co-substrates addition with microalgal biomass would have high prospects to ensure consistent biohythane productivity. Recent intervention of Agri-industrial wastes and sludge co-digestion with microalgae is reviewed to improve the C/N ratio of the mixture and robust biohythane productivity

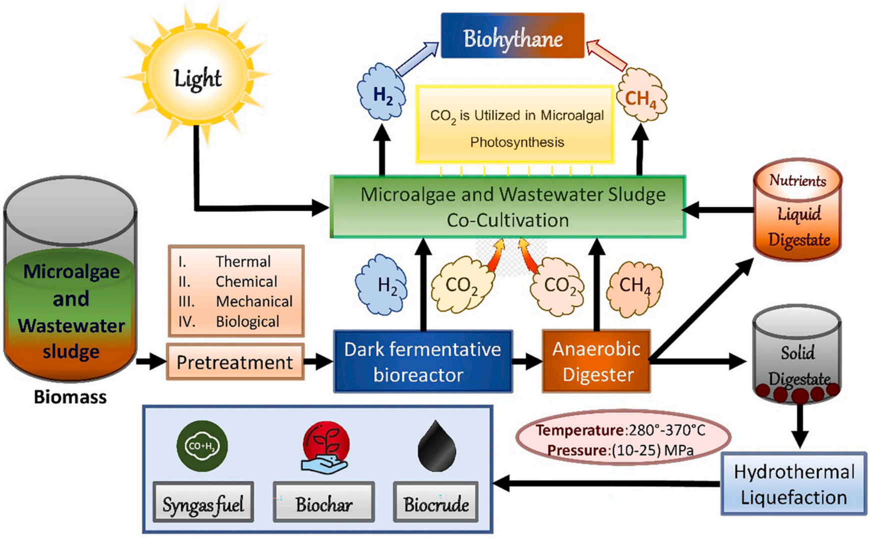

Fig. 5. Microalgae-wastewater sludge to bioenergy.

(Solé-Bundó et al., 2019b). In this context, co-digestion application in large scale plants is recommended to attain a cost-effective biohythane processing.

iii) The improvement of the energy-efficient pretreatment method (biological pretreatment) such as, innovate formations of enzymatic cocktails could be investigated in large scale operation to improve the biohydrogen productivity (Zabed et al., 2019).

iv) An innovative approach by combining two-processing steps (enzyme pretreatment+ co-digestion) could be applied in large scale plants to reduce operation costs and increase productivity.

v) The economic assessment, policy-related drivers and environmental impact of this approach in large scale implementation need more investigation. Vasco-Correa et al., (2018) reviewed that the policy regulations and incentives have been the prime facts behind the steady growth of the AD technology, in both developing and developed countries.

7.2. *Integrated biorefinery economy and circular economy: a zero-waste concept*

Digestate is an AD bioproduct that could either be considered a waste or alternative resource for generating bioresource (Barampouti et al., 2020). Since land disposal of the digestate adversely effected on soil fertility, it has become a major burden to manage as a waste (Nkoa, 2014). Alternatively, the liquid digestate could be used for microalgal biomass production (Hasan et al., 2021a, 2021b). Microalgae has an impressive ability to consume the micro and macronutrients from water and convert them into metabolites (lipids, proteins, and carbohydrates) (Ahmad Latiffi, 2018). Since microalgae requires high nutrient concentration in the growth medium, it has become economically unfeasible for commercial algae production. Anaerobic digestate could supply the necessary nutrients to enhance microalgal productivity and enhance the lipid concentration (Xia and Murphy, 2016). Khalekuzzaman et al. (2019) investigated that microalgae-digestate mixed culture enhanced biomass productivity (algal-bacteria symbiosis) and quality without additional nutrient supplements. Tan et al. (2020) applied anaerobic digestate in the algae culture media and observed 3.2 fold increase in the lipid concentration among microalgae species. In addition, researchers are also implementing novel concepts of microalgae-bacteria microbiome (Paddock et al., 2020), microalgae-microalgae consortia (Behera et al., 2020), and myco-algal consortia (Xu et al., 2017) on digestate to enhance biomass production and digestate treatment process. However, due to the variable nature of digestate parameters (C: N: P ratio, pH, and salinity), more study is required to optimize the digestate application into microalgal culture.

On the contrary, the solid digestate could be converted to bioenergy using physicochemical methods. Miliotti et al. (2020) used both pyrolysis and hydrothermal carbonization (HTC) technology on solid digestate. They observed 72% (w/w) conversion of solid digestate to hydrochar using HTC technology and suggested that the HTC of digestate was more effective than slow pyrolysis (Miliotti et al., 2020). Other thermal conversation strategies like HTL (Okoro and Sun, 2020) and gasification (Chang et al., 2020) are applied on solid digestate to form valuable products: biocrude, biochar, and syn-gas. Hasan et al. (2021a, 2021b) introduced an energy-positive HTL approach, where the photo anoxic baffled reactor (PABR) digestate (algal-bacteria culture) was utilized to produce biocrude. The PABR digestate derived biocrude, and biochar quality was better than standalone microalgae (Hasan et al., 2021a, 2021b). To conclude, the combined approach of biomass cultivation using liquid digestate and then bioenergy conversion from microalgae-digestate using HTL process is recommended for an integrated biorefinery economy and circular economy: a zero-waste concept. In this context, there are scopes for future studies to work on this approach to achieve environmental sustainability and economic viability.

7.3. *Biohythane biological purification: microalgal performance*

Microalgae possess an extraordinary photosynthetic-fixation ability (10-50 times more efficient than terrestrial plants), which contributes to about a 40% reduction of CO_2 from the atmosphere (Maity et al., 2014; Zhou et al., 2017). Studies suggested that the presence of CO_2 in the culture medium directly affects the metabolite formation of microalgae (Shahid et al., 2020; Zhou et al., 2017). Several microalgal strains (i.e., *Scenedesmus obliquus, Chlorella pyrenoidosa, Chlorella reinhardtii*) have an external carbonic anhydrase enzyme that utilizes CO_2 and bicarbonate to formulate organic biomass (Zhou et al., 2017). Therefore, microalgae culture could be utilized to uptake the CO_2 gas during biohythane production in the TSAD system (Miyawaki et al., 2020).

One of the challenges regarding biohythane application for transport fuel is the requirement of a limited CO_2 content (lower than 3%) (Hora and Agarwal, 2018). Seengenyoung et al. (2019) reported about 30-40% CO_2 concentration in the biohythane mixture of biowastes digestion. So, reduction of carbon dioxide is required to develop energy-dense biohythane. Recently, alkaline water absorption, polyethylene glycol absorption, membrane technology, and molecular sieves (activated

Table 4
Co-digestion efficiency of microalgae-wastewater integrated biomass.

Substrates	Mixing Ratio (Microalgae: sludge)	Methods						Highlights	Bio-CH_4 (mL CH_4/gVS)	Energy yield (MJ/kg VS) [a]	References
		Pre-treatment	Reactor Type	HRT	pH	Temperature (°C)	Inoculum				
Microalgae+ Sewage sludge	37:63	-	Batch-conical flask	35	6.9-7.0	37	WWTP digestate	Co-digestion of microalgae increased bio-CH_4 yield by 23%.	408	14.57	(Olsson et al., 2014)
Microalgae + Septic sludge	50:50	-	Batch-200mL serum bottle	30	7.0	35	Mesophilic digestate	The highest CH_4 yield was observed for the initial organic concentration of VS 20g/L.	327.1	11.46	(Lu et al., 2019)
Microalgae+ mixed sludge (primary +secondary)	2:1	Alkali pretreatment (NaOH)	Batch-500mL bottle	23	7-8	37	Anaerobic digestate	Co-digestion of microalgae increased bio-CH_4 yield by 12.4% than mono-digestion of microalgae	343	12.25	(Du et al., 2020)
(Microalgae + Primary sludge) + FOG	50:50	Thermal Pretreatment (75°C for 10h)	BMP= 250 mL glass bottle	60	-	35	WWTP digestate	FOG addition of 20% (VS) increased the C/N ratio of the mixture to 18 as well as bio-CH_4 production by 42%	334	11.92	(Solé-Bundó et al., 2020)
Microalgae + primary sludge	25:75	Thermal Pretreatment (120°C for 40 min)	BMP= batch reactor	30	7	35	WWTP sludge	Co-digestion and pretreatment increased bio-CH_4 yield by 15%	293.4	10.48	(Mahdy et al., 2015)
Microalgae + Secondary sludge	75:25	Thermal Pretreatment (120°C for 40 min)	BMP= 120mL glass bottle	30	7	35	WWTP sludge	Pretreatment increased CH_4 yield by 40%	150	5.35	(Mahdy et al., 2015)
Microalgae+ primary sludge	25:75	Thermal Pretreatment (75°C for 10h)	Batch	20	7.3	37	WWTP sludge	Co-digestion accelerated the AD process as well as increased CH_4 productivity by 2.9-fold.	460	16.43	(Solé-Bundó et al., 2018)
Microalgae +primary sludge	1:9	Thermophilic (55°C) aerobic (HRT=2d)	BMP= 250 mL serum bottle	35	7	35	WWTP (activated sludge) ISR=2	Co-digestion and thermophilic aerobic pretreatment increased bio-CH_4 yield by 36%.	308	11.01	(Damtie et al., 2020)
Microalgae (*Scenedesmus* sp.) + primary sludge	38:62	-	CSTR- 14L	30	6.9	35	WWTP sludge	The system achieved 73% biodegradability and high stability and was observed in terms of pH and volatile fatty acid.	435	15.53	(Serna-García et al., 2020)
Microalgae + primary sludge	25:75	-	CSTR- 2L	20	6	37	Primary sludge and digested sludge	Co-digestion increased bio-CH_4 production by 65% and removed organic contaminates by 90%	330	11.78	(Solé-Bundó et al., 2019a)
Microalgae+ WAS	1:25	Thermal pretreatment (60°C)	Batch-900mL stainless steel bottle	50	7-7.2	37	WWTP digestate	Thermal pretreatment yielded 34% lower bio-CH_4	101.7	3.63	(Avila et al., 2020)
Microalgae (*Chlorella* sp.) + WAS= 41:59 (VS)	41:59	-	Lab-scale digester (5L)	60	6.8-7.0	37	WWTP sludge	Co-digestion of Chlorella increased bio-CH_4 yield by 79%.	468	16.71	(Wang et al., 2013)

WAS=waste activated sludge, FOG= fat, oil and grease, VS= volatile solids, ISR= inoculum to substrate ratio, CSTR=continuous stirred tank reactor, WWTP=wastewater treatment plant.

[a] Energy yield is demonstrated as the total heating value of bio-CH_4.

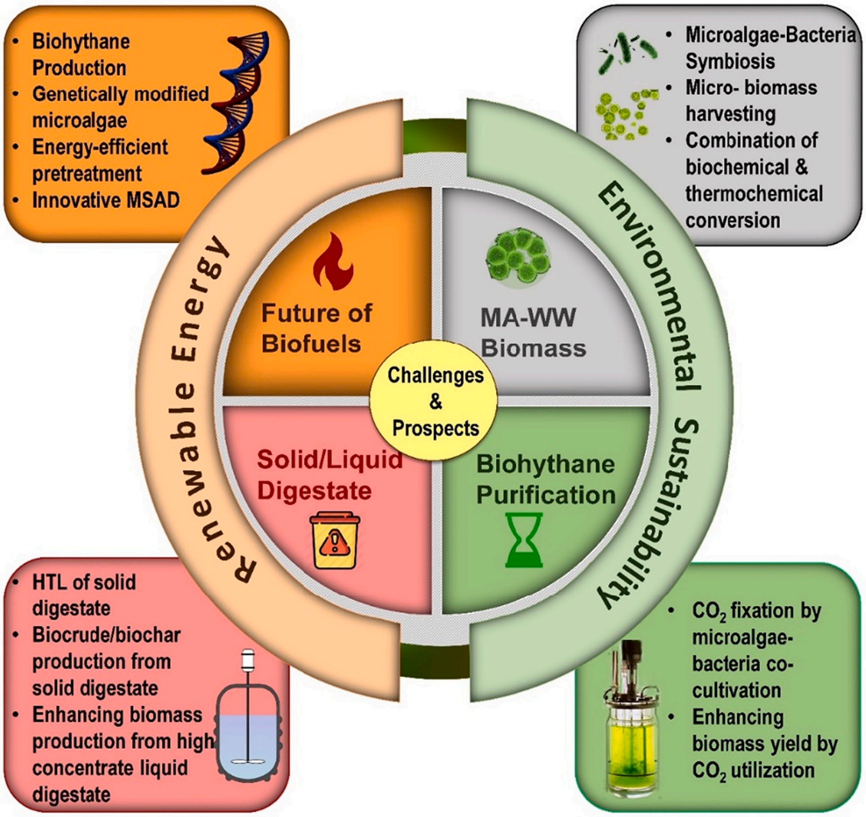

Fig. 6. Challenges and prospects of integrated MA-WW biomass biorefinery approach.

carbon) pressurized absorption technology have been implemented to remove CO_2 from the biogas mixture. However, these processes need significant energy input and cost (Ryckebosch et al., 2011). In contrast, microalgae-bacteria culture in wastewater could photosynthetically biofix the CO_2 and produce biomass, which serves as an energy-saving and economical approach (Kong et al., 2021). Again, proper selection of microalgal sp. is required for maximizing CO_2 consumption (Thi Nguyen et al., 2019). Thi Nguyen et al. (2019) reported that biogas applications in the microalgal-bacteria culture (*Scenedesmus* sp.) could remove up to 98.2% of carbon dioxide concentration from biogas. Therefore, a microalgae-bacteria-based purification system is suggested for ensuring economic biohythane productivity.

7.4. Perspectives on biohythane industrial scale up

Since the TSAD process provides superior waste to energy conversion efficiency than SSAD, industries around the world (Hythane company, Treviso, Sapporo Breweries Limited, etc.) have started to process biohythane at a commercial scale. Some of the challenging factors of two staged process are the requirement of additional energy for heating up two reactors, energy losses during fermentation stage and stage shifting process. There has been a limited study on mass-energy balance analysis on large scale production of biohythane. However, the theoretical energy recovery through the TSAD process is far superior than SSAD process. Treviso council designed full scale plant of biohythane production from wastewater sludge and organic wastes, in which they reported 8341 kWh overall energy production from 2200 m^3 waste feedstock per day. As per the large-scale plant energy and economic evaluation, the energy recovery of this approach is higher compared to provided energy where the instalment cost could be fully recovered within two years of operation. The energy recovery process could be enhanced through implementing a combined approach of biohythane-biocrude production from integrated TSAD-HTL process. Si et al., (2016) introduced a novel strategy for the large scale-up of biohythane-biocrude from lignocellulosic biomass through an integrated process of TSAD and HTL, where the maximum recovery of energy and carbon was up to 79% and 68%, respectively.

The key parameters of TSAD process are pH (5-6 for DF and 7-8 for AD), organic loading rate (6.0 gVS/L/d for DF and 2.0 gVS/L/d for AD), thermal condition (thermophilic for DF and mesophilic for AD), and substrate nutrient balance (C/N ratio of 20 to 30, N/P ratio of 7) that should be maintained during large-scale operation (Ding et al., 2018). During thermophilic DF process in large scale-up, the H_2 partial pressure in the liquid phase causes deactivation of hydrogenase enzyme, which could be solved by nitrogen sparging into DF reactor headspace and enhance H_2 production (Stanislaus et al., 2018). Another challenge is the pH balance and microbial consortium control of the DF reactor, which could be maintained through recirculation of heat treated methanogenic digestate with an optimum recirculation rate of 30 % (Thong et al., 2016). On the basis of wastewater sludge-microalgae co-digestion, the reactor configuration of integrated CSTR fermentation with anaerobic (e.g., ABR, UASB etc.) methanogenic reactor is recommended for effective recirculation (Thong et al., 2016). The reactor should be designed to provide improved mixing characteristics and reduction of overhead gas partial pressure for achieving maximum microbial growth rate. The stability of fermentative reactor could be maintained through CSTR by making sure of providing more HRT than the specific growth rate of bacteria and introducing a gravity settler after the CSTR for bio-H_2 producer retention. The fermentative bacteria grow well in suspended growth media with homogeneous mixing condition and so CSTR would be economical to achieve high bio-H_2 productivity. In terms of methanogenesis, the attached growth media is much effective than suspended growth media, which could form high-rate biofilm system to maximize VFA degradation and bio-CH_4 productivity (Demirel and Yenigün, 2002). At the same time an optimum level of pH (7.5) needs to be maintained by using buffering solution for reactor stability (Demirel et al., 2010). TSAD have been reported to support a superior stability and robustness of the methanogenic step which could be able to cope with higher organic loading rates when compared to conventional SSAD (Hans and Kumar, 2019). The anaerobic digestate sludge could be effectively utilized for biocrude production via HTL technology, which are currently implemented in large scale plants of EPA's Water Engineering Research Laboratory, Cincinnati, Ohio, USA and Organo Corp., Japan (Chen et al., 2020). Thus, the combined approach of wastewater sludge-microalgae to biohythane-biocrude industrial scale production is recommended for an integrated biorefinery economy and circular economy: a zero-waste concept.

The worldwide development of biohythane production from sludge-microalgae is on the rise due to the increasing rate of wet feedstock production (359.4 billion m^3/yr. of wastewater sludge) and the increasing demand of gaseous biofuels (Jones et al., 2021). In this perspective, there has been rapid development on the large-scale biogas fermentation technology that provides a platform for the progress of biohythane. The current large-scale biomethane could be easily converted to biohythane system by upgrading the reactor configuration. The beneficial prospects of biohythane over CNG fuel are fuel efficiency and pollutant reduction that could be the cleaner pathway of waste reduction and second-generation biofuel production.

8. Conclusions

The TSAD provides an optimized growth environment for anaerobes to maximize microbial efficiency among the anaerobic digestion processes. As a consequence, the co-generation of biohythane has a higher energy recovery potential than other conversion processes. The techno-economic assessment also suggests that biohythane is a higher efficient SOFC material than other bio-based fuels.

Microalgal biomass shows higher biohythane production potential for both bio-H_2 in DF and bio-CH_4 in AD. Addressing the challenges of microalgae cultivation, this study summarized the algae-digestate bacteria co-cultivation and recommended their beneficial symbiotic nature to enhance biomass productivity. However, microalgal metabolism to synthesis biopolymer enhances during algal-bacteria co-cultivation which results into hampering the production of bio-H_2. Hydrolytic enzyme pretreatment is reviewed to have higher potential to break the biopolymers and increase the bio-H_2 productivity. Another problem associated with microalgae is the low C/N ratio of biomass which creates inhibitory compounds during TSAD. The co-digestion of microalgae-wastewater sludge would increase the C/N ratio of the mixture to the optimum level, which is reviewed to increase biohythane production. This review also recommended algae-bacteria culture to be used for CO_2 removal from the biohythane mixture as an eco-friendly and cost-effective solution to purify biohythane.

The present review emphasized the prospects of an integrated biorefinery economy and circular economy: a zero-waste concept through TSAD of microalgae-wastewater sludge, HTL of solid digestate, and biohythane purification using algae-bacteria co-culture. The biological pretreatment and co-digestion of microalgae-wastewater sludge have achieved a higher energy conversion rate and reduced inhibitory metabolites; whereas, HTL is recommended to process high value products such as biocrude and biochar from digestate. The cogeneration of biohythane-biocrude using microalgae-wastewater sludge might be the double-edge solution against waste-sludge management and renewable energy production.

CRediT authorship contribution statement

Sadib Bin Kabir: Conceptualization, Methodology, Resources, Investigation, Data curation, Formal analysis, Software, Writing – original draft. **Md Khalekuzzaman:** Conceptualization, Funding acquisition, Project administration, Supervision, Formal analysis, Visualization, Writing – review & editing. **Nazia Hossain:** Writing – review & editing. **Mamun Jamal:** Writing – review & editing. **Md Asraful Alam:** Writing – review & editing, Supervision. **Abd El-Fatah Abomohra:** Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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