Alternate green approach of spent media utilization for hydrogen and for lipid production

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Abstract:

In a view to uplift the biodiesel industry as a major energy carrier, new approach of minimizing the waste and utilizing generated waste needs to be explored. The spent media generated from the co-culture system is cost effective and can serve as renewable supplement for mixed-culture based hydrogen (H_2) production and lipid production. Direct conversion of spent media along with crude glycerol (CG) at 20 g/L using heat-shock pretreated wastewater sludge resulted in 38.12 ±0.84 mmol/L of H_2. In another approach, the spent media was used as co-supplement along with fresh media at 3:2 for algal growth, resulting in 0.098 ±0.007 g/L of lipid. The spent media contained dead biomass, residual media nutrients, biomolecules and unutilized glycerol together acting as supplementary source during H_2 and lipid production. According to the closed system results, the H_2 produced ($1.47x10^9$ L of H_2) can be converted into energy ($1.87\ x10^4$ GJ) for electricity ($1.77\ x10^4$ GJ) and heat ($4.32\ x10^3$ GJ). The produced H_2 can be used as in-house energy source and the lipids can be used as third generation feedstock. The study explores the utilization of CG and spent media valorization towards an efficient closed system approach for a competitive biodiesel industry.

Keywords: biodiesel; crude glycerol; hydrogen; lipid; mixed-culture; photo-fermentation

1. Introduction

Significant success in renewable energy has resulted in the commercial utilization of biohydrogen in comparison to fossil fuel utilization with concerns of global climate change [1]. The advantages of hydrogen over commercial fuels resulted with strong support from government policies and increased incentives worldwide [1]. Across the world, a new initiative of bioconversion of crude glycerol (CG) (by-product of biodiesel industry) to hydrogen (H_2) production has been carried out to expand biodiesel industry [2, 3]. The characteristics of CG, such as low market value, feedstock availability [3], with increased reduction state in comparison to other organic wastes; make it suitable for microbial conversion to H_2 over other value-added products [4].

The bioconversion of CG to H_2 can be carried out using co-culture, mixed-culture and photo-fermentation systems. Each of the systems has advantages and disadvantages. The co-culture system works in harmony, reduces the fermentation time, performs complex functions and produces higher H_2 in comparison to mono-culture system [5]. The dark fermentation carried out using mixed-culture system has broader variety of potential substrate, including residuals and waste products during H_2 production [6]. Photo-fermentation offers typical advantages with high theoretical conversion ability and utilization of organic acids (acetate, butyrate) or solvents (acetone, butanol) produced during dark fermentation [6].

The accumulation of organic acids and solvents result in sharp drop in fermentation pH and limit H_2 production during dark fermentation. The spent media containing organic compounds and unutilized substrate with media components is of high interest as promising choice for value-addition [7, 8]. Researchers have carried out combined dark and photo-fermentation to use unconverted substrate/metabolites for complete utilization of chemical energy in spent media [6, 7, 9, 10]. Sustainable utilization of active biomass and spent media

resulted in improved H_2 production [11] along with one-pot green synthesis of nanoparticles [12]. The ethanol and beer producing industry spent waste is valorized into lactic acid by utilizing the free nitrogen content in wastes and eliminating the necessary addition of nitrogen supplement [13]. Production of ethanol can also be carried out by using a simple acid pretreatment step on waste algal biomass resulting in almost 2-fold increased yield in comparison to control experiment using glucose [14]. The spent media generated across different systems with purpose of production of value-added compounds as presented in Table 1.

Table 1 – Spent media generated across different systems for production of value-added compounds

Purpose	**Spent media type**	**Process details**	**Product**	**Ref.**
To complete utilization of chemical energy stored in spent media	After dark fermentation of H_2 production: unconverted metabolites	Photofermentation using *Rhodobacter sphaeroides* O.U.001	H_2 production: with 81% of the acetic acid utilization from spent media	[6]
Sustainable utilization of waste from H_2 production	After dark fermentation of H_2 production: spent and active biomass	Dark fermentation using *Enterobacter aerogenes*	H_2 production: improved from 13.37 to 57.98%	[11]
Faster mass scale one-pot green synthesis	After dark fermentation of H_2 production: waste	Bioreduction of silver ions into silver nanoparticles	Silver nanoparticle: improved formation with high purity	[12]

Purpose	Spent media type	Process details	Product	Ref.
	culture	(AgNPs)		
Bioconversion of waste algal biomass into ethanol	After the growth of algae: harvested algal biomass	Ethanol production using *Clostridium phytofermentans*	Ethanol production: 4.6 g/L	[14]
To resolve the problem of waste utilization	After ethanol production: distiller's grain	Dark- and Photo-fermentation for H_2 production	H_2 production: 1.5-3-folds higher	[7]
To reuse volatile fatty acids-rich spent medium	After H_2 production by dark fermentation	Dark- and Photo-fermentation for H_2 production	H_2 production: Maximum yield (58 mmol) from the spent medium during photo-fermentation	[9]
Valorization of waste substrates from bioethanol and beer production	After ethanol and beer production: wasted bread, wasted potato stillage and brewers spent grain hydrolysate	Lactic acid production by *Lactobacillus rhamnosus* ATCC 7469.	Lactic acid with 1.54 g/L/h production: appropriate use of free alpha amino nitrogen content in wastes	[13]

Purpose	Spent media type	Process details	Product	Ref.
Valorization of spent media for H_2 and lipid production	After H_2 production by dark fermentation	Mixed-culture for H_2 and photo-fermentation for lipid production	H_2 production: increased by 29.53% Lipid production: as low-cost media co-supplement	This study

H_2 production is only able to utilize 30-40% of substrate with remaining 60-70% used for metabolite production [1]. Bioconversion of CG (~22 kg) to 1 kg of H_2 generates around ~8700 L of spent media consisting of organic carbon, total nitrogen, media components, biomass, metabolites in distilled water [1], if unutilized at large-scale is challenging owing to the cost involved [9]. Thus, spent media utilization will minimize the additional media components usage. This study aimed at H_2 production by mixed-culture and lipid production by photo-fermentation using CG fermentation waste.

In the present study, the spent media obtained after co-culture system of H_2 production was proposed as media supplement during mixed-culture H_2 production. The mixed-culture system was studied for the first time during H_2 production with biodiesel primary sludge (BPS) as inoculum along with spent media and CG as substrate. In another approach, the fresh media was replaced with spent media and used for algal growth for lipid production. In the interest of algae as third generation feedstock for the biodiesel fuel and to minimize the cost of TAP (Tris-Acetate-Phosphate) growth media, spent media was used for *Chlamydomonas reinhardtii* growth during lipid production. In order to utilize maximum energy recovery from spent media, a closed system approach has been proposed.

2. Materials and Methods

Crude glycerol as substrate

The animal by-products from food processing, superstores and restaurants are recycled for biodiesel production from Rothsay, Canada [15]. The generated crude glycerol and wastewater sludge by Rothsay, Canada are used in this study. The CG comprised (*w/w*): 23.6% glycerol, 35.9% carbon, 5.7% moisture, 3.2% nitrogen, 3.1% ash, <1.0-0.5% methanol and 67.56% matter organic non-glycerol (MONG) [16]. The pH of the crude glycerol was around 3.4 ± 0.1 [4].

Chemicals and reagents used in this study are purchased from Fisher scientific, VWR and Lallemand, Canada [16].

Seed inoculum for mixed-culture

Rothsay, Canada carries out rigorous wastewater treatment prior to discharge treated effluent in local waterbodies [15]. The purification system generates biodiesel primary sludge (BPS) (settling solids), which was used as seed inoculum for mixed-culture system of H_2 production. The BPS was stored at 4 ^{0}C, prior to pretreatment to produce H_2 using CG. Likewise, wastewater secondary sludge (WSS) collected from Quebec Urban Community (QUC) wastewater treatment plant (WWTP) (Quebec, QC, Canada) was analyzed as possible seed inoculum along with BPS.

A comparative study of acid, alkali, chloroform, heat-shock and microwave pretreatment on wastewater sludge was carried out using CG as substrate. The increased H_2 production resulted in heat-shock pretreatment in comparison to other methods [4]. In this study, BPS and WSS was subjected to heat pretreatment. Around 50 mL of BPS and WSS was taken in two separate 150 mL serum bottles, pure nitrogen gas was sparged (3-4 min) to create anaerobic environment; the bottle was sealed using pre-inserted septa and transferred to pre-

set 100 ^{0}C Isotemp Standard Lab Ovens for 15 min [4]. The cooled treated BPS, WSS and mix 1:1 (BPS:WSS) was used as inoculum and transferred using sterile syringe at varying volumes for H_2 production.

Algae pre-culture media and inoculum development for photo-fermentation

The green algae, *Chlamydomonas reinhardtii* is being currently used in the H_2 production [5] and considered as model organism for accumulation of energy rich compounds, such as lipids [17] [18]. The green algae, *C. reinhardtii* was grown using 100 mL of TAP (Tris-Acetate-Phosphate) growth medium (Gibco®, ThermoFisher Scientific, USA) ready-to-use 1X with pH 7.0, under constant agitation of 60 rpm at 20 ± 1^{0}C with continuous illumination of 60-80 µmol/m^2/s throughout 7 days [19].

Hydrogen production using spent media by mixed-culture system

The optimum condition of 20 g/L crude glycerol (CG), 20% (*v/v*) inoculum size (InS) and pH 7.0 from our previous study using wastewater secondary sludge as seed inoculum was utilized [4]. Proposed addition of spent media during the fermentation was carried out for increased H_2 production. The spent media characteristics are presented in Table 2. The spent media obtained after H_2 production was used to make-up the final volume to replace the addition of distilled water. With the presence of unutilized CG in spent media, the CG concentration in the fermentation media was varied across 15, 20 and 25 g/L. The increasing concentration of CG (15, 20 and 25 g/L) was mixed with spent media to make-up the volume to 40 mL. A control experiment using distilled water in the absence of spent media was also carried out. The pH was set at 7.0, transferred to serum bottles, sparged with nitrogen, sealed with pre-inserted septa followed by sterilization at 121 ^{0}C for 15 min in autoclave. The pretreated sludge at 20% (*v/v*) inoculum size i.e 10 mL was transferred to the sterilized media

using sterile syringe under laminar hood to make-up the total working volume of 50 mL. The H_2 production was carried out at 150 rpm at 37 ^{0}C for five days and all the experiments were performed in triplicates. The presented values are the average of triplicates and error bars represent the standard deviation (±) values. During fermentation, at every 24 h, gas sample using a gas tight syringe (1 mL) was collected from the headspace into vacuumed sample vials for hydrogen analysis by gas chromatography (GC). Likewise, after five days, the fermented sample was analyzed for glycerol and end metabolite concentration by GC.

Table 2 – Characterization of spent media

Composition of spent media	
Ethanol (g/L)	0.58 ±0.18
Acetate (g/L)	2.03 ±0.06
Butyrate (g/L)	2.37 ±0.80
1,3-Propanediol (g/L)	0.92 ±0.39
Residual glycerol (g/L)	5.02 ±0.50
pH	5.56 ±0.13

Lipid production using spent media by Photo-fermentation

The TAP growth media is optimized for *C. reinhardtii* culture and is ready-to-use, eliminates the procurement of individual media components, trace elements with tedious media preparation steps. The TAP media was replaced from 50 to 0 mL (50, 40, 30, 20, 10, 0 mL) with addition of spent media (autoclaved) at different volumes from 0 to 50 mL (0, 10, 20, 30, 40, 50 mL) and final mixture was transferred to serum bottles under laminar flow

chamber. The experimental runs (1 to 6) was carried out in aerobic (for lipid production) in triplicates. At the end of incubation, lipid estimation was carried out.

Material and Energy balance calculation

An efficient closed system approach is designed to support the biodiesel industry. In order to valorize the crude glycerol into in-house self-sufficient energy source was evaluated using energy of produced hydrogen and mass balance of spent media. In this study, the energy and mass balance are calculated based on 45 million liter (average: 10-75 million L) of crude glycerol production across biodiesel industry in Canada [20]. Bioconversion of CG into H_2 using semi-continuous fermentation with capacity conversion (240-356 L of cumulative H_2/kg of CG) [21] are considered in the calculation. Further, energy value of produced H_2 equivalent in-terms of energy, electricity and heat [20, 22] are calculated in this study.

Analytical techniques

Hydrogen analysis by GC

During the mixed-culture system, the hydrogen gas sample collected was analyzed using gas chromatography (Varian 3800, USA) with a set-up of thermal conductivity detector (TCD). The PoraPLOT Q® column (Agilent technology, USA) of 3 m width under carrier gas nitrogen at flow rate of 3.5 mL/min was used. During the method run, the injector, column temperature and detector temperature are set at 100 ^{0}C. The area under the curve was converted to volume of gas produced (mmol) in consideration of the experimental conditions, such as temperature and atmospheric pressure [16].

End-metabolites/by-products analysis by GC-FID

The concentrations of glycerol and end-metabolites were determined using GC (7890B GC-Agilent, CA) with flame ionization detector (FID) system. The column used was ZB-WAX

plus with carrier helium gas at 1 mL/min flow rate in a 80−240 ^{0}C temperature profile for 8.4 min run time [16].

Estimation of lipid production

The total lipids at the end of fermentation was extracted from *C. reinhardtii* biomass and determined using gravimetric method as described in [23] [24]. Around 35 mL of fermented media was subjected to centrifugation (4000 *x* g) for 15 min, the cell pellet was separated from the supernatant. Around 800 µL phosphate buffer (0.05 M, pH 7.4) and 400 µm glass beads was added and transferred to cell disruptor for 10 min. To the lysed mixture, 800 µL phosphate buffer, 4 mL of chloroform, 2 mL of methanol was mixed and the lipid was extracted by 15 min of sonication. After sonication, 2 mL each of chloroform and methanol was added and the resulting mixture was made to settle for separation. The bottom organic phase containing the lipids was transferred and equal volume of 5% NaCl solution (1:1 *v/v*) was added. The solvent was subjected to nitrogen evaporation; the left over lipid was calculated and expressed in g/L of medium [23] [24].

3. Results and discussion

Hydrogen production using spent media by mixed-culture system

The mixed inoculum was composed of BPS and WSS at 1:1 ratio. The H_2 production using the optimized condition of (InS: 20% and pH: 7) in case of different CG concentrations of 15, 20 and 25 g/L are presented in the Table 3. The maximum H_2 production was around 38.12 ±0.84 mmol/L for WSS at 20 g/L of CG. The minimum H_2 production was around 18.96 ±0.13 for the mix at 15 g/L of CG as seen from Table 3.

Table 3 – Hydrogen (mmol/L) and 1,3-Propanediol (g/L) production across different seed inocula using variable crude glycerol concentrations (g/L)

Seed inoculum type	Crude glycerol (g/L)	Hydrogen (mmol/L)	1,3-Propanediol (g/L)
Biodiesel primary sludge (BPS),	15	24.51 ±0.20	2.83 ±0.31
	20	27.09 ±0.83	5.62 ±0.11
	25	22.39 ±0.23	6.72 ±0.51
Wastewater secondary sludge (WSS)	15	25.44 ±0.62	3.17 ±0.49
	20	38.12 ±0.84	5.46 ±0.37
	25	33.04 ±0.61	6.52 ±0.26
MIX at 1:1 (BPS:WSS)	15	18.96 ±0.13	1.87 ±0.08
	20	24.06 ±0.45	2.98 ±0.09
	25	19.68 ±0.52	3.45 ±0.14

In case of BPS as seed inoculum, the maximum H_2 production was around 27.09 ±0.83 mmol/L with minimum of around 22.39 ±0.23 mmol/L. Across the three seed inoculum types, the H_2 production increased from 15 to 20 g/L. However, the H_2 production decreased with further increase CG at 25 g/L. The CG at 20 g/L was found to be optimum for the seed inoculum for the increased H_2 production. The optimum condition of (CG: 20 g/L, InS: 20% and pH 7.0) in case of heat treated WSS without spent media resulted in 29.43 ±0.71 mmol/L of H_2 production [4]. In this study, the spent media containing the unutilized CG along with media components benefited with 29.53% increased H_2 production (38.12 ±0.84 mmol/L) .The results matched 32.5% increased H_2 production obtained during use of spent media along with CG by *Enterobacter aerogenes* [11]. The volatile fatty acid rich spent medium acts as effective feedstock for subsequent H_2 production [9]. The spent media contains dead biomass, residual media nutrients, biomolecules and unutilized glycerol together which act as supplementary source for the mixed-culture system for H_2 production.

219 The advantage of mixed-culture system to grow on broader choice of organic waste feedstock
220 requires easy and simple pretreatment conditions. The ability to reuse the spent media during
221 mixed-culture system uplifts the H_2 production making it economical. The impurities in CG
222 have increased inhibition effect on co-culture at concentrations of 15-20 g/L and above [4].
223 However, the seed inoculum from WSS nullified the inhibition effect at 20 g/L of CG with
224 increased H_2 production. The choice of WSS as seed inoculum along with heat-shock
225 treatment proved to be the best combination for the utilization of spent media along with CG
226 as substrate for increased H_2 production. In the case of seed inoculum of BPS, the H_2
227 production reached a maximum of 27.09 ±0.83mmol/L at 20 g/L of CG. The objective of
228 using the BPS was to identify the microbial community able to degrade glycerol at higher
229 concentration, as BPS is in contact with residual glycerol after biodiesel production.
230 However, the BPS possessed the ability to produce higher 1,3-propanediol (1,3-PD) (6.72
231 ±0.51 g/L) across other seed inocula as seen in Table 3. While monitoring the H_2 production
232 in most cases, production of 1,3-PD is also determined as they are important metabolites of
233 the glycerol fermentation pathway. The production of 1,3-PD increased during the glycerol
234 fermentation as reductive pathway was favored over oxidative pathway with decreased
235 production of H_2 [16]. This was true as CG was 25 g/L, H_2 production decreased to 22.39
236 ±0.23 mol/L with increased production of 1,3-PD reaching a higher value of around 6.72
237 ±0.51g/L in case of BPS as seed inoculum. The ability to degrade glycerol at higher
238 concentration and produce a value-added compound 1,3-PD, in one way or the other will help
239 the biodiesel industry. The sludge mix (BPS:WSS) at 1:1 ratio as seed inoculum was also
240 investigated for H_2 production. The maximum H_2 production was around 24.06 ±0.45
241 mmol/L and maximum 1,3-PD production was around 3.45 ±0.14 g/L in case of sludge mix
242 as seen from the Table 3. In order to exploit the property of H_2 production from WSS and
243 1,3-PD from BPS, the mixed seed inoculum was investigated. The ratio of 1:1 was not

sufficient in exploiting the property of both the seed inocula. A combination of different ratios can be tested for increased H_2 and 1,3-PD production.

The WSS is the final repository of various complex microorganisms possessing the property of working at higher substrate concentration with ability to degrade complex substrate and hence providing capability to reutilize the spent media with ease [4, 25]. The sludge generated from wastewater treatment plant is composed of microbial matter beneficial for anaerobic digestion during H_2 production along with H_2 consuming microorganisms [26, 27]. Heat-shock pretreatment has been currently tested as a simple pretreatment step to screen and accelerate growth rate of H_2-producing species for increased H_2 production [27, 28].

Lipid production using spent media by algae

C. reinhardtii emerged as model organism for the synthesis of bioenergy carriers for the efficient conversion of light, water and CO_2 into renewable energy applications, such as H_2 and lipids [18]. *C. reinhardtii* is gaining attention to test cultivation strategies in increasing lipid yields for biodiesel production [17]. The growth conditions of *C. reinhardtii* were optimized by [19] and used for the photofermentation using different volumes of spent media for lipid production as presented in Table 4. Lipid production using *C. reinhardtii* at different volumes of spent and fresh media (TAP growth media) was carried out with analyses of metabolites are presented in Table 4.

Table 4 –Experimental runs using varied volumes of spent (mL) and fresh media (mL), with the percentage of utilization for acetate, butyrate and glycerol at the end of photofermentation (7-days) for lipid production

Experimental Runs	**Spent Media**	**Fresh Media**	**Acetate utilization**	**Butyrate utilization**	**Glycerol utilization**	**Lipid production**

	(mL)	(mL)	(%)	(%)	(%)	(g/L)
1	0	50	70.00 ±1.37	3.58 ±1.50	26.52 ±3.25	0.045 ±0.006
2	10	40	95.39 ±0.47	25.52 ±1.79	38.84 ±1.06	0.067 ±0.006
3	20	30	97.44 ±0.56	28.45 ±1.14	42.62 ±2.12	0.072 ±0.002
4	30	20	99.03 ±0.38	47.88 ±0.73	55.66 ±1.18	0.098 ±0.007
5	40	10	98.81 ±0.41	41.56 ±1.45	28.92 ±1.38	0.036 ±0.004
6	50	0	96.80 ±0.42	16.50 ±1.29	23.23 ±0.49	0.010 ±0.002

The purpose was to reduce the utilization of fresh media (FM) and utilize the spent media (SM) during lipid production. The maximum lipid production was around 0.098 ±0.07 g/L for the mixture of (SM: 30, FM: 20) and the minimum was around 0.010 ±0.02 g/L in case of (SM: 0, FM: 50). In the presence of completely fresh media (50 mL), the lipid production was around 0.045 ±0.006 g/L in comparison to 0.010 ±0.002 g/L with complete spent media (50 mL). With the increase in the concentration of the spent media from (0 to 30 mL), the production of lipid increased from 0.045 to 0.098 g/L. However, with further increase from 30 mL of spent media, the lipid production decreased reaching a minimum of 0.010±0.002 g/L. The spent media composition with organic/solvents and unutilized glycerol at minimum concentration tend to favor the growth of *C. reinhardtii*. With further increase in the volume of the spent media, the concentration of these compounds increased resulting in an inhibition of the growth of *C. reinhardtii* with decreased lipid production. The maximum lipid production of 0.098 ±0.007 g/L was on higher side in comparison to (0.05 g/L) [24] and matched the results across different studies [18] [29]. The highest lipid productivity in case of run 4 was around 14 ±0.007 mg/L/day considering the algal biomass. The lipid productivity was within the range (0.54 to 16.2 mg/L/day), obtained across *Scenedesmus* spp. (green microalgae) grown in fermented swine [30] and artificial wastewater [31].

The lipid productivity obtained in this study is very low in comparison to other microalgae grown in various wastewater conditions across the study [32]. The microalgae can be cultivated on variety of carbon sources, such as municipal, agricultural and industrial wastewater, unlike yeasts requiring sugars, amino acids and nutrient supplement for lipid production [32] [33]. The microalgae growth and lipid content depends on various factors, such as choice of algae, nutrient starvation, temperature change, heavy metal stress, light irradiation, genetic engineering, cell harvest, photobioreactor with efficient mass and light transfer [34] [35] [36]. High lipid content occurs under environmental stress, such as nutrient limitations, which often result in low cell growth or may lead to lower lipid productivity [36]. The future of microalgae lipid production depends on the improvement in the cultivation using costly genetically engineered organisms and energy intense harvesting technologies [17]. However, the advantage of using spent media for microalgae growth is economical and sustainable, further optimization of above factors can be carried out to increase the lipid productivity.

The spent media is composed of acetate and butyrate, which are utilized as substrates during photofermentation. In the case of *C. reinhardtii* growth media, the external addition of organic acids is carried out along with complex media and micronutrients during the lipid production. *C. reinhardtii* possesses the ability to grow on acetate and was supplemented with glacial acetic acid with 20 mM (1.2 g/L) of carbon source in the minimal medium [37]. In order to determine the metabolite utilization across the spent media during lipid production, analysis of acetate, butyrate along with glycerol concentration before and after lipid production was carried out. The results of the acetate, butyrate and glycerol utilization percentage (%) at the end of photofermentation (7-days) for lipid production are presented in Table 4.

The results across the lipid production suggested that the mixture volume for run 4 (SM: 30, FM: 20) produced maximum lipid in comparison to other mixture volumes. In case of run 4, the percentage utilization for glycerol was around 55.66%, butyrate was around 47.88% and acetate was highest with 99.03%. The percentage utilization of these compounds supports the results with lipid production across other mixture volumes. The metabolites, ethanol and 1,3-PD was also analyzed, however there was not much change in the percentage utilization (data not shown). The percentage of glycerol, acetate and butyrate utilization increased with the spent media volume till 30 mL. However, with further increase in the volume from 40 to 50 mL, there was a decrease in the percentage utilization, similar to lipid production as presented in Table 4. The optimum concentration of acetate for the growth of *C. reinhardtii* was around 20 mM (1.2 g/L) [37]. In case of volume mixture of run 5 and 6, the acetate limits the optimum conditions and inhibits the growth resulting in decreased lipid production. In the case of glycerol, the optimum concentration was around 30-50 mM [5], which in the case of run 4 was within the limits. However, for run 5 and 6, the concentration of glycerol reached the limiting concentration resulting in decreased lipid production (0.036 ±0.004 and 0.010 ±0.002 g/L). The optimum mixture volume (3:2) of spent media (30 mL) and fresh media (20 mL) resulted in the increased lipid production along with maximum percentage utilization of the metabolites from the spent media.

The spent media from the dark fermentation utilized around 60-70% of substrate for metabolite generation during H_2 production [1]. The presence of acetate and butyrate in the spent media, tend to act as carbon source and help towards the growth of *C. reinhardtii* for lipid production. The effective approach of utilizing the spent media will bring down the cost of microalgae cultivation and decrease the overall cost, making microalgae derived biodiesel competitive.

Efficient closed system approach for biodiesel industry

Treatment plants are facing increasing challenges in disposal of excess sludge due to rapidly shrinking landfills, stringent environmental standards, awareness from governing bodies and increasing disposal cost [25, 26]. Researchers are exploring the sludge treatment and disposal methods for maximum energy recovery using various integration strategies [26, 38]. The proposed closed system is an alternative approach for sludge stabilization with reduction in the volume and weight of excess sludge through sustainable harvest into biofuels.

In a proposed efficient closed system for biodiesel industry as represented in Fig. 1, the approach was to minimize the waste generated and efficiently resource it for H_2 production and uplift biodiesel industry.

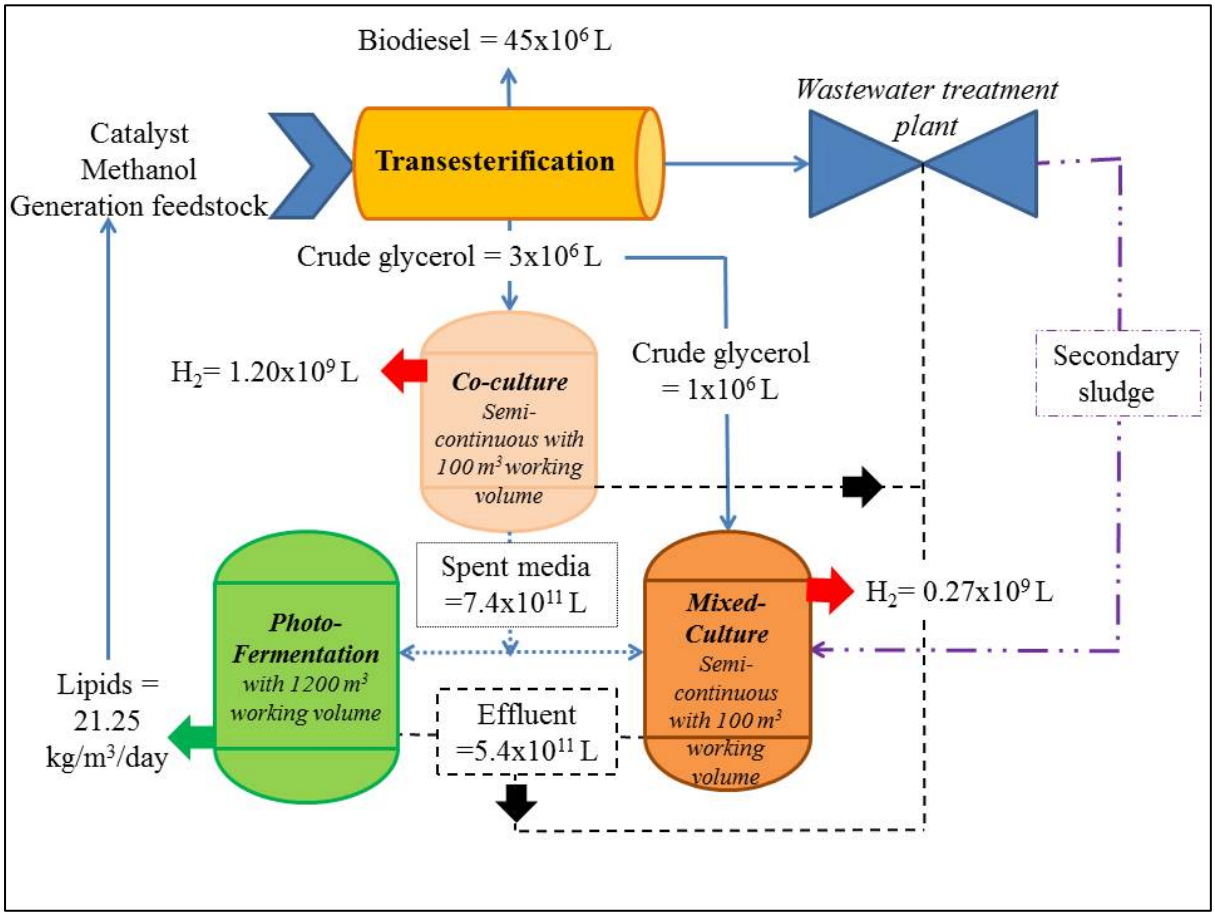

Fig. 1 – Efficient closed system approach for biodiesel industry for valorization of by-product crude glycerol into hydrogen production by co-culture system, utilization of the generated spent media by mixed-culture for hydrogen and by photo-fermentation for lipid production.

In this study, the spent media obtained after co-culture system of hydrogen production was utilized for media preparation instead of distilled water for the mixed-culture system of hydrogen. The efficient closed system can use the secondary wastewater sludge as seed inoculum with simple heat-shock treatment. The mixed-culture successfully resulted in utilization of crude glycerol, spent media to produce H_2, which can be used as in-house energy fuel for biodiesel industry. In another approach of closed system, the spent media was successfully replaced by the fresh media and also resulted in increased lipid production. The utilization of spent media helped to minimize the use of fresh media, thereby decreasing the media cost for lipid production. In addition, the produced lipid can be used as third generation feedstock for the biodiesel industry.

Bioconversion of (~22 kg) CG to 1 kg of H_2 utilizes 30-40% of substrate and generates ~8700 L of spent media available as potential for energy recovery. With efficient closed system, the recovered energy can be utilized as fuel or electricity or heat to create an in-house self-sufficient energy source [38]. This is based on an assumption that a biodiesel industry with capacity of 45 million liter production generates CG of around $4x10^6$ L per year [15]. In case of detailed methodology of bioconversion of 1 kg of CG into H_2 [21, 39], material balance of microalgae cultivation [40] and energy balance equivalent [20] can be consulted. About 75% of CG (i.e $3x10^6$ L) is used for semi-continuous of H_2 production using co-culture system with capacity of (356 L of cumulative H_2/kg of CG) produces around $1.2x10^9$ L of H_2 with $7.4x10^{11}$ L of spent media as seen in Fig. 1. The spent media is divided into half and utilized for semi-continuous H_2 production using mixed-culture system with capacity of (240 L of cumulative H_2/kg of CG) producing around $0.27x10^9$ L of H_2. According to the effective

closed system results, the H_2 produced ($1.47x10^9$ L of H_2) can be converted into energy (1.87 $x10^4$ GJ) for electricity (1.77 $x10^4$ GJ) and heat (4.32 $x10^3$ GJ). The spent media ($3.7x10^{11}$ L), can be used for microalgae cultivation with capacity of 1200 m^3 will produce algae at 50 $kg/m^3/d$ with lipid content of 35-50% (*w/w*) [40] as seen in Fig. 1. The produced lipid can be used as third generation feedstock for biodiesel industry.

The sludge treatment cost for 100 m^3 reactor is around $270,864/year to reduce the organic loading rate in the effluent [5, 39]. Using effective closed system, the organic loading rate with H_2 production by co-/mixed-culture is reduced to 30-40% and further 60% reduction can be obtained by photofermentation. The effective closed system was encouraging in terms of crude glycerol utilization, H_2 production, spent media reuse, sludge as inoculum, photo-fermentation for lipid production and sludge treatment. The decreasing market value of crude glycerol can be raised with the approach of efficient closed system. The small- and medium-scale biodiesel industry approach is to recycle around 99-100% of its input into value-added products. Thus, efficient closed system can help to reach these figures requiring minor production modification and the long term result will uplift the small- and medium-scale biodiesel industry.

4. Conclusions

The spent media generated during dark fermentation containing organic compounds and unutilized substrate with media components presents promising choice for waste utilization. The spent media can be used across different platforms to generate value-added chemicals. In this study, the spent media is used to replace distilled water used as a component of media preparation during H_2 production by mixed-culture system. The heat-shock pretreatment of wastewater sludge at 20% (*v/v*) inoculum with crude glycerol at 20 g/L resulted in increased H_2 production of around 38.12 ±0.84 mmol/L. In another approach, the spent media was

replaced with fresh media across *C. reinhardtii* growth during lipid production. The mixture volume of spent media (30 mL) and fresh media (20 mL) resulted in 0.098 ±0.007 g/L of lipid production. The spent media was thus used for both mixed-culture and photo-fermentation for H_2 and lipid production. The effective closed system approach of utilizing crude glycerol, H_2 production, spent media valorization, sludge as inoculum, photo-fermentation for lipid production and effluent treatment can make the biodiesel industry competitive in the biofuels market.

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Figures:

Fig. 1 – Efficient closed system approach for biodiesel industry for valorization of by-product crude glycerol into hydrogen production by co-culture system, utilization of the generated spent media by mixed-culture for hydrogen and by photo-fermentation for lipid production.

Tables:

Table 1 – Spent media generated across different systems for production of value-added compounds

Table 2 – Characterization of spent media

Table 3 – Hydrogen (mmol/L) and 1,3-Propanediol (g/L) production across different seed inocula using variable crude glycerol concentrations (g/L)

Table 4 –Experimental runs using varied volumes of spent (mL) and fresh media (mL), with the percentage of utilization for acetate, butyrate and glycerol at the end of photofermentation (7-days) for lipid production