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Identification of Microbial Community of WAS in Trickling Packed Bed Reactor Produced Biohydrogen and Studying the Effect of Hydrolysis by TiO_2 Photocatalysis

[1]Ali K. Al-Banaa, [1]Mohammad A. Atiya and [2]Nadhem H. Hayder
[1]Department of Biochemical Engineering,
[2]Department of Biotechnology, University of Baghdad, Baghdad, Iraq

Abstract: In this research, a Trickling Packed Bed Reactor (TPBR) is configured to produce biohydrogen (bio-H_2) using hydrolysis of an aqueous solution of Waste Active Sludge (WAS) as a substrate in a pilot heterogeneous nanoparticles photocatalysis (UV+TiO_2). The microbial community in the reactor system was detected which. The results of COD removal% in the photoreactor in TiO_2+UV, UV and dark after 6 h of operation are equal to 73, 55 and -18.4%, respectively with the presence of spore-forming and gram-negative bacteria. Also, the results show that the acid method was better than the heat one in preparing inoculum for TPBR. And the optimum condition for bio-H_2 production in TPBR is at HRT 8h and pH 5.5 with the presence of microbial community represented by *Citrobacter freundii*, *Bacillus* sp., *Clostridium* sp., *Klebsiella oxytoca*, *Serratia ficaria and Pseudomonas putida*. The bio-H_2 volume and COD removal% reach 7.92 mL and 69.55%, respectively in control pretreatment. While *Rhizobium radiobacter*, *Klebsiella pneumoniae* sp. *pneumoniae*, *E. coli, Bacillus* sp. and *Clostridium* sp. were indicated in the UV pretreatment that leads to achieving bio-H_2 volume and COD removal % of 22.5 mL and 71%, respectively, the microbial community of *Klebsiella pneumonia* sp. *pneumoniae, Burkholderia mallei, Enterococcus columbae*, *Bacillus* sp., *Clostridium* sp. and *Enterobacter aerogenes* were indicated in the TiO_2+UVpretreatment method with H_2volume and COD removal% reaching 39.6 mL and 80%, respectively. The maximum bio-$H_2$was produced after supplying organic shock rate, becoming 72 mL after 24 h in the batch state.

Key words: TPBR, COD, pretreatmen, shock rate, microbial , nanoparticles

INTRODUCTION

The microbial produced bio-H_2 can be either photosynthetic or non-photosynthetic. Facultative or obligate anaerobes lead to fermentative bio-H_2 production. Dark fermentation is one of the propitious ways to produce the bio-H_2 from different organic biomass using the mixed population of microorganisms. Microorganisms can concentrate and recover energy from aqueous organic resources such as industrial waste water and different sludge in a usable whichform (Lee *et al*., 2011). Organic wastes were applied for H_2 production such as waste activated sludge, cereal and food waste. Besides, the indigenous microorganisms can be used as hydrogen producers with no need to any addition of inoculum (Garcia *et al*., 2012). Many studies have employed suspended culture systems such as CSTR to produce bio-H_2, since, these systems are relatively simple and easy to operate. However, the results of wash-out cells limited the increase of biomass in these systems (Ahn *et al*., 2005). The employment of biofilm reactors appears to be a better approach for fermentative H_2 production, since, biofilms accommodate higher biomass and fermentation rate (Junior *et al*., 2015).Many researchers used immobilized biofilm on carrier materials to increase the production of bio-H_2 by reducing lag phase (Garcia *et al*., 2012; Barca,*et al*., 2015; Hastuti *et al*., 2016). The main parameters that affected the bio-H_2 production in bioreactor were pH and HRT. The reported optimum pH for H_2 production is in the range of pH 6-8, this range of pH is represented as supporting the growth of many Hydrogen-Producing Bacteria (HPB) (Poleto *et al*., 2016). HPB contains the essential enzyme as hydrogenase which plays the most important role in the H_2 production. Hydrogenase is optimized at a pH range of 6-6.5 (Bernardo *et al*., 2015). pH plays a critical role in sustaining the growth of HPB and the activity of hydrogenase in H_2 production. On the other hand, the effect of HRT on Bio-H_2 production yield was increased with the decrease in HRT by increasing the activity of hydrogenic activity which is also, increased with the decrease in HRT (Tanisho, 2001). HRT is considered an impact factor in the selection of microorganisms by the effect on the growth rates which required mechanical

Corresponding Author: Ali K. Al-Banaa, Department of Biochemical Engineering, University of Baghdad, Baghdad, Iraq

dilution caused by continuous volumetric circulation (Hawkes *et al*., 2007). An extensive fermentation time is unfavorable for H_2 production caused by the shifting from acidogenesis to methanogenesis reaction. For satisfactory H_2 yields, the optimum HRTs were between 8 and 14 hr for a wide variety of substrates (Mohan, 2010). By maintaining short HRTs (2-10 h), the methanogenesis was effectively suppressed (Ren *et al*., 2005). However, the HRT is affected by several factors including the type and composition of the substrate, type of microorganism organic loading rate and the system redox condition (Chen *et al*., 2009). Microbial diversity was observed from different inoculum sources (Jianlong and Yanan, 2016). Different substrates were used to study the production of H_2 from these microbes including glucose, glycerol, sucrose, lactose, xylose, cellobiose and industrial waste (Chandrasekhar *et al*., 2015). The greatest disadvantage of using WAS as an inoculum was the presence of methanogenesis and solventogenesis bacteria as consumed biohydrogen. Many strategies were used to increase biohydrogen production by separating acidogenesis from methanogenesis and solventogenesis in WAS such as mechanical, chemical, thermal, biological and physiochemical methods by Advanced Oxidation Process (AOPs) and alkaline (Pilli *et al*., 2016). These methods were put into operation to select the microorganisms by the supplemented harsh condition on the system which helped increase the bio-H_2 production by reducing the consumed bio-H_2 microbial (Hernandez-Mendoza *et al*., 2014).

AOPs reveal great potentiality for enhancing the hydrolysis of macromolecular components in WAS because of its generation of highly reactive hydroxyl radicals (•OH) that could oxidize various organics quickly and non-selectively (Ljubas, 2005; Arlos *et al*., 2016). TiO_2 has proven to be the most suitable photocatalyst because of its high chemical stability and strong photocatalytic activity as well as being inexpensive and nontoxic (Friedmann *et al*., 2010). Although, the reaction mechanism of AOPs in general is the generation of highly reactive •OH, the photocatalytic degradation of organics over TiO_2 particles occurs mainly via the formation of holes (hvb^+). AOPs using strong oxidizing agents show significant potential to accelerate the hydrolysis of macromolecular components as the generation of highly reactive hydroxyl radicals (•OH). However, from the viewpoint of energy saving and environmental conservation, developing a more cost-efficient and environmental friendly pretreatment is essential (Yang *et al*., 2012).

The information on the microbial community in which dark fermentative H_2-producing reactors is necessary to better understand and improve the process. However, only a few studies on the microbial community in such reactors have been reported (Moreno-Andrade *et al*., 2015; Dessi *et al*., 2017), although, many studies have reported physicochemical aspects of the process (Gadhe *et al*., 2015; Zhang *et al*., 2014). The main objective of this research is to investigate the microbial community in a mesophilic TPBR with various conditions of HRTs and pH, pretreated with TiO_2+UV photocatalyst.

MATERIALS AND METHODS

About 5 L of Waste Active Sludge (WAS) sample was collected from a dry tank of Al-Rustumiah Waste Water Treatment Plant in South Baghdad, Iraq, every week. The sample was stored in a refrigerator at 4°C. The TiO_2 photocatalyst was provided by Cheng Du, Ltd., China. The properties of the photocatalyst are as follows: TiO_2 type anatase, active surface area 60-80 m^2/g with size 25 nm, before using it as a photocatalyst, the XRD test is used for ensuring its properties, then prepared as a solution by impregnation method by dissolving it with deionized water, mixing it by ultrasonic probe (Q500 Sonicator, Qsonica-LTD, China). Nutrient agar and MacConkey agar were purchased from Hi-Media and Sigma Aldrich India.

Mineral Salt Medium (MSM) for trickling bioreactor: This medium was prepared from the following components and was contained (g/L of distilled water): yeast extract 0.5 g, NH_4NO_3 0.1 g, $CaCO_3$ 0.5 g, $NaHPO_4.12H_2O$ 0.02 g and $(NH_4)_2SO_4$ 0.002 g (Prema and Niladevi, 2008).

Analytical procedures: Chemical Oxygen Demand (COD) was measured depending on the standard method (Chen *et al*., 2009). The pH value was measured using a pH meter (INOLAB 7110, WTW Co., Germany). The gas composition H_2 was detected by a GC (GC-2014, Shimadzu, Japan) using TCD and molecular sieve column with nitrogen gas as a carrier. The types of bacteria were indicated by Vetik (VITEK 2 Compact System) (Ligozzi *et al*. (2002)). This device is used to confirm the identification of isolates and test their sensitivity to antibiotics.

Photoreactor: A lab-scale fluidized photoreactor was used for the hydrolysis of WAS where the UV irradiate was used to energized photocatalysis.

This photoreactor contains 5 L mixing tank, stock pump (12 L/h) (Germany), UV reactor stainless steel cylinder 2.4 L with dimension 85×6.5 cm (Fig. 1) and

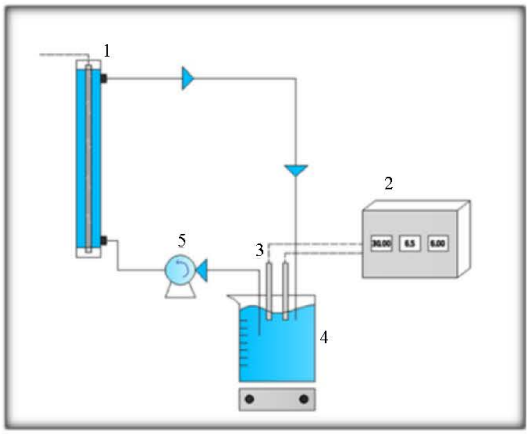

Fig. 1: The fluidized bed photoreactor: 1) Photoreactor; 2) Column; 3) Control box; 4) temperature probe and 5) Mixing tank and stock pump

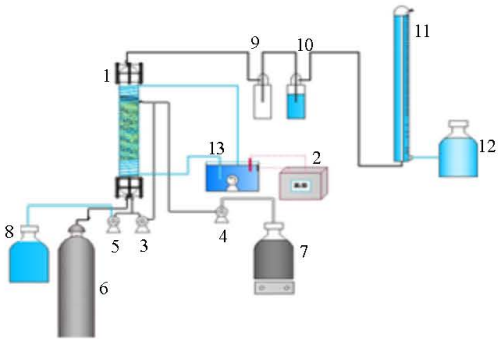

Fig. 2: Anaerobic down flow packed bed reactor TPBR, 2: Temp. control box; 3-5) Peristaltic pump; 6) N_2 gas cylinder; 7) Feeding tank; 8) Effluent tank; 9) Trap gas column; 10) Washer gas column; 11) Measuring gas cylinder; 12) Storage tank and 13) Water bath

irradiation UV lamp (40 W, 254 nm, Philips) with average light intensity of 5.5 W/m². The photoreactor also, contains temperature probe (China).

Identification of microbial community in hydrolyzed WAS in photoreactor: To compare the hydrolysis of WAS in three different pretreatments (control, UV and TiO_2 photocatalyst) on bacteria community in the photoreactor, firstly, raw WAS was loaded in the photoreactor at pH (6.5) with concentrations 5% (v/v) operated in the dark. The solution was mixed by a magnetic stirrer for 1 h, then loaded to fluidized bed photoreactor circulated in the dark for 1 h. Finally, it was operated for 6 h and the samples were taken (5 mL) every 2 h. COD and types of bacteria were detected. Firstly, the hydrolyzed WAS with concentration 5% v/v was loaded into the TPBR to determine the optimum operation condition and identified the microbe in every operational stage. Besides, to study the effect of UV on the types of bio-H_2 producing bacteria, raw WAS was loaded in the photoreactor circulation for 1 h in the dark, then irradiated for 6 h with volume (5 mL) taking a sample every 2 h. COD and types of bacteria were detected. Finally, for studying the effect of TiO_2 photocatalyst on the types of bio-H_2 producing bacteria, raw WAS was loaded in the UV-photoreactor with the presence of TiO_2 nanoparticles (5 mg/L) in pH of 6.5. In the mixing tank, TiO_2 was used as a dosage to raw WAS solution with stirring for 1 h, then loaded in the photoreactor circulating for 6 h with UV radiation. The samples (5 mL) were taken every 2 h to study the effect of photocatalysis on COD and types of bacteria.

Preparation of inoculum: Firstly, hydrolyzed controlled WAS at concentration 5 % (v/v) was mixed with 7.5% (v/v) of fresh WAS separate. Secondly, the solution was treated under harsh conditions including acidifying or heating, to inhibit methanogenesis microorganisms. In the case of hydrolyzed controlled WAS, the first harsh condition was achieved by taking 5% (v/v) of WAS after adding the inoculum and mixing with 4 M of HCl (Ligozzi *et al*., 2002). The solution was stirred for 0.5-1 h, then placed in the refrigerator at 4°C for 24 h. Finally, the hydrolysate was neutralized with 3 M of sodium hydroxide (NaOH) and packaged in a packed bed reactor that is loaded with MSM containing 1 g/L lactose at pH 7.0 for 2 days for immobilization purposes. The acidifying of hydrolyzed UV and TiO_2 WAS was done under similar conditions of control but without adding the inoculum. Another condition for inhibiting the methanogenesis was tested by heating the solution by using autoclaving at 100°C for 15 min (Chen *et al*., 2012).

Anaerobic Trickling Packed Bed Reactor (TPBR): The cylinder of packed bed reactor was constructed from a plastic material and covered with the rmostable Teflon at each end of the reactor as shown in Fig. 2. The reactor with dimension H = 60 cm, D_o= 8 cm, D_i = 7 cm, total volume 2.3 L and working volume 1.25 L was filled with ceramic ball with a diameter of 2 cm as a carrier material with the actual volume of the liquid being about 1.25 L. The bottom of the reactor has two points, the first one was divided into two parts, the first for the effluent exit and the other for recycling the reactor by peristaltic pump. The second point is for the nitrogen gas flushing and finally the top of the reactor has one outlet for collecting

the gas. The feeding and effluent are pumped into two reactors by one intelligent flow peristaltic pump with pump head DG6-4 (6 roles-4 channels) with flow rate 0.00016-26 mL/min/channel (Gold Pump, China) and a peristaltic pump was used for circulation flow in the reactor. The gas outlet from the bioreactor is connected to the trap column used as a trap for gasses, the outlet from the trap column enters the washer gas column containing NaOH 0.2 M. The bubble gas outlet from the washer column passes by measuring gas column. Finally, the reactor was surrounded by a coil jacket and covered with an aluminum paper protective layer.

Identification of bacteria in WAS for each pretreated method: A serial dilutions method was achieved from each sample that was collected from control, photolysis and catalysis pretreatment methods. A 100 μL from dilution 10^6 was translated on a nutrient agar and MacConkey agar for a cultivation containing microbes, then identified by VITEK technique to determine the genus and/or species of bacteria that produced bio-hydrogen in each pretreated method.

Correlation of bacteria types with bio-H_2 production yield in TPBR: To determine and identify microbes in the optimum condition for TPBR produced bio-H_2, the TPBR was operated at different pretreated conditions of WAS. To immobilize WAS that contains microbes on a ceramic ball of TPBR, hydrolyzed controlled WAS 5% v/v with the addition of inoculum separately was loaded in TPBR packaging with MSM and lactose as a substrate (Fig. 2) for 2 days at different HRT including 48, 24, 12 and 8 h with different pH including 5.5-7. For each condition, COD removal %, hydrogen volume and identification of the types of microbes producing bio-H_2 were determined and identified. On the other hand, shocking and starvation strategies by the batch state were employed for microorganisms by increasing or decreasing OLR.

RESULTS AND DISCUSSION

Identification of microbes in hydrolysis WAS by using photoreactor: In dark reaction pretreatment was at concentration 5% was loaded in photoreactor at pH 6.5 used as a control hydrolysis. The result in Fig. 3 has shown that the lower removal efficiency of COD reached -18.4%. Also, the results indicated the presence of *citrobacter freundii*, *Bacillus* sp., *Clostridium* sp., *Klebsiella oxytoca*, *Serratia ficaria* and *E. coli* isolated and identified by using VITEK technology. The results of COD removal% and COD removal were illustrated in

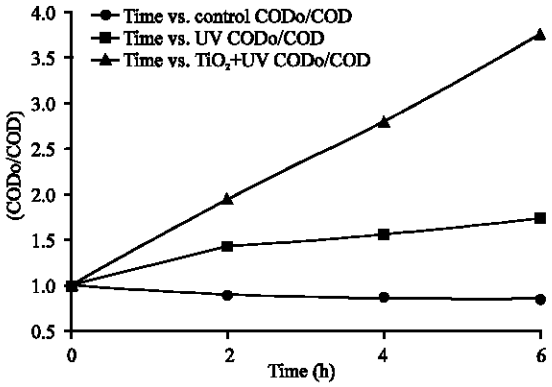

Fig. 3: The COD fegradation in photoreactor with TiO_2+UV

Table 1: The relationship between COD removal (%) and pH in the photoreactor

Pretreatment	Reactor operation	pH	COD removal (%)
Control photoreactor	6 h in dark	6.5-6.4	-3.58
UV-photoreactor	6 h radiated	6.3	42
TiO_2 photoreactor	6 h radiated	5.8	73

Table 1 and Fig. 3 showing the effect of mechanical hydrolysis by a pump in the photoreactor which were in good agreement with the results of (Alemahdi *et al*., 2015; Sharmila *et al*., 2017) while the results from the bacteria presence agree with the results of (Li, 2013) by the presence of negative gram bacteria.

The results from WAS in the UV pretreated in the photoreactor for 6 h of irradiation indicated that the types of bacteria were activated as a mixed culture represented by Rhizobium radiobacter, *Bacillus* sp., *Clostridium* sp. *Klebsiella pneumonia* sp., *Pneumonia* and *E. coli*. On the other hand, the result illustrated in Fig. 3 showed that the COD was increasing with the increase in the exposure period of WAS to the pretreated UV and reached 42% after 6 hr of irradiation. Table 1 and 2 show similar results observed in the research of (Govindammal and Parthasarathi, 2013). Finally, was with a dosage of 5 mg/L TiO_2 photocatalyst was operated for 6 h of irradiation showing that the COD removal % increased with time to be 73% at the end of 6 h irradiation. The increase in the removal of COD is due to the increase in the insoluble intracellular substances by a radical agent produced by the photoreaction at the surface of semiconductor photocatalyst under the exposure of UV light (Jingxin *et al*., 2017; Kavitha *et al*., 2015). The result of hydrolysis is in good agreement with the research of (Alemahdi *et al*., 2015; Lima *et al*., 2015).

The mixed culture that was activated in this stage is represented with *Bacillus* sp., *Clostridium* sp., *Klebsiella pneumonia* sp., *pneumonia*, *E. coli* and *Enterobacter*

Table 2: Microbes identified by VITEK in the photoreactor and TPBR

Microorganisms	Type of aeration	C1	U1	T1	1P	2P	3P	4P	5P	6P	7P	Activity	References
Klebsiella Pneumonia ssp. *Pneumonia*	Facultative anaerobic	-	+	+	+	-	-	-	-	+	+	HPB	Gadhe *et al.* (2015)
Bacillus sp.	Obligate aerobes or facultative anaerobes	+	+	+	+	+	+	+	+	+	+	HPB	Gadhe *et al.* (2015), Zhang *et al.* (2014)
Clostridium sp.	Obligate anaerobes	+	+	+	+	+	+	+	+	+	+	HPB	Gadhe *et al.* (2015), Qaim *et al.* (2017)
E. coli	facultative anaerobic	+	+	+	+	-	+	-	+	+	+	HPB	Gadhe *et al.* (2015), Prema and Niladevi (2008)
Citrobacter freundii,	Facultative anaerobic	+	-	-	-	+	+	+	+	-	-	HPB	Gadhe *et al.* (2015)
Citrobacter amalonaticus	Facultative anaerobic	-	-	-	-	+	-	-	-	-	-	HPB	Gadhe *et al.* (2015)
Klebsiella oxytoca	facultative anaerobic	+	-	-	-	-	-	+	+	-	-	HPB	Gadhe *et al.* (2015)
Rhizopium radiobacter	Facultative aerobic	-	+	-	-	-	-	-	-	-	-	PHE	Gadhe *et al.* (2015)
Burkholderia mallei	Facultative anaerobic	-	-	-	-	-	-	-	-	-	+	HPB (NFB)	Kumar *et al.* (2014)
Enterococcus columbae,	Facultative anaerobic	-	-	-	-	-	-	-	-	-	+	HPB	Bielen *et al.* (2013)
Enterobacter aerogenes	Facultative anaerobic	-	-	+	-	-	-	-	-	-	-	HPB	Bielen *et al.* (2013)
Serratia ficaria	Facultative anaerobic	+	-	-	-	-	-	-	+	-	-	AHE	Lee *at al.* (2011)
Pseudomonas putida	Facultative anaerobic	-	-	-	-	-	-	+	+	-	-	AHE	Lee *at al.* (2011)

C1: Control pretreatment in photoreactor, U1: pretreatment in photoreactor, T1: pretreatment in photoreactor, *1P: TBPR HRT 48 pH 6.3, *2P: TBPR HRT 24 pH 6.1, *3P: TPBR HRT 12 pH 6.3, *4P: TBPR HRT 8 pH 5.8, *5P: TPBR organic shocking rate pH 5.5, *6P: TPBR UV pretreated HRT 8 pH 5.5, *7P: TPBR TiO_2+UV pretreated HRT 8 pH 5.5, HPB: hydrogen production bacteria, PHE: Produced Hydrolysis Enzymes, NFB: N_2-fixing bacteria and AHE: Associated hydrogenase enzyme

aerogenes. The results of the bacteria shown in Table 2 exhibited higher tolerance than the bacteria isolated when exposed to UV light with TiO_2 with better survival rate in this condition. The difference in photolysis and photocatalytic inactivation of bacteria is typically imputed to the variance in cell wall structure between gram-negative and gram-positive bacteria. Gram-negative bacteria have a triple-layer cell wall with an inner membrane, a thin peptidoglycan layer and an outer membrane whereas the gram-positive bacteria have a thicker peptidoglycan without an outer membrane. Also, this may have related to different affinities for photocatalyst and cell wall of bacteria. From this, it can be concluded that the rate of photocatalytic antibacterial activity is a command not only by cell wall thickness but also by the morphology of cell envelope and resistance of the outer membrane to the Reactive Oxygen Species (ROS) produced on the surface of the photocatalyst (Sharmila *et al*., 2015).

Detection of bacteria isolated in inoculum pretreated with acid and heat: The identification of bacteria isolated using the VITEK system in the current study showed that the acidic method was the best for hydrolysis WAS in control pretreatment (Skorb *et al*., 2008) due to the activation of acidogenesis bacteria such as the isolates *Citrobacter freundii, Klebsiella oxytoca, Serratia ficaria*, *Bacillus* sp. and *Clostridium* sp. and the inhibition of the methanogenic bacteria while the heating method was activated for *Citrobacter freundii*, only in the same pretreatment. pH is considered as a key factor in the impact on the outcome of H_2 fermentation and it influences the metabolic pathways, the activity of the hydrogenase enzyme (s) as specified in a mixed culture (Mohan *et al*., 2008). pH significantly affects and is able to restrict the methanogen growth and regulate the shift from hydrogenases to disadvantageous solventogenesis. The methanogenesis and propionogenesis are well known and undesired phenomena for bio-H_2 production due to their H_2 consuming characteristics. pH below 4.5 significantly ejects the hydrogen oxidizing methanogens and adaption of the granular-sludge (Lin *et al*., 2012). pH variation can affect H_2 production, microbial population shift, cell morphology and structure as a consequence, essential to find the most suitable pH conditions, so as to obtain efficient hydrogen production (Mohan *et al*., 2008).

Correlation of bacteria types with bio-H_2 production yield in TPBR: The control acidic WAS at a concentration of 5% was loading in TPBR to determine the best concentration of WAS as well as the types of microbial genera that produce maximum bio-H_2 yield. According to the results illustrated in Fig. 4-6 and Table 2, the COD removal % and higher H_2 volume reached 71.4% and 7.9 ml at 8 h HRT and 5.5 pH, respectively with the appearance of bacteria flora *Citrobacter freundii*, *Bacillus* sp., *Clostridium* sp., *Klebsiella oxytoca and Pseudomonas putida* (Table 2) while when TPBR operated as a fed-batch system at 48 h HRT and 6.8 pH decreasing in COD removal, H_2 production reached 54.1% and 6.72 mL, respectively (Fig 4) with the appearance of new bacteria

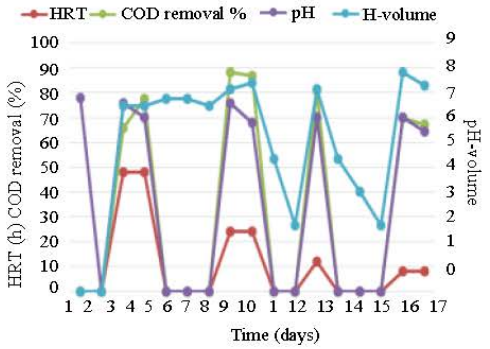

Fig. 4: Relationship between HRT, pH with COD and (H) volume production by mixed culture

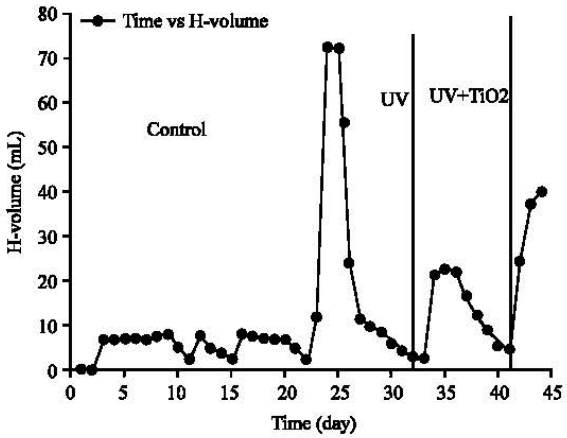

Fig. 5: Hydrogen volume produced along 44 days in TBPR

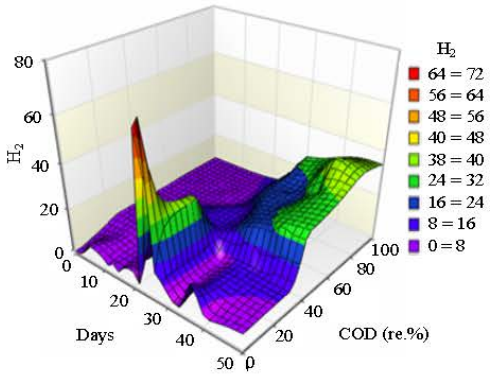

Fig. 6: Response surface plot showing the interactive effect of relationship between COD removal (%) and H_2 (mL) produced in TPBR along 44 days of operation

isolates including *Klebsiella pneumonia* sp., Pneumonia, *Bacillus* sp., *Clostridium* sp. and *E. coli*. However, semi-continuous operation of TPBR increased COD removal% reaching 78.7% and the H_2 volume was equal to 7.56 mL at 24 h HRT in 6.1 pH with the appearance of bacteria genera *Citrobacter freundii, Citrobacter amalonaticus*, *Bacillus* sp. and *Clostridium* sp. whereas COD removal % was equal to 75.5% and the H_2 volume reached 7.32 mL in semi-continuous operation at 12 h HRT and 6.2 pH with the existing *Citrobacter freundii, Bacillus* sp., *Clostridium* sp., and *E. coli*. According to the results, the optimum HRT and pH for producing bio-H_2 gas from mixed culture WAS were at 8 h and 5.5, respectively, at a concentration of 5% of WAS. However, high H_2-yield was gained at initial pH range of 5.5-7.0. Finally, to investigate the effect of increasing Organic Loading Rate (OLR) in TPBR at optimum condition (WAS 5%, HRT 8 and pH 5.5), the reactor was loaded with lactose at a concentration of 3 g/L with an optimum condition at HRT 8hr and pH 5.5. The results were shown in Fig. 5 and Table 2 in which the COD removal and H_2 volume reached 55.5% and 11.64 mL, respectively with the appearance of *Citrobacter freundii*, *Bacillus* sp., *Clostridium* sp., Klebsiella oxytoca, *E. coli*, *Serratia ficaria* and *Pseudomonas putida* bacterial isolates. On the other hand, the maximum H_2 volume observed reached 72 mL after 24 h in the batch operational state. Based on this phenomenon, many studies have tried to improve the bio-H_2 yield through fixing pH of a reactor at around pH 5.5 (Hernandez-Mendoza *et al*., 2014; Calusinska *et al*., 2015). Christopher (Chandrasekhar *et al*., 2015) found that the initial pH of 5.9 was optimized for anaerobic microflora which gave a maximum bio-H_2 production of 33.2 mL H_2/L h and a yield of 0.83 mole H_2/mole glucose. The changes in pH influence the metabolic activity of bacteria producing H_2 and the fermentative process in general because pH affects the activity of the hydrogenase enzyme as well as the metabolic routes. The metabolic pathways to produce H_2 cause a decrease in pH during the exponential growth phase of the bacteria (Barca *et al*., 2015) clearly indicating the acidogenic nature of the microbiological activity. This drop in pH is important because the lower pH helps in the reduction of methanogenesis. The effect of HRT on bio-H_2 production is illustrated in Fig. 4, the hydrogen yield was found to increase with the decrease in HRT.

To determine the correlation of bacterial types with bio-H_2 production yield, the data used for this study was taken from the optimum condition for gas production at HRT 8 and pH 5.5. The results showed that bacterial types synergism with bio-H_2 production is represented by *Citrobacter freundii*, *Bacillus* sp., *Clostridium* sp., *Klebsiella oxytoca* and *Pseudomonas putida* with increasing the H_2 yield, reaching the maximum value

because these bacteria play a key role in converting the complex substrate to the simplest one. It can be noted that NADH produced during anaerobic glycolysis is rarely used for hydrogen production by the bacteria of the family Enterobacteriaceae, due to the absence of specific coenzymes such as ferredoxin oxidoreductase (Rossi *et al*., 2011). Nevertheless, NADH must still be oxidized back to NAD, otherwise, the anaerobic glycolysis will be in intermission. Many microorganisms as *Serratia ficaria and Pseudomonas putida* solve this problem by slowing or stopping the pyruvate dehydrogenase activity and using pyruvate or one of its derivatives as an electron and hydrogen acceptor for the re-oxidization of NADH in a fermentation process. As a result, the oxidation-reduction state must be balanced through the NADH consumption to form the abundance of mixed acids and alcohols, most of which are the hydrogen-containing reduced products, accompanied by the formation of formate (Bielen *et al*., 2013). However, the result showed that the COD removal% decreased which is similar to the results reported by (Lee *et al*., 2011) where a short HRT led to low efficiency of carbohydrate removal and increased in hydrogen yield which might be attributed to the increased availability of fresh substrates to the microbes leading to the appearance of new microbes (Kumar *et al*., 2014).

Table 3: Bio-hydrogen producing operational processing TPBR with relationship between HRT, hydrogen volume, VHPR (H_2/COD) and COD removal% along 44 days of operation

Days	Operational	HRT h	H (mL)	Yield (H_2/COD (mL/g.L^{-1}))	COD removal (%)
1	I mm*	0	0	-	0
2	I mm*	0	0	-	0
3	Control 35.7	48	6.72	8.4	
4	Control 54.1	48	6.72	4.66	
8	Control 81	24	7.32	1.37	
9	Control 78.7	24	7.56	1.46	
12	Control 75.5	12	7.32	1.48	
16	Control 71.4	8	7.92	1.63	
17	Control 69.1	8	7.44	1.57	
23	*2organic shocking rate 55.5	8	11.64	1.49	
24	*3	0	72.00	0	0
25	*3 0	0	72.00	0	
34	UV	8	21.00	3.9	74
35	UV	8	22.50	4.4	71
36	UV	8	21.75	4.9	69.8
42	TiO_2	8	24.00	4.7	74
43	TiO_2	8	36.96	10	75.4
44	TiO_2	8	39.60	10.1	80

*1 Immobilization, *2Increasing concentration of lactose from 1-3 g/L to shown the effect of shocking OLR strategy,*3batch state

Detection of microbial flora in TPBR pretreated with photolysis: To study the effect of UV on the types of bio-H_2 producing bacteria, hydrolyzed WAS at a concentration of 5% was loaded in the photoreactor at 8 hr HRT and 5.5 pH for 72 h. The results revealed that the types of bacteria tolerated advanced oxidation by UV photocatalysis (5.5 W/m^2, 254 nm) included Rhizobium radiobacter, *Klebsiella pneumonia* ssp., *Pneumonia, E. coli*, *Bacillus* sp. and *Clostridium* sp. Synergism with COD removal % and hydrogen volume, COD removal % reached 71% and H_2 volume is equal to 22.5 mL (Fig. 6 and Tables 2 and 3). As a result of consuming lactose as a substrate and any other material in MSM used in a bioreactor, *Rhizobium radiobacter* also, plays a key role in the advanced oxidation reaction of WAS, it is considered as a good resource for the mass production of catalase for the treatment of hydrogen peroxide containing waste water (Sui *et al*., 2017; Nakayama *et al*., 2008), besides its resistance to radiation having multiple copies of its genome and rapid DNA repair mechanisms. It usually repairs breaks in its chromosomes within 12-24 h by a 2-step process synergism with the activation of high tolerant bacteria of UV as bio-hydrogen producer (Nakayama *et al*., 2008; Rastogi *et al*., 2010). Spore-forming bacteria like *Bacillus* sp. and *Clostridium* are 5-50 times more resistant to UV radiation than the corresponding growing cells. This elevated spore UV resistance is due to, DNA repair in particular, SP-specific repair, during spore germination and the photochemistry of DNA within spores as UV generates few, if any, cyclobutane dimers but rather a photo product called spore photoproduct (SP; 5-thyminyl-5,6-dihydrothymine). The unfamiliar UV photochemistry of spore DNA is largely due to its saturation with a group of Small, Acid-Soluble Proteins (SASP) which are unique to spores and whose binding alters the DNA conformation and thus, its photochemistry. These reasons made these bacteria increase their resistance to UV radiation and in turn increase H_2 volume to 22.47 mL and VHPR to 4.9 H_2-mL/COD-g/L (Table 3).

Detection of microbial flora in TPBR pretreated with photocatalysis: To determine the effect of UV and TiO_2 on the types of bio-H_2 producing bacteria, hydrolyzed WAS was loaded in UV-photoreactor at optimum operation

conditions including WAS 5%, $TiO_2$5mg/L, HRT 8 h and pH 5.5. The VITEK results indicated that the types of bacteria tolerated advanced oxidation by UV and TiO_2 photocatalysis were including *Klebsiella pneumonia* ssp., *Pneumonia, Burkholderia mallei*, *Enterococcus* columbae, *Bacillus* sp., *E. coli*, *Clostridium* sp. and *Enterobacter aerogenes* synergism with COD removal value and higher H_2 volume. COD removal % and hydrogen volume reached 80% and 39.6 mL, respectively (Fig. 6 and Tables 2 and 3). Also, the results show that bio-H_2 volume reached 39.6 mL and VHPR reached 10.1 (H_2-mL/ COD-g/L) (Table 3).

The presence of N_2-Fixing Bacteria (NFB) Burkholderia mallei helped produce bio-H_2 using *Burkholderia* sp. (*Burkholderia unamae and Burkholderia tropica*) isolated from two sugar cane crops in Mexico to produce bio-H_2 and determine their H_2 production capacities. The results illustrated the ability of both strains to produce bio-H_2. The maximum bio-H_2 was observed when the medium was composed of 1% v/v molasses, enriched with $FeSO_4$ (0.2 g/L), Na_2MoO_4(0.2 g/L) and cysteine (0.02 g/L) under a partial vacuum (air 20% v/v). Under these conditions, the HPR obtained was 24.64 mmol H_2/L when using *Burkholderia* unamae. (Terrazas-Hoyos *et al*., 2014) reported that *Burkholderia mallei* had the ability to produce catalyzed enzymes which are able to convert oxalate, acetoacetate and CoA as a substrate to form finally C6-CoA, so, it could be used as a source for bio-H_2 production of other bacteria. Also, the same results in the presence of the bacteria *Bacillus* sp. and *Burkholderia* sp. were observed by Kumar *et al.* (2014) when the bioreactor with OLR = 30 and pH 4-5.5 was operated while the results in the presence of spore-forms bacteria *Bacillus* sp. and *Clostridium* sp. were observed by Park *et al.* (2015) in which the number of bacterial spore-strains stayed constant when the fermentation performance recovered from various disturbances of the environment such as shock loading, alkalization, acidification and starvation (Oh *et al*., 2016; Kumar *et al*., 2015).

However, the effect of TiO_2 photocatalytic pretreatment accelerated the hydrolysis of macromolecular components of WAS to smaller molecular weight hydrolysates which are more readily metabolized by microorganisms in consequent anaerobic digestion (Li *et al*., 2013), so that, it helps increase the biofilm community produced bio-H_2 which finally, increased the H_2 to become the maximum value in continuous states in TPBR.

CONCLUSION

The most elevated yield hydrogen production rate and COD removal % were 10 mL/g L^{-1} and 80%, respectively when TPBR was operated with WAS pretreated by TiO_2 photocatalyst at an HRT of 8 h. Our results demonstrate the increase in microbial community produced hydrogen with the presence of N_2-fixing bacteria. Likewise, the outcome has demonstrated that the types of bacteria that had the ability to tolerate UV+ nano-photocatalyst, starvation, photolysis and organic shocking rate were spore-forming and gram-negative bacteria. The consequence of organic shocking rate technique is the highest hydrogen production of 72 mL/day with an increase in the microbial community and in the presence of associated hydrogenase enzyme bacteria when the reactor worked in starvation as batch operation.

REFERENCES

Ahn, Y., E.J. Park, Y.K. Oh, S. Park and G. Webster *et al*., 2005. Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production. FEMS. Microbiol. Lett., 249: 31-38.

Alemahdi, N., H.C. Man, N.A. Rahman, N. Nasirian and Y. Yang, 2015. Enhanced mesophilic bio-hydrogen production of raw rice straw and activated sewage sludge by co-digestion. Intl. J. Hydrogen Energy, 40: 16033-16044.

Arlos, M.J., M.M. Hatat-Fraile, R. Liang, L.M. Bragg and N.Y. Zhou *et al*., 2016. Photocatalytic decomposition of organic micropollutants using immobilized TiO_2 having different isoelectric points. Water Res. J., 101: 351-361.

Barca, C., A. Soric, D. Ranava, M.T. Giudici-Orticoni and J.H. Ferrasse, 2015. Anaerobic biofilm reactors for dark fermentative hydrogen production from wastewater: A review. Bioresour. Technol., 185: 386-398.

Barca, C., D. Ranava, M. Bauzan, J.H. Ferrasse and M.T. Giudici-Orticoni *et al*., 2016. Fermentative hydrogen production in an up-flow anaerobic biofilm reactor inoculated with a co-culture of *Clostridium acetobutylicum* and *Desulfovibrio vulgaris*. Bioresour. Technol., 221: 526-533.

Bernardo, R., T. Tonia and S. Sara, 2015. Bio-H_2 & Bio-CH_4 through Anaerobic Digestion, Part of the Green Energy and Technology Book Series. Springer, London, England, UK., ISBN:978-1-4471-6431-9, Pages: 218.

Bielen, A.A.M., M.R.A. Verhaart, J. Van Der Oost and S.W.M. Kengen, 2013. Biohydrogen production by the thermophilic bacterium Caldicellulosiruptor saccharolyticus: Current status and perspectives. Life, J., 3: 52-85.

Calusinska, M., C. Hamilton, P. Monsieurs, G. Mathy and N. Leys *et al*., 2015. Genome-wide transcriptional analysis suggests hydrogenase and nitrogenase-mediated hydrogen production in *Clostridium butyricum* CWBI 1009. Biotechnol. Biofuels J., 8: 1-27.

Chandrasekhar, K., Y.J. Lee and D.W. Lee, 2015. Biohydrogen production: Strategies to improve process efficiency through microbial routes. Intl. J. Mol. Sci., 16: 8266-8293.

Chen, W.H., S. Sung and S.Y. Chen, 2009. Biological hydrogen production in an anaerobic sequencing batch reactor: PH and cyclic duration effects. Intl. J. Hydrogen Energy, 34: 227-234.

Chen, W.Y., J. Seiner, T. Suzuki and M. Lackner, 2012. Handbook of Climate Change Mitigation. Springer, New York, USA., ISBN:9781441979926, Pages: 2130.

Dessi, P., A.M. Lakaniemi and P.N. Lens, 2017. Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70°C. Water Res. J., 115: 120-129.

Friedmann, D., C. Mendive and D. Bahnemann, 2010. TiO_2 for water treatment: Parameters affecting the kinetics and mechanisms of photocatalysis. J. Appl. Catal. B. Environ., 99: 398-406.

Gadhe, A., S.S. Sonawane and M.N. Varma, 2015. Enhanced biohydrogen production from dark fermentation of complex dairy wastewater by sonolysis. Intl. J. Hydrogen Energy, 40: 9942-9951.

Garcia, R.E., V.L. Martinez, J.I. Franco and G. Curutchet, 2012. Selection of natural bacterial communities for the biological production of hydrogen. Intl. J. Hydrogen Energy, 37: 10095-10100.

Govindammal, M. and R. Parthasarathi, 2013. Production and characterization of bio surfactant using renewable substrates by Pseudomonas fluorescence isolated from mangrove ecosystem. J. Appl. Chem., 2: 55-62.

Hastuti, Z.D., C.Y. Chu, M.A. Rachman, W.W. Purwanto and E.L. Dewi *et al*., 2016. Effect of concentration on biohydrogen production in a continuous stirred bioreactor using biofilm induced packed-carrier. Intl. J. Hydrogen Energy, 41: 21649-21656.

Hawkes, F.R., I. Hussy, G. Kyazze, R. Dinsdale and D.L. Hawkes, 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int. J. Hydrogen Energy, 32: 172-184.

Hernandez-Mendoza, C.E., I. Moreno-Andrade and G. Buitron, 2014. Comparison of hydrogen-producing bacterial communities adapted in continuous and discontinuous reactors. Intl. J. Hydrogen Energy, 39: 14234-14239.

Hernandez-Mendoza, C.E., I. Moreno-Andrade and G. Buitron, 2014. Comparison of hydrogen-producing bacterial communities adapted in continuous and discontinuous reactors. Intl. J. Hydrogen Energy, 39: 14234-14239.

Jianlong, W. and Y. Yanan, 2016. Biohydrogen Production from Organic Wastes. In: Part of the Green Energy and Technology Book Series, Jianlong, W. and Y. Yanan (Eds.). Springer, Switzerland, Europe, pp: 197-268.

Junior, A.D.N.F., C. Etchebehere and M. Zaiat, 2015. Mesophilic hydrogen production in Acidogenic Packed-Bed Reactors (APBR) using raw sugarcane vinasse as substrate: Influence of support materials. Anaerobe, 34: 94-105.

Kavitha, S., T. Saranya, S. Kaliappan, S.A. Kumar and I.T. Yeom *et al*., 2015. Accelerating the sludge disintegration potential of a novel bacterial strain Planococcus Jake 01 by CaCl2 induced deflocculation. Bioresour. Technol., 175: 396-405.

Kumar, G., B. Sen, P. Sivagurunathan and C.Y. Lin, 2015. Comparative evaluation of hydrogen fermentation of de-oiled Jatropha waste hydrolyzates. Intl. J. Hydrogen Energy, 40: 10766-10774.

Kumar, G., J.H. Park, M.S. Kim, D.H. Kim and S.H. Kim, 2014. Hydrogen fermentation of different galactose-glucose compositions during various Hydraulic Retention Times (HRTs). Intl. J. Hydrogen Energy, 39: 20625-20631.

Lee, D.J., K.Y. Show and A. Su, 2011. Dark fermentation on biohydrogen production: Pure culture. Bioresour. Technol., 102: 8393-8402.

Li, D., 2013. TiO_2 photo-catalytic degradation of waste activated sludge and potassium hydrogen phthalate in wastewater for enhancing biogas production. Ph.D Thesis, University of Tsukuba, Tsukuba, Japan.

Li, D., Y. Zhao, Q. Wang, Y. Yang and Z. Zhang, 2013. Enhanced cbiohydrogen production by accelerating the hydrolysis of macromolecular components of waste activated sludge using TiO_2 photocatalysis as a pretreatment. Open J. Appl. Sci., 3: 155-162.

Ligozzi, M., C. Bernini, M.G. Bonora, M. de Fatima, J. Zuliani and R. Fontana, 2002. Evaluation of the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram-positive cocci. J. Clin. Microbiol., 40: 1681-1686.

Lima, C.S., K.A. Batista, A.G. Rodriguez, J.R. Souza and K.F. Fernandes, 2015. Photodecomposition and color removal of a real sample of textile wastewater using heterogeneous photocatalysis with polypyrrole. Solar Energy, 114: 105-113.

Lin, C.Y., C.H. Lay, B. Sen, C.Y. Chu and G. Kumar *et al*., 2012. Fermentative hydrogen production from wastewaters: A review and prognosis. Intl. J. Hydrogen Energy, 37: 15632-15642.

Ljubas, D., 2005. Solar photocatalysis-a possible step in drinking water treatment. J. Energy, 30: 1699-1710.

Mohan, S.V., 2010. Waste to Renewable Energy: A Sustainable and Green Approach Towards Production of Biohydrogen by Acidogenic Fermentation. In: Sustainable Biotechnology: Sources of Renewable Energy, Singh, O.V. and S.P. Harvey (Eds.). Springer, Dordrecht, Netherlands, Europe, ISBN:978-90-481-3294-2, pp: 129-164.

Mohan, S.V., V.L. Babu and P.N. Sarma, 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresour. Technol., 99: 59-67.

Moreno-Andrade, I., J. Carrillo-Reyes, S.G. Santiago and M.C. Bujanos-Adame, 2015. Biohydrogen from food waste in a discontinuous process: Effect of HRT and microbial community analysis. Intl. J. Hydrogen Energy, 40: 17246-17252.

Nakayama, M., T. Nakajima-Kambe, H. Katayama, K. Higuchi and Y. Kawasaki *et al*., 2008. High catalase production by *Rhizobium radiobacter* strain 2-1. J. Biosci. Bioeng., 106: 554-558.

Oh, J., I. Hwang and S. Rhee, 2016. Structural insights into an oxalate-producing serine hydrolase with an unusual oxyanion hole and additional lyase activity. J. Biol. Chem., 29: 15185-15195.

Park, J.H., G. Kumar, J.H. Park, H.D. Park and S.H. Kim, 2015. Changes in performance and bacterial communities in response to various process disturbances in a high-rate biohydrogen reactor fed with galactose. Bioresour. Technol., 188: 109-116.

Pilli, S., T.T. More, S. Yan, R.D. Tyagi and R.Y. Surampalli, 2016. Fenton pre-treatment of secondary sludge to enhance anaerobic digestion: Energy balance and greenhouse gas emissions. Chem. Eng. J., 283: 285-292.

Poleto, L., P. Souza, F.E. Magrini, L.L. Beal and A.P.R. Torres *et al*., 2016. Selection and identification of microorganisms present in the treatment of wastewater and activated sludge to produce biohydrogen from glycerol. Intl. J. Hydrogen Energy, 41: 4374-4381.

Prema, P. and K.N. Niladevi, 2008. Immobilization of laccase from Streptomyces psammoticus and its application in phenol removal using packed bed reactor. World J. Microbiol. Biotechnol., 24: 1215-1222.

Qian, C.X., L.Y. Chen, R. Hui and X.M. Yuan, 2011. Hydrogen production by mixed culture of several facultative bacteria and anaerobic bacteria. Prog. Nat. Sci. Mater. Intl. J., 21: 506-511.

Rastogi, R.P., Richa, A. Kumar, M.B. Tyagi and R.P. Sinha, 2010. Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. J. Nucleic Acids, 10.4061/2010/592980

Ren, N., Z. Chen, A. Wang and D. Hu, 2005. Removal of organic pollutants and analysis of MLSS-COD removal relationship at different HRTs in a submerged membrane bioreactor. Intl. Biodeterior. Biodegrad., 55: 279-284.

Rossi, D.M., J.B. De-Costa, E.A. De Souza, M.D.C.R. Peralba and D. Samios *et al*., 2011. Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. Intl. J. Hydrogen Energy, 36: 4814-4819.

Sharmila, V.G., P. Dhanalakshmi, J.R. Banu, S. Kavitha and M. Gunasekaran, 2017. Effect of deflocculation on photo induced thin layer titanium dioxide disintegration of dairy waste activated sludge for cost and energy efficient methane production. Bioresour. Technol. J., 244: 776-784.

Sharmila, V.G., S. Kavitha, K. Rajashankar, I.T. Yeom and J.R. Banu, 2015. Effects of titanium dioxide mediated dairy waste activated sludge deflocculation on the efficiency of bacterial disintegration and cost of sludge management. Bioresour. Technol., 197: 64-71.

Skorb, E.V., L.I. Antonouskaya, N.A. Belyasova, D.G. Shchukin and H. Mohwald *et al*., 2008. Antibacterial activity of thin-film photocatalysts based on metal-modified TiO_2 and TiO_2: In_2O_3 nanocomposite. Appl. Catal Environ., 84: 94-99.

Sui, H., J. Dong, M. Wu, X. Li and R. Zhang *et al*., 2017. Continuous hydrogen production by dark fermentation in a foam SiC ceramic packed up-flow anaerobic sludge blanket reactor. Can. J. Chem. Eng., 95: 62-68.

Tanisho, S., 2001. A Scheme for Developing the Yield of Hydrogen by Fermentation. In: Biohydrogen II, Miyake J., T. Matsunaga and A.S. Pietro (Eds.). Pergamon Press, Oxford, England, UK., pp: 131-140.

Terrazas-Hoyos, H., E. Portugal-Marin, E. Sanchez-Salinas and M.L. Ortiz-Hernandez, 2014. Evaluation of the potential hydrogen production by diazotrophic Burkholderia species. Intl. J. Hydrogen Energy, 39: 3142-3151.

Yang, S.S., W.Q. Guo, G.L. Cao, H.S. Zheng and N.Q. Ren, 2012. Simultaneous waste activated sludge disintegration and biological hydrogen production using an ozone/ultrasound pretreatment. Bioresour. Technol., 124: 347-354.

Zhang, K., N.Q. Ren and A.J. Wang, 2014. Enhanced biohydrogen production from corn stover hydrolyzate by pretreatment of two typical seed sludges. Intl. J. Hydrogen Energy, 39: 14653-14662.