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Optimization (Substrate and pH) and Anaerobic Fermentative Hydrogen Production by Various Industrial Wastes Isolates Utilizing Biscuit Industry Waste as Substrate

Gujuluva Hari Dinesh[1,2], Karthik Sundaram[3], Kulanthaisamy Mohanrasu[1,2], Ramu Satheesh Murugan[1,2], Puthamohan Vinayaga Moorthi[4], Tondi Rajan Angelin Swetha[2], Gopal Selvakumar[5] and Alagarsamy Arun[2]*

[1]Department of Energy Science, Alagappa University, Karaikudi, India.
[2]Department of Microbiology, Alagappa University, Karaikudi, India.
[3]Department of Microbiology, PSG Arts College, Coimbatore, India.
[4]Department of Human Genetics and Molecular Biology, Bharathiyar University, Coimbatore, India.
[5]Department of Microbiology, DDE, Alagappa University, Karaikudi, India.

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The bio hydrogen (H_2) production by anaerobic digestion of industrial waste is beneficial one due to the availability of proteins and carbohydrates as potential substrate for biological H_2 production. An anaerobic fermentative route is a promising method of bio-hydrogenation. The microbial isolates from various industrial wastes (dairy, sugar and food) were assessed for their potential bio H_2 production. The selected individual isolates (F1 - *Bacillus subtilis* and A3 -*Bacillus subtilis*) and their cocultures were used for the optimization of bio H_2 production utilizing various concentration of biscuit industry waste as substrate at various pH conditions. The mixed consortium which displayed the higher bio H_2 production was selected for the detailed analysis of the 3L fermentation studies using 90% Organic Loading Rate (OLR) substrate at pH 6.5. Significantly higher Hydrogen Yield (HY) of 0.87 mol H_2/mol glucose on 16th day of incubation was observed.

Keywords: Food waste, substrate, pH, Bio hydrogen, Fermentation, *Bacillus subtilis*.

The indiscriminate use of fossil fuels has polluted the environment and also have exhausted the limited fuel reserves necessitating searching for alternative energy. H_2 is one of the promising potential alternative clean energy[1]. Though various methods are available for H_2 production, Das and veziroglu [2] highlighted the importance of biological H_2 production as it is usually operated at amphient temperature and atmospheric pressure. Microbial H_2 production is an attractive process for supplying the significant share of the H_2 energy required for the near future[3].

A high organic load containing industrial waste water that dispersed in to the natural water system without any proper treatment primarily has a number of negative effects on the inhabitants. The high organic content present in the system is being used as a source of energy by various indigenous microbial populations[4-6]. Effluent from various industries such as food, sugar, paper, dairy and pharmaceutical contains huge volume of waste water with a very high organic content[7]. The current physical and chemical methods of treatments are effective only to a particular extent as the disadvantage in the production of sludge during processing and the high processing charges makes it inconvenient in the industry point of view[8, 9].

* To whom all correspondence should be addressed.
E-mail: arunalacha@gmail.com

Industries have already started utilizing the microbes by deploying them in the activated sludge process. Their reduction efficiency is on par with the physical and chemical treatment methods with a significant reduction in the processing charges. In contrast with a proper knowledge on the value added utilities, more useful products could be evolved out of the waste water treatment system, which could not only solve the problem of pollution, but also aid in reducing the overall processing of the industry. Fermentative H_2 production is the alternative way for H_2 production[10, 11].

Bio H_2 is one such value added product that could be evolved out of the industrial waste water with the proper exploitation of the effective microbes[5, 6, 12]. The overall metabolic activity of the microbes present in the environment would be less owing to a least difference in the energy levels between the organic content and the compound that is formed after the breaking down of organic content. With the production of H_2, the industry could be self-sustained in their energy production. H_2 gives the maximum amount of energy when burnt and this heat energy could be efficiently converted to electrical energy. In addition, on combustion H_2 gives out pure water, which again does not lead to any global warming or pollution[13]. In this study, H_2 producing microbes were enriched and isolated from various industrial waste water. The basic idea behind it is that the microorganism gets acclimatized to the harsh environment with the capability of utilizing the organic content present in the wastes effectively than that of the non-native microbes. Among the enriched isolated H_2 producers, the maximum H_2 evolving microorganisms were selected and were used for further studies. The selected bacterial strains were characterized by conventional microbial characterization studies and 16S rRNA sequencing analysis. Optimal pH for bio H_2 production was found to be different depending on the type of inoculation and substrate used in the study[14]. As the major obstacle in large scale bio H_2 is their optimized environment for microbial metabolic activity, hence the different OLR and pH of the food industry waste were optimized using individual and mixed cultures. The best optimized condition was further used for scale up in 3L fermenter.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were procured with highest purity available and were purchased from Himedia, India. All the experiments were done in triplicates.

Collection of Various Industrial Wastes

Properly pretreated mixed anaerobic sludge of various industrial wastes such as dairy, sugar, food industry were collected and used as a seed for the isolation of H_2 producing microorganisms. Effluent collected from the industries were stored at 4°C to prevent the oxidation of organic content present in it and was used for further treatment trials for H_2 production[15].

Enrichment and Isolation of Hydrogen Producing Microorganism

The spore forming bacterial strains (*Clostridium* sp and *Bacillus* sp, etc) are those which were involved in the production of biogas. In order to make sure that the H_2 producing strains were enriched and selectively isolated from the effluent source, the samples were subjected to rigorous pretreatment. The microorganisms present in the collected industrial wastes were enriched by subjecting them to a cyclic heat shock[16] and acid treatment methods[15]. To restrain the growth of bacteria and simultaneously to selective enriched H_2 producing acidogenic bacteria, 50 ml of various industrial wastes were subjected to cyclic pretreatment sequences (four times) changing between heat-shock at 100°C by keeping in oven for 2 h and acid by adjusting the pH of the content to pH 3 using 88% Orthophosphoric acid and kept for 24 h. After heat and acid treatment, a loopful of treated samples was aseptically streaked on thioglycollate agar plates. The plates were anaerobically incubated at 37°C for 24 h in an anaerobic chamber. After incubation the morphologically different microorganisms grown in thioglycollate agar plates were selected for further studies.

The oxygen tolerances of the isolated microorganisms were assessed by growing in thioglycollate medium with 1% percentage of agar deep tubes. The isolates were inoculated on the thioglycollate agar deep tubes and incubated

at 27°C for 24 h. The growth pattern indicates whether the isolates are aerobic or microaerophilic or facultative anaerobic (or) obligate anaerobic organisms.

Isolation of potential Bio hydrogen producing microbes

The bio H_2 production by the isolated microorganisms in liquid culture was determined using 100 ml of serum bottles containing 50 ml of sterile nutrient broth medium. Serum bottles were aseptically inoculated with 100µl of 24h bacterial isolates individually, as consortium. Treated and untreated industrial wastes were also used as Inoculum on nutrient broth. The serum bottles were capped with butyl rubber stopper and clamped with aluminum cap using crimper. The serum bottles were subjected to anaerobic environment by sparging nitrogen gas in the head space for 5 min. The serum bottles were incubated at 37°C at 150 rpm in incubator shaker (at 150 rpm) for 72 h. The biogas produced was analysed by using modified hungate technique[17] and the gas composition was analysed using gas chromatography (Shimadzu GC 2014) equipped with thermal conductivity detector (TCD). Column was packed with porapak Q tube (80/100 mesh) and nitrogen gas was used as the carrier gas. The operational temperatures of the injection port, oven and the detector were 100^0C, 80^0C and 150^0C respectively. The biogas was manually injected and the injection volume was about 1 ml. Based on the gas volume and composition the higher bio H_2 producing microorganisms were selected for further studies.

Identification of the Isolates

The selected pure isolates were identified based on their microscopic, morphological, biochemical characters[18] and partial sequencing of their 16S rRNA. The isolation of DNA was done according to Janardhanan and Vincent[19]. The partial 16S rRNA was amplified according to Rochelle *et al*[20]. Partial DNA obtained from PCR was sent for sequencing service. The sequences of the partial 16S rRNA were compared with the 16S rRNA sequence available in the public nucleotide databases at the National Center for Biotechnology Information (NCBI) by using its World Wide Web site (http://www.ncbi.nlm.nih.gov), and the BLAST (basic local alignment search tool) algorithm.

Substrate characterization

The biscuit industry waste was used as a substrate for bio H_2 production. The waste was collected from Indian foods, Madurai, Tamilnadu, India. The various physiochemical properties such as pH, oxidation-reduction potential (ORP), total volatile fatty acids (VFA), alkalinity, chemical oxygen demand (COD), total solids (TS), total suspended solid (TSS), volatile suspended solid (VSS), protein and glucose of the waste were analysed in triplicates according to the standard methods[21].

Optimization studies

The major obstacle in large scale production of bio H_2 by the microbes is their optimized environment for their best metabolic activity. Microorganisms with higher bio H_2 production were further used for the optimization studies. The optimization studies were carried out by shake culture method using serum bottles. The analysis of H_2 production efficiency of the microbes (individually and mixed) was carried out using food industry waste (50 ml) under different organic loading rate (OLR) (50%, 60%, 70%, 80%, 90% and 100%) and pH (5, 5.5, 6, 6.5 and 7). The selected individual bacteria or mixed or effluent used as inoculum were prepared in sterile nutrient broth and after 24h of incubation, 2% of the culture was added to the 48 ml of the production medium. The production medium in each serum bottles consisted of sterile food industry waste with different OLR and pH levels. The serum bottles were capped with butyl rubber stopper and clamped with aluminum cap using crimper. The serum bottles were subjected to anaerobic environment by sparging nitrogen gas in the head space for 5 min. The serum bottles were incubated at 37°C at150 rpm in incubator shaker for 72 h. The biogas produced and gas composition were analysed as referred before.

Fermentation studies

Microbial isolates with higher bio H_2 production were used for the fermentative bio H_2 production in 3L fermenter. Batch fermentation was carried out in a 3 litre fermentor (Lark-hygiene plus, India) with a working volume of 2.5 litre. The bioreactor was equipped with pH, temperature and dissolved oxygen controllers. The optimized substrate concentration and pH was maintained at 37°C during fermentation. The

headspace was sparged with N_2 gas to generate anaerobic condition. The batch mode condition was operated to a maximum period of 21 days. Samples were taken at every 24 h intervals and were used for analytical methods and the biomass was calculated. Uninoculated media was used as control. The gas produced during the fermentation process was passed through an acidic solution with pH of 3, in order to prevent dissolution of biogas as described by Ren *et al*[22]. The volume of the biogas from the experiment was measured using water displacement method and analysed for gas composition as described earlier.

The cumulative bio H_2 production profile from batch fermentation was calculated by modified gompertz equation[23].

$$H_{(t)}=P.exp\{-\exp[\frac{Rm.e}{p}(\lambda - t)+1)]\}$$

Where $H_{(t)}$ is the cumulative H_2 production (ml), P is the maximum H_2 production (ml), Rm is the maximum H_2 production rate (ml/h), ë is the lag phase time (h) ('e' is 2.718), and t is the incubation time (h).

Volumetric H_2 production rate (HPR) (ml l^{-1} h^{-1}) was calculated from cumulative H_2 production (ml/l) divided by fermentation time (h). Hydrogen yield (HY) (mol H_2/mol glucose) was calculated as total molar amount of H_2 divided by molaric amount of consumed glucose (as reducing sugar). The total molaric amount of H_2 (mol/l) was calculated using ideal gas law; total molaric amount of H_2 (mol/l) = Cumulative H_2 production (l) divided by RT. Where, R=0.0821 atm K^{-1} mol^{-1} and T=303 K.

RESULTS AND DISCUSSION

All the values studied in this work are mean of three replicates with a standard deviation <3 %. After proper enrichment process to inhibit H_2 consuming bacteria, 5 different bacterial isolates were isolated. Among the 5 different organisms that grew on the solid thioglycollate agar, three have been isolated from the dairy waste (A1, A2 and A3) one each from the sugar industry (S1) and food industry (F1) wastes respectively. Based on the oxygen tolerance all the isolates were identified to be facultative anaerobes.

Wang and Wan [14] and Xiao *et al*[24] reported that the maximum H_2 production of the mesophilic bacteria will occur at 37°C. So the fermentation studies were carried out at 37°C. Five bacterial isolates were used to study their bio H_2 production potential in nutrient medium[25] under anaerobic condition. The total biogas and composition of the biogas produced by the isolates were studied. The biogas produced contained only H_2 and CO_2 indicating that pretreatment process had effectively removed the methanogens. Based on the gas volume and gas analysis by GC, among the isolates F1 had shown a maximum bio H_2 production of 104 ml H_2/L and the isolate A3 shown 60 ml H_2/L. All the other organisms such as A1, A2 and S1 had shown 16 ml H_2/L.

In the treated/untreated industrial wastes as inoculum, 3 ml, 3.5 ml of gas evolved with 40% : 60% (H_2 : CO_2) and 20% : 20% : 60% (H_2 : CO_2 : methane) respectively. In treated waste as inoculum, very less level of H_2 gas evolved confirms that the organisms are not acclimatized to the new nutrient environment to produce higher H_2 gas production. The untreated waste as inoculum also had similar level of H_2 gas evolved along with methane. This result highly indicates the need of pretreatment for better H_2 production with suppressed level of methanogen activity. Kim *et al*[26] and Daims *et al*[27] also highlighted the importance of pretreatment for higher bio H_2 production.

The partial sequences of 16S rRNA gene were determined for all the five isolates as they all shown bio H_2 production. Based on the results of 16S rRNA partial gene sequence comparison with

Table 1. Characterization of effluent used as substrate for hydrogen production

Parameter	Values
pH	5.625±0.12
ORP	139±9.90
TS (mg/l)	1375±176
TSS (mg/l)	2230±42
VSS (mg/l)	350±70
Alkalinity(mg/l)	3915±332
VFA (mg/l)	4277±219
COD (mg/l)	6912±123
BOD(mg/l)	6.7±0.37
Glucose (g/L)	0.5±0.14
Protein (g/L)	0.31±0.07

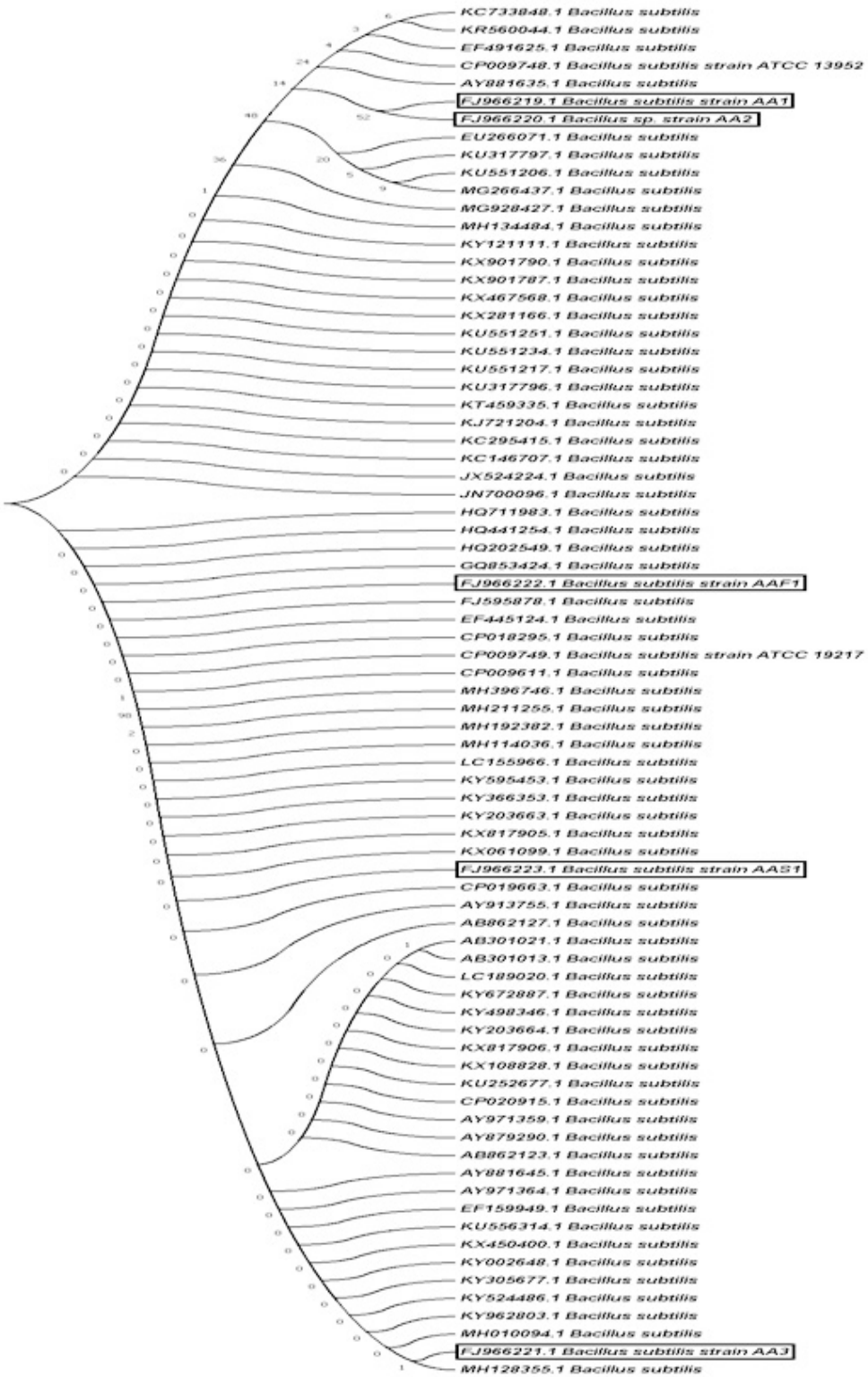

Fig. 1. Phylogenetic tree of Bio-Hydrogen producing *Bacillus subtilis* strains

existing database in Gene Bank indicates that the isolated bacterial strains belonged to the genus Bacillus. The sequence of A1, A2, A3, F1 and S1 strains had a 97%, 97%, 99%, 99% and 100% identity respectively with *Bacillus subtilis*. Based on the morphological, cultural and biochemical characteristics, the organisms were also further confirmed. The nucleotide sequence such as A1, A2, A3, S1 and F1 have been deposited in the Gen bank database under accession number FJ966219, FJ966220, FJ966221, FJ966222 and FJ96223 respectively (Fig. 1).

As the F1 and A3 isolates are having higher bio H_2 producing capability they were (individual, mixed culture and effluent) selected for further optimization studies using food industry wastes with different OLR and pH. The temperature was maintained at 37˚C, as the isolates are mesophiles in virtue. The physiochemical character of the food industry waste is tabulated (Table 1).

The biscuit industry waste used in the study is rich in carbohydrate content and thus it is very much suitable for bio H_2 production. Lee *et al*[28] and Hu *et al*[29] also suggested that the food waste is suitable for bio H_2 production. Lay *et al*. [30] found that carbohydrate give 15 times greater bio H_2 production compared to lipid and protein utilization. The food waste as it contains very high COD concentration (7000 mg/l), it is best suited for bio H_2 production.

The important criteria to optimize the media for higher bio H_2 production are OLR and pH. pH is one of important parameter influencing bio H_2 production produced from food waste[30]. Bio H_2 production using food waste has been wildly tested either by batch or continues mode[24, 29, 31, 32, 33]. To our knowledge there is no study on bio H_2 production using biscuit industry waste.

The condition such as OLR (50-100%) and pH (5.0, 5.5, 6.0, 6.5 and 7.0) were optimized for the bio H_2 production by the selected isolates (F1 & A3). Among the two isolates F1 (0.70 mol H_2/mol glucose) and A3 (0.67 mol H_2/mol glucose) (80% H_2 and 20% CO_2) were displayed higher bio HY in 90% OLR at pH 6.5 for 48 h. Mixture consortium (F1 & A3) was shown 0.82 mol H_2/mol glucose of HY in 90% OLR at pH 6.5 for 48 h. In effluent used as inoculam, 0.39 mol H_2/mol glucose of HY in 90% OLR at pH 6.5 for 48 h was observed (Fig. 2). The biogas produced by the individuals, selected consortium isolates contained only H_2 and CO_2. The increasing VFA level and decreased level of COD are the useful indicators in monitoring H_2 production. The present results corroborate well with the results of Cheng *et al*[34] and Teng *et al*[35].

Lee *et al*[36] also observed maximum H_2 production at pH 6 to 7. Valdez and varaldo[37] emphasized that the pH has larger impact on bio H_2 production, which directly affects the hydrogenase activity. Khanna *et al*[38] found pH 6.5 as the optimized pH for maximum bio H_2 production using *Enterobacter cloacae*. The H_2 production is usually linked by increase in volatile fatty acids by the metabolism of hydrogenase producing microorganisms [39].

The effect of initial pH adjustment and autoclaving of food waste for bio H_2 production was studied by Hu *et al*[29]. Elbeshbishy *et al*[40] and Hu *et al*[29] observed that the pretreatment (pH and autoclaving) could enhance the solubility of organic content of the food waste. In the present study we used the autoclaved substrate. The mixed

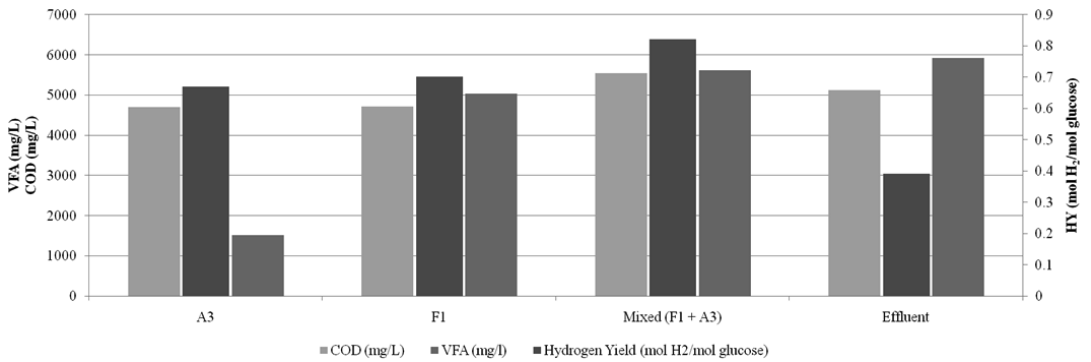

Fig. 2. The maximum results of Hydrogen Yield, COD and VFA in 90 % of Biscuit industry wastewater at pH 6.5 during the optimization studies

consortium which displayed the higher bio H_2 production was selected for the detailed analysis of the batch fermentation studies using 90% OLR substrate at pH 6.5. Khanal *et al*[41] emphasized the pH control is an important factor to suppress H_2 consumers. The biogas produced (ml) was calculated after the composition analysis using gas chromatography excluding other gases. During the course of fermentation study the evolvement of the H_2 was observed. The maximum production of H2 was seen up in 16th day of incubation (0.87 mol H2 /mol glucose) (Fig.3). A steady decrease of COD and steady increase of VFA confirms the acidogenic bio H2 process.

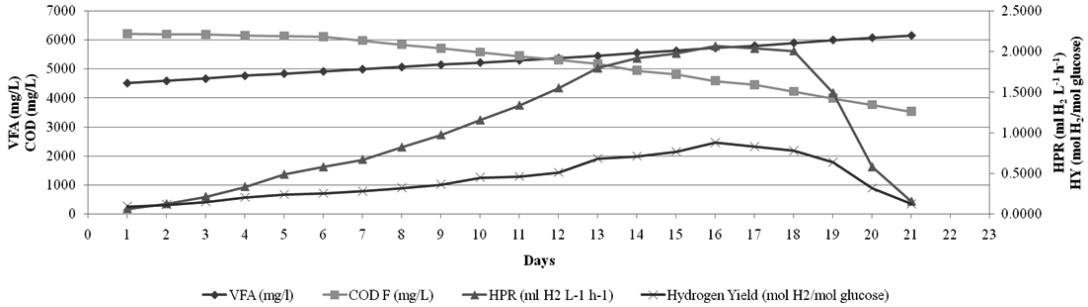

Fig. 3. Fermentation studies on Biohydrogen Production in Food industrial wastewater (90 %) at pH 6.5 using Mixed Isolate (F1 and A3) as inoculam

In the present fermentation study, when observing H_2 production, a lag phase (2 day) followed by incessant log phase (up to 16th day) and decline phase was observed. Similar kind of result was seen by Fan *et al*[42]. Lay *et al*[43] observed log phase when the bacterial cells are transferred to superior environment. From our study we observed a maximum HY of 0.87 mol H_2/mol glucose at 16th day of incubation utilizing 90% (OLR) food industry waste at pH 6.5 by bacterial consortium. This result has to be applied further for industrial level higher rate H_2 production.

CONCLUSION

Fermentative bio H_2 production using biscuit industry waste as a fermentation medium by mixed anaerobic consortia isolated from industrial waste was carried out. The screening study was first carried out to select potential bio H_2 producer, among the 5 different isolates strains F1 (*Bacillus subtilis*) and A3 (*Bacillus subtilis*) showed commendable production of H_2 gas. The present study successfully found the natural microbial inhabitant of industrial waste with potential bio H_2 production efficiency. Maximal level of H_2 production was observed at a substrate concentration of 90% at pH 6.5 by the mixed consortium of all the isolates. The optimized was used for 3L batch fermentation study using the mixed consortium of F1 (*Bacillus subtilis*) and A3 (*Bacillus subtilis*). A maximum cumulative H_2 production of 49.58 ml/L was observed at 16th day of incubation. There was a relationship between bio H_2 content, the COD reduction and increasing VFA ratio is significant indication of industrial wastes into useful products. Further industrial scale H_2 production has to be evolved in future.

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