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Original Research

Exploitation of dark fermented effluent of cheese whey by co-culture of *Rhodobacter sphaeroides* and *Bacillus firmus* for photo-hydrogen production

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Abstract: In this study photo-hydrogen production from cheese whey dark fermentation (DF) effluent by the co-culture of *Rhodobacter sphaeroides* –NMBL-01 and *Bacillus firmus* – NMBL-03 has been reported. The effect of pH, initial chemical oxygen demand (COD) and the concentration effect of $FeSO_4.7H_2O$ on photo-hydrogen production have been investigated. The end products of dark fermentation effluent of cheese whey were mainly comprised of soluble organic acids, i.e. butyric acid and lactic acid. The batch process was carried out under light intensity of 2.5 kLux at 32 ± 2°C without any addition of extra carbon and nitrogen source. The single parameter optimization studies revealed optimum pH 6.5, initial COD 4.71 g/L and supplementation of Fe^{2+} concentration 100 mg/L. The maximum cumulative hydrogen production and yield were found to be 469 ± 45.8 ml H_2/L and 146.56 ± 14.31 ml H_2/g COD reduced (67.9% reduction in COD) respectively. The mutual interactions among the process parameters were also investigated by three factorial Box-Behnken design of response surface methodology. The optimized experimental values were found concurrent with the calculated values obtained from the theoretical model.

Key words: Co-culture; Photofermentation; Cheese whey wastewater.

Introduction

Hydrogen as a clean and an efficient carrier of fuel (143GJ/ton) is widely being accepted to be a potential substitute for fossil fuels. The challenge today is to produce it renewably and in large scale, through environmentally benign processes (1, 2). Some of the physico-chemical factors which significantly affect biological hydrogen production are pH, carbon source, C/N ratio, phosphate levels as well as the nature of microbial flora (3, 4). Photosynthetic hydrogen production is driven by nitrogenase activity which simultaneously converts molecular nitrogen (N_2) to ammonia (NH_3). On the other hand H_2-producing activity of photofermentative bacteria is strongly inhibited in the presence of excess nitrogen and unfavourable pH (5-7). A sequential process of dark fermentation (DF) and photofermentation (PF) by photosynthetic purple non sulfur bacteria (PSB) has not only tremendous potential for H_2 production but also provides a promising way for bioprocess development and complete conversion of sugars (5, 8-10). Therefore, the optimized production of hydrogen lies in understanding the hydrogen metabolism i.e. the responses with regard to the change in the environment of the culture.

Cheese whey being a waste of cheese manufacturing industry has been used as a cheap raw material for production of fuels (ethanol, methane, hydrogen) by photo/dark fermentation of organic acids (lactic acid, butyric acid and acetic acid), carbohydrate (5-6% w/v lactose), protein (0.8-1%), and fat (0.06%) contents present therein (11). The influence of initial pH on the hydrogen yield in a batch process using *Clostridium* strain from diluted cheese whey has been investigated (12). Although there are several reports of biohydrogen production using cheese whey as substrate by dark fermentation but to best of our knowledge, so far, there is no report of further conversion of organic acids (which are by-products of cheese whey dark fermentation) to photohydrogen. This conversion of organic acids present in the dark fermentation effluent could be achieved by photofermentation either by the pure suitable photosynthetic bacterial (PSB) strain or a suitable co-culture. The advantage of the co-culture (photo- and dark fermenter) is that phototroph utilizes the in situ generated organic acids (i.e. butyric/lactic acids) produced by the fermenting bacteria and nullifies its inhibitory effects thereby making the process sustainable till all carbon sources exhausted. Simultaneously, in the same batch culture the emitted carbon dioxide by dark/photo fermenter is fixed by the phototrophs and gets fermented by the dark fermenting organism. Moreover, the overall success of the photofermentation process also depends on the strain of the used photosynthetic bacteria (PSB) (5, 13).

In the present study, photohydrogen production from DF effluent of cheese whey as substrate using the co-culture of *Rhodobacter sphaeroides* –NMBL-01 and *Bacillus firmus* – NMBL-03 has been reported. Our previous work with *R. sphaeroides*-NMBL-01 strain has given promising results (5) which has been used for biohydrogen production experiments. The RSM was used to maximize yield and rate of hydrogen gas production

from DF effluent of cheese whey wastewater.

Materials and Methods

Bacterial strains and growth conditions

In the current study *Rhodobacter sphaeroides* NMBL-01 which is a photosynthetic purple non sulfur bacteria (PSB) (5) has been employed for photofermentation along with the heterotrophic partner microbe *Bacillus firmus* NMBL-03 (Gene Bank Accession no.: HE610429.1). The later was isolated from local sludge, purified by clonal selection method and characterized by 16S rRNA gene sequencing using universal primers 27 F and 1492 R (Eurofins, Bangalore, India). This co-culture (*Rhodobacter sphaeroides* NMBL-01 and *Bacillus firmus* NMBL-03) was grown together for 8-10 sub-cultures and used for optimized photo-hydrogen production using DF effluent of cheese whey wastewater.

Substrate and media composition

The DF effluent of cheese whey wastewater (from milk industry) with chemical oxygen demand (COD) value of 80g/L was obtained as gift from B.H.U., India and used as such in the current study. The co-culture used in the current study was cultured and maintained in DF effluent containing mineral solution, trace elements and vitamin solutions as reported earlier (5). The effect of initial pH of the media on hydrogen production was studied at different initial pH values i.e. 6.0, 6.5, 6.8, 7.0, 7.3, 7.5 and 7.8 (maintained by addition of 0.1 M NaOH). The DF effluent was diluted in folds of 4, 8, 12, 14, 17 and 20 using the mineral-trace-vitamin solution (5) to examine the photohydrogen evolution at different COD values. To study the effect of Fe^{2+} ions (in 0.1, 1, 50, 100, 150, 300 and 500 mg/L concentrations) on hydrogen production, filter sterilized $FeSO_4.7H_2O$ was used in place of Fe (III) citrate in the solution of trace elements. After autoclaving the media for 15 min at 103.5 kPa (15 psi) pressure and 121 $^oC \pm 2\ ^oC$ temperature, filter sterilized vitamin solution was added.

Experimental conditions

The batch experiments were performed in air-tight 120 mL serum bottles containing 55 mL of inoculated medium under stirring condition (50 rpm) (5). Initial cell concentration was kept at 0.40 g dcw/L (48 h grown cells). The experimental set up was maintained at 32 $^oC \pm 2\ ^oC$ and illuminated with a 200W tungsten lamp to provide a uniform light intensity of 2.5 kLux at the surface of batch reactors. The experiment was monitored for 3 days. The produced hydrogen was collected in 20 mL gas tight disposable syringes. The colony forming unit per mL (cfu/mL) was calculated on day 0, day 1 and day 3 to know the population dynamics of the co-culture using serial dilution plating technique.

First strand cDNA synthesis and its analysis

12 h and 24 h grown co-cultures at different pH values were used for messenger RNA (mRNA) isolation using RNeasy® Protect Bacteria Mini Kit from Qiagen. DNase treatment was performed according to the protocol provided by manufacturer. Purity and concentration of DNA-free RNA samples were determined by the absorbance measurements at 260 nm and 280 nm on UV-Vis spectrophotometer (Labomed, USA). 100 ng mRNA was used for synthesis of first strand cDNA with primer of *nif*H(5'-AGACCGTGTGGGGCGTGCTG-CACGATATT-3') using first strand cDNA synthesis kit (First Strand cDNA Synthesis Kit # 1611 # 1612 from Fermentas) according to procedure provided by manufacturer (14). The concentration of first strand cDNA was determined spectrophotometrically at 260 nm.

Single parameter optimization

Single parameter optimization with respect to different initial pH values, dilution folds (i.e. % dilutions) of DF effluent of cheese whey substrate and iron (II) sulfate concentrations was done in 120 mL serum bottles containing 55 mL media under strict anaerobic illuminated conditions as illustrated above.

Multiple parameter optimizations

Multiple parameter optimizations with respect to initial pH, initial COD and Fe (II) sulfate concentrations were studied by using three factorial Box-Behnken design (15). Actual levels of the chosen variables and 12 unique sets with a triplicate on the centre point of experimental design are given in Table 1. Response Surface Methodology (RSM) was used to interpret the significant interactions existing between the chosen independent variables using MINITAB 15 statistical software based on second degree polynomial equation (15).

Analytical method

The pH of the culture medium was measured with Eutech (Merck) pH meter. The final pH of the media was taken at the end of experiment i.e. 3rd day. The bacterial cell concentration was measured by UV-Vis spectrophotometer using the standard curve where one

Table 1. Coded and actual levels of variables chosen for the statistical design of experiment.

Factors	Levels	Variables
pH	-1	6.0
	0	6.5
	+1	7.0
Dilution fold	-1	14
	0	17
	+1	20
Fe (II)sulfate	-1	50 mg/L
	0	100 mg/L
	+1	150 mg/L

unit of optical density at 660 nm corresponded to 0.50 g dcw/L medium. Bacteriochlorophyll-a concentration present in the whole cell was determined according to the method al., 2012 (16). The COD content of cheese whey was measured according to standard APHA protocols (17). The collected gas was analyzed with a gas chromatograph (Agilent 7890) equipped with a thermoconductivity detector and a capillary column (HP-PLOT/Q). Nitrogen gas served as carrier and pure hydrogen gas served as standard. The oven temperature was 90 °C, and the temperature of detector and injector as 100 °C and 70 °C respectively. The analysis of organic acids was done through HPLC (Agilent 1200) fitted with C-18 column using 0.05 M KH_2PO_4 buffer (adjusted pH 2.5 using phosphoric acid with a flow rate of 0.15 mL/min).

Results and discussion

Effect of pH on hydrogen production

The optimization of pH for photohydrogen production by co-culture of *Rhodobacter sphaeroides* and *Bacillus firmus* using spent media of dark fermentation (DF effluent) of cheese whey wastewater has been investigated. At initial pH 6.5 and COD (5.71 g/L), maximum cumulative hydrogen (213 ± 25.56 mL H_2/L) with yield 57.41 ± 2.53 mL/g COD reduced for biomass 1.08 ± 0.12 g/L and bacteriochlorophyll-a 16.95 ± 1.25 mM (Table 2) using initial COD (5.71 g/L) of the DF effluent was obtained. The results showed that the initial pH has pronounced effect on the yield of hydrogen production, biomass and bacteriochlorophyll-a concentration (Table 2). The analysis of cfu/mL during the whole experimental tenure showed the values $1X10^8$ and $1X10^8$ for *Rhodobacter sphaeroides* and *Bacillus firmus* respectively. This indicates that both the cultures grew at 1:1 proportion for the experimental duration without any drastic change in final pH which is the main advantage of co-culture. The first strand of cDNA of *nif*H transcript was synthesized to investigate the effect of pH on expression of *nif*H gene which is coding dinitrogenase reductase (subunit of nitrogenase enzyme containing iron as a cofactor) (18). To best of our knowledge it is the first report on *nif*H gene expression at different pH. In our investigation the concentration of first strand cDNA of *nif*H gene's transcript was found maximum (5.5 µg/mg) at initial pH 6.5 supporting the high yield of obtained hydrogen (data not shown).

pH has pronounced effect on cell biomass and hydrogen production. Reportedly, the maximum biomass yield (0.38 g/L) in case of *Rhodobacter sphaeroides* O.U. 001 was obtained at pH 7.0 (7). However, the end pH increased to 8.25 and 8.66 when the initial media pH values were adjusted at 6.5 and 7.0 respectively (7). Due to this the available light energy within the culture through absorption and scattering effects decreases. Notably, high concentration of biomass and bacteriochlorophyll-a significantly affects the light penetration reaching to the active cells facilitating nitrogenase catalysed photo-hydrogen production (7, 14).

In the present study we have obtained highest biomass (1.85 ± 0.26 g/L) at pH 7.8. The final pH increased from 6.84 to 7.3 and this pH range is most suitable for optimum nitrogenase expression and activity. The observed pH range in the present study may be attributed mainly due to the co-culture of heterotroph, *B. firmus* (as sugar utilizer and organic acid producer) and PSB, *R. sphaeroides* (as organic acid utilizer for sustained H_2 production) without increase in alkalinity beyond 7.3. In comparison to the two-step process of sequential dark fermentation and photofermentation, co-cultures of anaerobic and photosynthetic bacteria are more efficient and cost effective for hydrogen production (8). Evidently, co-cultures of *Clostridium butyricum* and the photosynthetic bacterium *Rhodopseudomonas* sp. RV produced 7 mol H_2/mol hexose (8). Co-cultures of *Clostridium acidisoli* and *Rhodobacter sphaeroides* were used (13) to optimize substrate concentration, initial pH and inoculum's ratio of *C. acidisoli* to *R. sphaeroides* as 11.42 g/L sucrose, 7.13, and 0.83 respectively for efficient hydrogen production. A comparison of some of the relevant data from the literature with the findings of the present work is given in Table 3.

Effect of dilution of spent media (DF effluent of cheese whey wastewater) on hydrogen production

The HPLC analysis of DF effluent of cheese whey wastewater showed high concentration of butyric acid and lactic acid as main organic acids alongwith dissolved ammonium. Therefore, dilution of the effluent is a mandatory step to achieve optimized hydrogen production. The spent media was diluted (% dilution) 4 (25%), 8 (12.5%), 12 (8.3%), 14 (7.1%), 17 (5.9%) and 20 (5%) folds with mineral-trace-vitamin solution maintaining optimized initial pH at 6.5 to study its effect on cumulative hydrogen production and yield. In pres-

Table 2. Effect of initial medium pH on final pH, cumulative hydrogen produced, yield of H_2 production (mL H_2/g COD reduced), bacteriochlorophyll-a concentration (mM) and biomass (g/L) by photofermentation of 14 fold diluted dark fermentation spent media of cheese whey (5.71 g/L COD) using co-culture of *R. sphaeroides*-NMBL-01 and *Bacillus firmus*-NMBL-03 at temperature 32 °C ± 2 °C under illumination of 2.5 kLux in a batch process. The hydrogen production was monitored for 3 days.

Initial pH	Final pH	Cumulative hydrogen produced (ml/L)	Yield (mL H_2/g COD reduced)	Bacteriochlorophyll-a concentration (mM)	Biomass (g/L)
6.0	6.9 ± 0.20	175 ± 17.00	47.17 ± 4.58	13.20 ± 1.10	1.10 ± 0.15
6.5	6.84 ± 0.20	213 ± 25.56	57.41 ± 6.89	16.95 ± 1.25	1.08 ± 0.12
6.8	7.1 ± 0.18	153 ± 9.40	41.24 ± 2.53	20.15 ± 1.82	1.30 ± 0.13
7.0	6.99 ± 0.30	147 ± 9.20	39.62 ± 2.48	23.05 ± 1.81	1.45 ± 0.15
7.3	7.13 ± 0.20	142 ± 9.80	38.27 ± 2.64	21.30 ± 1.90	1.45 ± 0.17
7.5	7.3 ± 0.34	144 ± 8.50	53.14 ± 3.14	19.90 ± 1.80	1.57 ± 0.21
7.8	7.28 ± 0.28	149 ± 7.20	40.16 ± 1.94	16.90 ± 1.82	1.59 ± 0.26

Table 3. Comparison of hydrogen yields with the literature studies.

Inoculum	Carbon substrate	Yield of H_2	Organic loading rate	Reference
Clostridium saccharoperbutylacetonicum	Cheese whey	2.8 mmol H_2/mol lactose	89.3 g/COD in batch	Ferchichi et al., 2005
Mixed fermentative seed sludge	Cheese whey	-	20 g COD/L/d	Castello et al., 2009
Anaerobic fermentation	Cheese whey powder	8.1 mmol H_2/g COD	10 g/L lactose In batch	Azbar et al. ,2009
E. cloacae IIT-BT 08	Cheese whey	10.3 mmol H_2/g COD	10 g/L COD in batch	Khanna et al. , 2011
Anaerobic sludge	Cheese whey powder	1.03 mol H_2 /mol glucose	20 g total sugar/L	Kargi et al., 2012
Co-culture of *R. sphaeroides*-NMBL01 and *Bacillus firmus*-NMBL03 (photofermentation)	Dark fermentation product of fresh cheese whey waste water	146.56 mL (6.54 mmol) H_2/g COD reduced	4.71 g/L COD In batch	This study

ence of 17 fold diluted effluent, maximum cumulative hydrogen (265 ± 22.5 mL/L) and yield (86.60 ± 7.35 mL/g COD reduced) with dry biomass of 1.22 ± 0.08 g/L was obtained (Table 3). However, at lower dilutions the biomass was high (ranging from 1.28-1.9 g/L) while at higher dilutions it decreased (0.55 g/L ± 0.045 at 20 folds dilution). Butyric acid was also detected in addition to lactic acid as end metabolite in 4, 8 and 12 fold diluted effluent. The presence of butyric acid results in increased biomass (because it is precursor of PHB biosynthesis, a storage polymer in PSB) and higher pH at end which is unfavourable for hydrogen production due to less light penetration and limited electrons supply for nitrogenase .

It has also been reported (10, 19) that the initial substrate concentration and the inhibitory concentration of nitrogen significantly influence the yield of hydrogen production. Therefore, in order to decrease the concentration of VFA and/or nitrogenous compounds dilution of the spent media of dark fermentation before photofermentation is practiced. At 40% dilution of dark fermentation effluent, no hydrogen evolution was achieved using *R. capsulatus*. However, hydrogen production increased in the 40-60% dilution range and further decreased with increase in dilutions (9).

Effect of Fe (II) sulfate on hydrogen production

Photofermentative and fermentative hydrogen productions and consumptions are known to be derived by the actions of nitrogenase and hydrogenase enzymes (20). Several electron carriers of the photosynthetic electron transport system (ETS) are known to contain iron as necessary cofactor such as ferredoxin (21). Photofermentation is strongly associated with the ETS through which the bacteria produce energy necessary for nitrogenase catalysed hydrogen production (22). There are several reports about the effect of external Fe^{2+} (or Fe (II)) addition on the biological hydrogen production by pure or mixed cultures through dark or photofermentation (23, 24). The maximum hydrogen yield 131.9 mL/g sucrose at 800 mg/L $FeCl_2$ was obtained in fermentative hydrogen production (25). Similarly, H_2 production from the glucose media was increased from 194 to 302 mL after increasing the concentration of Fe^{2+} from 0 to 300 mg/L (6). Maximum yield of hydrogen production i.e. 2.73 mol/mol sucrose at 1600 mg/L $FeSO_4$ concentration was observed (26). Photofermentative hydrogen production process is also significantly affected by the iron supplementation. Hydrogen production increased linearly in the range of 0 to 1.6 mg/L Fe^{2+} and reached maximum at 2.4 mg/L Fe^{2+} by *Rhodobacter sphaeroides* (21). About 3 fold increase in the hydrogen production from *Rhodobacter capsulatus* after addition of 5 mg/L Fe (III) citrate was observed (21). In a recent study the addition of Fe^{3+} (0.1 mM) improved hydrogen production into 3 folds by *R. capsulatus* (10). A significant improvement in COD removal efficiency (48.1%) after supplementation of Fe^{2+} has also been reported (27). We have studied the effect of Fe (II) sulfate concentration (0.1, 1.0, 50.0, 100.0, 150, 300.0 and 500.0 mg/L) on

Table 4. Effect of fold dilution of dark fermentation spent media of cheese whey (80 g/L COD) on final pH, cumulative hydrogen produced (mL/L), yield of H_2 production (mL H_2/g COD) and biomass (g/L) by photofermentation using co-culture of *R. sphaeroides*-NMBL-01 and *Bacillus firmus*-NMBL-03 at temperature 32 °C ± 2 °C under illumination of 2.5 kLux in a batch process. The hydrogen production was monitored for 3 days.

Fold dilution of spent media	Final pH	Total hydrogen produced (mL/L)	Yield (mL H_2/g COD)	Biomass (g/L)
4	8.99 ± 0.20	20 ± 4	1.54 ± 0.31	2.9 ± 0.62
8	8.45 ± 0.22	32 ± 5.2	4.92 ± 0.8	1.8 ± 0.41
12	7.98 ± 0.18	124 ± 15	23.85 ± 2.88	1.65 ± 0.24
14	6.87 ± 0.25	213 ± 24.60	57.41 ± 6.63	1.08 ± 0.12
17	7.05 ± 0.15	265 ± 22.50	86.60 ± 7.35	1.02 ± 0.080
20	6.88 ± 0.15	75 ± 5.6	28.85 ± 2.15	0.55 ± 0.045

Table 5. Effect of $FeSO_4$ concentration on final pH, average hydrogen production rate (mL/L/d), cumulative hydrogen produced (mL/L), yield of H_2 production (mL H_2/g COD reduced), biomass (g/L) and % COD reduction by photofermentation of 17 fold diluted dark fermentation spent media of cheese whey (COD 4.71 g/L) keeping initial medium pH 6.5 using co-culture of *R. sphaeroides*-NMBL-01 and *Bacillus firmus*-NMBL-03 at temperature 32°C ± 2 °C under illumination of 2.5 kLux in a batch process. The hydrogen production was monitored for 3 days.

Concentration of Fe^{2+} (mg/L)	Final pH	Average rate of hydrogen production (mL/L/d)	Total hydrogen produced (mL/L)	Yield (mL H_2/g COD reduced)	Biomass (g/L)	COD reduction (%)
0.1	7.06 ± 0.1	22 ± 2.2	110 ± 11.0	51.89 ± 5.19	0.90 ± 0.08	45.00
1.0	7.05 ± 0.12	33.6 ± 3.04	168 ± 15.2	61.54 ± 5.57	0.95 ± 0.11	57.90
50	7.15 ± 0.15	59 ± 5.16	295 ± 25.8	96.41 ± 8.46	1.12 ± 0.12	64.90
100	7.12 ± 0.11	93.8 ± 9.16	469 ± 45.8	146.56 ± 14.31	1.29 ± 0.15	67.90
150	7.15 ± 0.12	73.8 ± 5.0	369 ± 25.0	123 ± 8.33	1.4 ± 0.19	63.69
300	7.16 ± 0.10	48.2 ± 9.25	241 ± 18.5	120.5 ± 9.25	1.35 ± 0.18	61.57
500	7.15 ± 0.11	37.6 ± 3.16	188 ± 15.8	76.73 ± 6.45	1.15 ± 0.21	52.02

Table 6. A three factor Box-Behnken design matrix and respective theoretical model based results.

V1	V1c	V2	V2c	V3	V3c	Cumulative hydrogen production (mL/L)
6.5	0	14	-1	150	+1	200.625
6.5	0	17	0	100	0	417.667
6.5	0	17	0	100	0	417.667
6.5	0	20	+1	150	+1	328.625
6.0	0	20	+1	100	0	142.125
7.0	+1	17	0	50	-1	134.750
6.0	-1	17	0	50	-1	213.500
7.0	+1	20	+1	100	0	175.875
6.5	0	14	-1	50	-1	264.375
6.0	-1	14	0	100	0	200.125
6.0	-1	17	0	150	+1	223.250
6.5	0	20	+1	50	-1	87.375
6.5	0	17	0	100	0	417.667
7.0	+1	14	-1	100	0	166.875
7.0	+1	17	0	150	+1	302.500

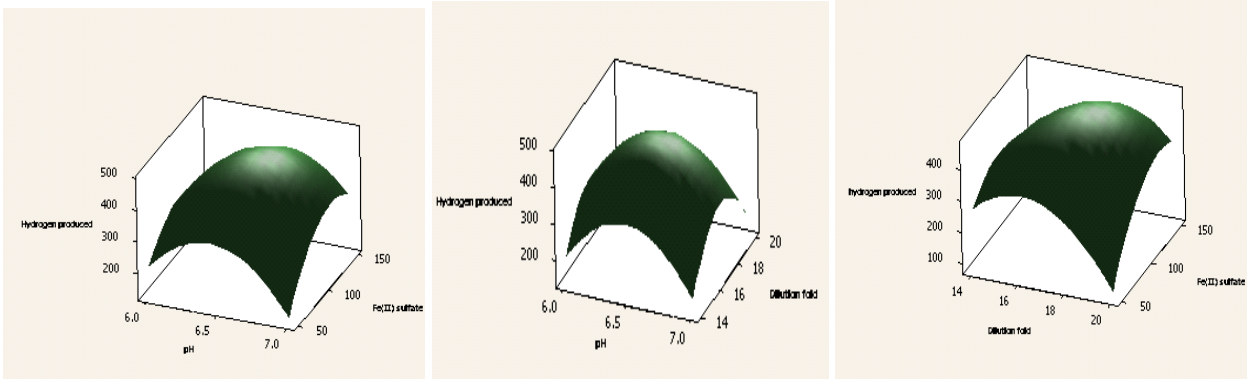
H_2 yield, biomass and % COD reduction (Table 4) by keeping initial pH 6.5 and using 17 fold diluted effluent (optimized conditions having COD 4.71 g/L). The optimum yield i.e. 146.56 ± 14.31 mL H_2/g COD reduced with 67.9 % COD reduction and biomass 1.29 ± 0.15 g/L at 100 mg/L Fe (II) sulfate concentration was observed. It may be concluded that with supplementation of Fe (II) sulfate at 100 mg/L, yield of hydrogen production increased by 1.69 folds. The comparative yield of biohydrogen with respect to present study has been documented in Table 6.

Hydrogen production by multiple parameter optimizations

The optimized parameters (pH, dilution of effluent and Fe^{2+} concentration) obtained from single parameter studies helped in designing the experiment for multiple parameter optimizations by a three factorial Box-Behnken design. The design of matrix and experimental results are shown in Table 1 and 5. Based on single parameter optimization results, pH was altered from 6 to 7 in steps of 0.5 unit, dilution fold of substrate from 14 to 20 fold in steps of 3 folds and Fe (II) sulfate concentrations from 50 mg/L to 150 mg/L in steps of 50 mg/L and hydrogen production was measured.

The response surface plot obtained by MINITAB 15 based on Fe (II) sulfate concentration and pH as independent variables on hydrogen production is shown in Figures 1a-1c. A significant increase in hydrogen production could be achieved by increasing Fe (II) sulfate concentration from 50 mg/L to 100 mg/L. After this concentration level a decrease in hydrogen production suggest that Fe(II) is inhibitory. Similarly, a significant increase in hydrogen production in the pH range of 6.0 to 6.5 was observed but further increase in pH to 7.0 decreased hydrogen production, implying that pH 6.5 is the optimum value. Therefore, 100 mg/L Fe (II) sulfate is the optimum concentration for hydrogen production at initial pH 6.5 (Figure 1a).

The surface response curve obtained with respect to dilution fold and pH is shown in Figure 1b. A significant increase in hydrogen production by increasing dilution fold of spent media from 14 to 18 fold and decreases further beyond 18 fold was achieved. The response curve of Fe (II) sulfate concentration and dilution fold on hydrogen production is shown in Figure 1c. It shows that there was an increase in hydrogen production from 14 fold to 16 fold dilutions of spent media in combination with increase in Fe (II) sulfate concentration from 50 mg/L to near 150 mg/L. However at 20 dilution fold



(a) (b) (c)

Figure 1. Surface Plot of hydrogen produced (mL/L) (a) vs Fe(II) sulfate (mg/L) and pH, Hold value: Dilution fold 17 (b) vs Dilution fold and pH, Hold value: Fe(II) sulfate 100 mg/L (c) vs Fe(II) sulfate (mg/L) and Dilution fold, Hold value: pH 6.5.

there is a drastic decrease in the produced hydrogen, suggesting that higher dilutions beyond 16 fold are not suitable for the growth of the co-culture due to drastic decrease in COD required for growth.

Thus optimized value of process parameters obtained through single parameter optimizations i.e. pH, dilution fold of spent media and Fe (II) sulfate concentration were 6.5, dilution fold 17 and 100 mg/L Fe (II) sulfate respectively with maximum hydrogen production 469 ± 45.8 mL/L and yield of 146.56 ± 14.31 mL/g COD reduced. However, the theoretical model of multi parameter optimization gave the maximum point of the model at 455.99 mL/L corresponding to pH 6.49, dilution fold 16.61 and 93.43 mg/L Fe (II) sulfate concentration. Therefore we have been successful in getting the experimental values quite close to the theoretical values. Therefore, optimized values of pH, dilution fold and Fe (II) sulfate concentration obtained through theoretical model can be used for scale up studies using this co culture for conversion of spent media of dark fermented cheese whey water.

The results from this study have shown for the first time the optimized photofermentation conditions for hydrogen production using dark fermentation products of cheese whey waste water by co-culture. The best conditions obtained for photofermentation using co-culture of *Rhodobacter sphaeroides* –NMBL-01 and *Bacillus firmus* – NMBL-03 were pH 6.5, dilution 17 fold (4.71 g COD/L) and supplementation of Fe^{2+} concentration 100 mg/L for maximized yield of 146.56 ±14.31 mL H_2/g COD reduced with 67.9 % COD reduction. The optimized process parameters found were in close vicinity to the values obtained by Box-Behnken design of multi parameter optimization. This co-culture might prove as potential co-culture for wide range of sugar/organic acid rich biorefinery waste conversion and hydrogen production for upscaling.

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