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Investigating dark fermentative hydrogen production from sucrose and molasses

The effects of initial VSS, COD and pH

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Abstract— The objective of this study was to investigate optimum initial chemical oxygen demand (COD), volatile suspended solids (VSS) and pH values leading to the highest dark fermentative hydrogen gas production in batch reactors. To this purpose, two sets of batch reactors were operated with either sucrose or sugar-beet molasses. An experimental design approach (Response Surface Methodology) was used. The results revealed that, when sucrose was used, hydrogen yield (HY) decreased as the $COD_{initial}$ increased (10 to 50 g/L), while HY increased as the $pH_{initial}$ increased (4 to 7). Maximum yield of 2.3 mol H_2/mol $sucrose_{added}$ was achieved at $pH_{initial}$ and $COD_{initial}$ of 7 and 10 g/L, respectively. The studied initial ratio of substrate to VSS (4, 12, 20 g COD/g VSS) had no effect on HY. Hydrogen production rate (HP) increased with the increase in $pH_{initial}$ from 4 to 7. Its maximum (10.7 mL $H_2/(L_{reactor}.hr)$) was achieved at a $COD_{initial}$ concentration of 26.6 g/L. When molasses was used, $COD_{initial}$ was the only variable affecting HY and HP. Maximum of both was achieved at $COD_{initial}$ of 10 g/L. $VSS_{initial}$ (2.5, 5.0, 7.5 g/L) had no effect on HP and HY. This study also indicated that molasses, for containing potential intrinsic microorganism, might be more suitable to support/trigger homoacetogenesis than sucrose.

Keywords— ***Dark Fermentation, Biohydrogen, molasses, homoacetogenesis, Response Surface Methodology.***

I. INTRODUCTION

Finding alternative ways of energy production other than fossil fuels is the most popular research area recently. Hydrogen attracts particular attention because of its high energy content, eco-friendly production methods and no greenhouse gases release when burned. The traditional ways of hydrogen production like photolysis of water or photo-fermentation are energy intensive and not environment friendly [1]. Hydrogen production from renewable natural wastes via biological methods namely dark fermentation, therefore suggest a more sustainable method of hydrogen production. In terms of recovering bioenergy and utilization of carbohydrate rich wastes dark fermentation stands out as a more sustainable method.

Classical experimental designs that investigate various variables on a particular objective usually depend on one factor at a time way of approaching the issue. But this experimental design approach misses the interaction of various variables that might change the magnitude of the objective [2]. This experimental design is also time consuming since it requires a number of experiments to cover different levels of a variable [3]. Therefore using a statistical design approach like Response Surface Methodology (RSM) reduces the number of experiments, includes the interactions of the variables in the resulting objective and provides a statistically sound set of results [4].

Molasses with its high carbohydrate content stands out as an excellent fuel for dark fermentative hydrogen production. Most of the sugar content of molasses is known to be sucrose [5]. Therefore, in this study, firstly sucrose was used as a substrate in batch dark fermentation reactors (Set-A). Set-A was designed with RSM and operated to investigate the effect of initial Chemical Oxygen Demand (COD) concentration, the substrate (in terms of COD) to initial Volatile Suspended Solid (VSS) ratio (S/X_0) and the initial pH on hydrogen production. According to the results of Set-A, another batch set (Set-B) with molasses as the substrate was designed with RSM and operated to investigate the effect of initial COD, VSS and pH values on hydrogen production. In literature, parameters such as initial pH and COD are frequently investigated [6, 7, 8]. Usually pH values from 4.5 to 6.0 are studied with the optimum value around 5.5 [6]. With anaerobic mixed cultures and carbohydrate based substrates, the initial COD values studied in batch reactors ranged from 0.3 to 96 g/L [7]. The optimum initial COD values, leading to highest hydrogen production were reported to be between 1.1 and 10 g/L [8, 9]. Yet, unlike the initial COD and pH, the parameters such as initial VSS concentration or Substrate to VSS ratio (S/X_0) and their effects on hydrogen production were not investigated in detail. To our knowledge, this study is the first to investigate the combined effect of initial COD, VSS (in turn Substrate to VSS ratio, S/X_0) and pH on dark fermentative hydrogen production. It was also aimed in this study to determine the optimum initial conditions (in terms of initial COD, VSS and pH) leading to the highest dark fermentative production from sucrose and molasses.

II. MATERIALS AND METHOD

A. Seed Sludge and Pretreatment

Sludge for the inoculation of the reactors was obtained from the Anaerobic Digesters of the Ankara Wastewater Treatment Plant. This sludge had a total suspended solids (TSS) value as 42888±702 and a VSS value of 20288±218 mg/L. It is common practice to apply pre-treatment to anaerobic sludge to eliminate methanogens and select acidogenic hydrogen producers [8]. Heat treatment has been mentioned successful in inactivation of hydrogen consumers and supporting *Clostridium* like hydrogen producers [10]. Therefore, the seed sludge used in this study was heat treated at 105^{o}C for an hour [11]. Initial pH of the seed sludge was 8.6.

B. Substrate and Basal Medium

In Set-A, sucrose was used as the carbon source. A basal medium was added to the reactors to provide appropriate nutrient concentrations for bacterial growth. The basal medium ingredients are given in Table 1.

TABLE I. BASAL MEDIUM USED IN THE REACTORS WITH SUCROSE AS THE SUBSTRATE (SET-A)

Chemical	***In reactor composition (mg/L)***
$MgSO_4.7H_2O$	400
$FeCl_2.4H_2O$	40
KH_2PO_4 and K_2HPO_4	300
Cysteine	10
NH_4Cl	400

Molasses, sugar industry by-product, is a renewable source which can be converted to hydrogen by dark fermentation due to its high organic content (>80% sucrose). Molasses was gathered from Ankara Etimesgut Sugar Factory and stored in 20^{o}C prior to use. The properties of the molasses can be seen in Tables 2. In second set of reactors, Set-B, molasses was used as the substrate. Additional nutrients were not added in reactors of Set-B.

C. Analytical Methods

TSS and VSS were measured by following Standard Methods (2540 A, B, C, D) (APHA, AWWA and WEF 2005). COD of filtered samples were measured according to an EPA approved reactor digestion method (for sCOD range of 0-1500 mg/L) (Hach Water Analysis Handbook, 2012). For sCOD measurements, Aqualytic AL 38 heater and PC Multidirect Spectrophotometer were used. Daily gas production of the reactors was measured with water displacement device [12]. The headspace gas composition of the reactors were measured with a gas chromatograph (GC) (Thermo Scientific Co.) employing a Thermal Conductivity Detector (TCD). Helium was the carrier gas. Injector, detector and oven temperatures were 50^{o}C, 80^{o}C and 35^{o}C, respectively. Volatile Fatty Acid (VFA) types and compositions were measured by an HPLC (SHIMADZU 20A) using a refractive index detector with a sample volume of 10 µL. VFAs measured were lactic, formic, acetic, propionic, iso-butyric, butyric and iso-valeric acids. HPLC protocol is as follows; oven temperature is 66^{o}C; mobile phase is HPLC grade 0.085 M sulfuric acid solution with the flow rate of 0.4 mL/minute. VFA concentrations were calculated according to the calibration curves prepared by the standard VFA solution (SupelCo VFA Standard Mix). To calculate the total VFA concentration, every acid composition is divided by its molecular weight and multiplied by the molecular weight of acetic acid. Therefore the addition of all these values gives the total VFA concentration in terms of acetic acid (HAc) [13].

The experimental sets were designed using Table 3 and the result parameters, called response, that are to be maximized were defined. In this study two responses were defined, i.e. hydrogen production yield (HY) and hydrogen productivity (HP).

To model HY and HP, Response Surface Methodology (RSM) was used. MiniTab (Minitab Pro 16.1.0.0) was used to employ RSM. Using MiniTab software, the effect of the independent variables (initial COD, pH and X_o (or S/X_o)) on each response was evaluated. The calculated response values for both responses were analyzed to find the effects of pre-defined independent variables. Then, by MiniTab, experimental results were used to produce appropriate models for the responses and their 3-D graphs. To investigate the validity of these models, ANOVA tables were modified for the models. The independent variable points that result in the maximization were calculated using the response optimization tool of RSM.

TABLE II. PROPERTIES OF MOLASSES USED IN SET-B

Characterization of Molasses				**VFA Composition of Molasses**	
Parameter (unit)	***Values***	***Parameter (unit)***	***Values***	***VFA Type***	***Concentration (mM)***
TSS (g/L)	140 ± 25	Ni (mg/L)	0.11	Lactic	567±101
VSS (g/L)	121 ± 24	Fe (mg/L)	6.88	Formic	- [b]
COD (g/L)	1331 ± 130	Cu (mg/L)	0.01	Acetic	294±15
sCOD (g/L)[a]	1153 ± 23	Al (mg/L)	0.00	Propionic	387±17
TKN (g/L)	16 ± 0	Mo (mg/L)	0.00	Iso-Butyric	25±2
TAN (mg/L)	700 ± 40	W (mg/L)	0.00	Butyric	28±3
Alkalinity (g/L)	27 ± 2	Se (mg/L)	0.00	Iso-Valeric	- [b]
Co (mg/L)	0.00	K (mg/L)	7300	Total VFA[c]	1313 ± 367
Zn (mg/L)	0.00				

[a] sCOD: Soluble COD; TAN: (NH4+-N + NH3-N)
[b] Not detected
[c] tVFA, in terms of HAc

TABLE III. FACTORS AND LEVELS USED IN THE BOX-BEHNKEN DESIGN OF SET-A AND SET-B

Independent Variable	***Symbol***	***Range and Levels***		
		-1	0	1
pH	X0	4	5.5	7
COD	X1	10	30	50
S/X_o (Set-A)	X2	4	12	20
X_o (Set-B)	X2	2500	5000	7500

D. *Experimental Procedure*

Batch experiments were conducted in two sets, Set-A and Set-B. Both of the sets were designed with RSM to investigate the effects of initial pH value, initial COD and initial VSS (or Substrate to VSS ratio, S/X_o) concentrations on dark fermentative hydrogen production. The design factors and levels of each of these independent variables (initial pH, COD and S/X_o (or X_o)) in both sets are presented in Table 3.

Set-A was conducted in 100 mL reactors with 60 mL working volumes. Set-B was conducted in 250 mL reactors with 150 mL working volume. All reactors were inoculated with the substrate (sucrose or molasses), pretreated sludge and the basal medium (only for Set-A) in required amounts. The initial pH values of the reactors were adjusted with 2M NaOH and HCl solutions. All reactors were incubated at 35±2^{o}C and constantly stirred at 125 rpm. Gas production and headspace gas compositions were measured every day.

Table 4 indicates the set-up of Set-B. As seen in Table 4, 13 reactor types were conducted with 2 to 6 replicas with respect to the Box-Behnken design approach [10]. Box-Behnken design method is more commonly used when the reactor sets are to be constructed only once since it needs less design points than other methods [14]. Set-A also had 13 reactor types, with 2 to 6 replicas. In total, both sets were composed of 30 reactors. The results were given taking the average of the results of each reactor type conducted in replicas.

III. Results and Discussion

A. *Results of SET-A*

The reactors in Set-A, where sucrose was used as substrate, were operated for 6 days.

The H_2 production abilities of the 30 reactors were evaluated using RSM for the two described responses HY and HP. A model was produced for each response. For HY, model 1 (Equation 1) was defined as follows;

$$HY\ (mol\ H_2/mol\ sucrose_{added}) = -3.30051 + 1.47150 * pH - 0.0160232*COD - 0.0828632*pH*pH + 0.000758894*COD*COD - 0.0110417*pH*COD \quad (1)$$

TABLE IV. BOX-BEHNKEN DESIGN WITH THREE INDEPENDENT VARIABLES AND CORRESPONDING EXPERIMENTAL RESULTS OF SET-B

Run Order	***Variables***			***Response***		***Other Items***		
	iCOD	***iVSS (X)***	**pH**	***HY***[a]	***HP***[b]	***Final pH***	***Suction (at hour)***	***Final tVFA***[c]
1	30	5000	5.5	373.6	897.7	4.1		120.2
2	30	7500	4	1324.7	3154.9	3.86		95.1
3	30	5000	5.5	589.5	1558.3	4.74	441	59.0
4	50	2500	5.5	178.5	453.1	3.84	441	163.2
5	30	7500	7	937.7	2233.1	5.24	282	97.9
6	30	7500	4	389.2	1268.9	3.89		111.6
7	30	2500	7	235.4	428.5	3.82		79.0
8	30	5000	5.5	749.5	1218.0	3.95	323	114.1
9	50	5000	7	197.9	6204.1	3.85	369	198.8
10	10	5000	7	1773.5	8562.2	5.6	109	43.5
11	50	2500	5.5	748.8	1900.6	3.79		103.9
12	30	2500	7	852.7	1772.9	3.95		124.2
13	10	5000	4	3166.3	2839.8	4.85	109	38.3
14	50	7500	5.5	1184.6	551.6	4.25		195.4
15	10	2500	5.5	2074.6	2958.1	4.77	157	36.0
16	30	2500	4	1652.4	5387.0	3.38		138.5
17	30	2500	4	895.2	1363.2	3.62		83.2
18	10	2500	5.5	1028.7	1731.4	4.9	133	21.0
19	30	5000	5.5	489.1	245.4	4.06		99.4
20	30	7500	7	285.6	593.8	5.39	323	77.1
21	10	7500	5.5	2870.5	5819.2	5.26	60	37.3
22	30	5000	5.5	161.1	557.4	4.79		68.3
23	50	7500	5.5	217.3	4105.1	3.95		208.1
24	50	5000	4	99.1	393.2	3.49	282	37.0
25	10	7500	5.5	1559.7	10709.9	5.26	60	33.0
26	50	5000	7	1563.1	600.2	3.93	282	269.1
27	10	5000	4	2600.5	6502.9	4.74	205	33.1
28	10	5000	7	3251.1	3642.4	5.59	85	38.6
29	30	5000	5.5	366.0	1594.5	4.75		46.5
30	50	5000	4	196.0	497.4	3.83		143.8

[a] Yield (mol H2/g CODadded)*10^6
[b] Productivity(Production Rate) (mL H2/ mL rxn vol*hour)*10^6
[c] tVFA, in terms of HAc

This model (Equation 1) is highly significant with all p values smaller than 0.05. R-Sq (R^2) value is 94.76%, therefore, explaining that much of the response values. The ANOVA result of the model is given in Table 5. According to model (1), pH, COD and their interactions pH*pH, COD*pH and COD*COD are the only parameters that have a significant effect on HY. The results of this analysis indicated that S/X_o

variable had no effect on resulting HY. Contour and Surface Plots of the HY model are given in Fig. 1. According to Fig. 1, at COD lower than 20 g/L, yield increases with increasing pH. This finding was consistent with the results of other studies [9, 15].

RSM was further used to calculate the maximum point for HY, which is calculated to occur at pH= 7 and COD= 10 g/L by the model. The maximum response (HY) value achieved was calculated as 2.08240 mol H_2 / mol sucrose.

TABLE V. BOX-BEHNKEN DESIGN WITH THREE INDEPENDENT VARIABLES AND CORRESPONDING EXPERIMENTAL RESULTS OF SET-A

for HY				**for HP**			
Term	***Coef***	***T-value***	***P-value***	***Term***	***Coef***	***T-value***	***P-value***
Constant	0.6665	12.917	0.000	Constant	-0.033995	-64.340	0.000
pH	0.3431	9.035	0.000	iCOD	0.000195	33.010	0.003
COD	-0.6244	-16.441	0.000	S/X	0.000355	24.020	0.025
pH*pH	-0.1864	-3.345	0.003	pH*COD	0.011916	63.040	0.000
COD*COD	0.3036	5.446	0.000	COD*COD	-0.000004	-38.270	0.001
pH*COD	-0.3312	-6.168	0.000	S/X*S/X	-0.000015	-25.050	0.020
				pH*pH	-0.000888	-51.910	0.000
R-Sq = 94.76% R-Sq(pred) = 90.99% R-Sq(adj) = 93.66%				R-Sq = 89.58% R-Sq(pred) = 82.47% R-Sq(adj) = 86.86%			

For the second response, HP, the following model (Equation 2) was obtained;

Productivity (mL $H_2/L_{reactor}$. hour) = -0.0333995 + 0.000195 * COD + 0.000355* S/X + 0.011916 * pH – 0.000004*COD*COD – 0.000015*S/X*S/X – 0.000888 * pH* pH (2)

This model (2) is highly significant with all p values smaller than 0.05. 89.58% of the data can be explained using this model (R-Sq 89.58%). The ANOVA result of the model is given in Table 5. Contour and Surface Plots of the HP model are given in Fig. 2. Model (2) indicated that, unlike for HY, S/X_o parameter had significant impact on HP. According to the surface and contour plot pairs (Fig.2), HP increases significantly as pH increases from 4 to 7. The maximum of HP is calculated at COD 26.566 g/L; S/X_o = 11.758 and pH=6.697 with the obtained model. The model calculates the maximum HP occurring at these three values as 10.7 mL $H_2/L_{reactor}$.hour.

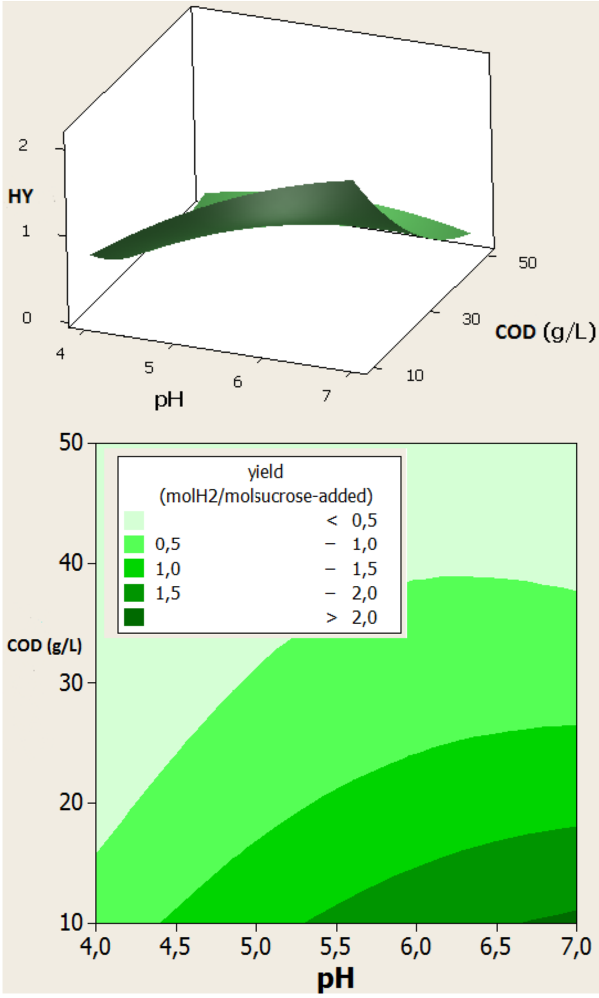

Fig. 1. Surface and Contour Plot for HY of Set-A

Initial VFA concentrations of all reactors at the beginning of the operation were less than 0.1 mM (data not shown). The final VFA concentration analysis for the reactor pairs showed that lactic acid was produced at the highest amounts in all of the reactors. It is known that lactic acid production is a H2 consuming pathway (Equation 3). Therefore, lactic acid production and its accumulation might have resulted in lower hydrogen yield values. Because lactic acid bacteria are resistant to low substrate concentrations and tolerate low pH values (pH<5) [16, 17], the low pH values of the reactors (3.4 to 3.8, measured at the end of the study) did not negatively affect their growth and abundance.

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + H^+ \quad (3)$$

The highest yield of 2.3 mol H_2/mol sucrose was observed in the reactor with the highest acetic and butyric acid production (13.6 and 10.4 mM, respectively), as expected. At higher pH's (5.5 and 7) acid production was high as is the H_2 production since they are simultaneously produced. Considering all the reactors had almost the same final pH values (3.4 to 3.8), the higher initial pH values resisted pH drops for a longer time and delayed the system inhibition therefore producing more H_2. It was also concluded from Set-A that, as expected, COD removal was not significant for the dark fermentation batch studies (only in the range of 10-20%, data not shown).

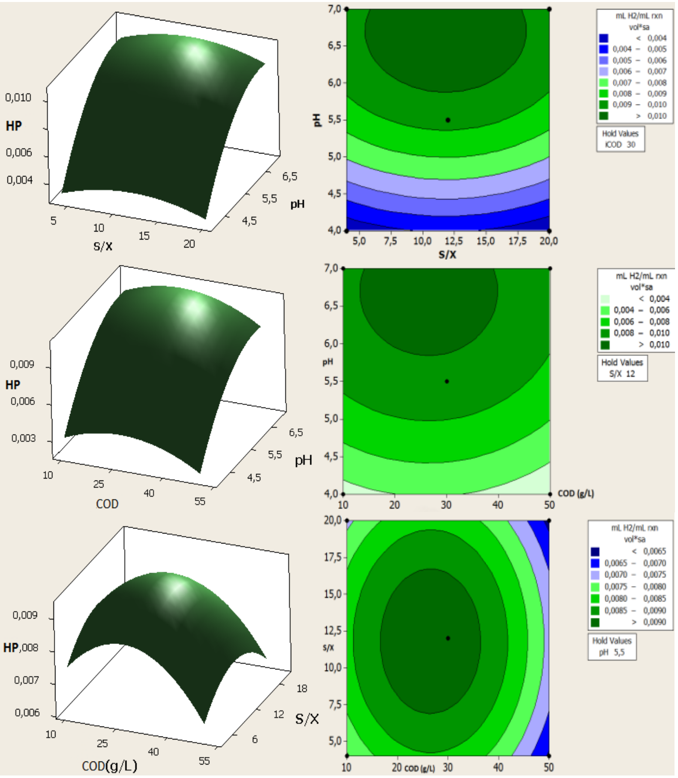

Fig. 2. Surface and Contour Plot for HP vs. various independent parameters of Set-A

B. Results of SET-B

Set-B reactors were incubated for 17 days and the results are given in Table 4. In this part of the study, a special condition is observed in the reactors that had not been observed in the previous set. Out of 30 reactors conducted, 17 ones started to suction after 60 to 400 hours of operation. Table 5 presents the results of Set-B in terms of yield and productivity, and indicates the reactors that started to suction.

From Table 4, it is evident that the reactors that do suction generally have higher final pH values than the others. This, along with the presence of suction in the reactors, was attributed to the presence of a new pathway; namely, homoacetogenesis [18]. Homoacetogenesis is one of the biggest concerns of the stability of dark fermentation studies and continues to be an unresolved issue [16]. Most of the recent dark fermentation studies mention its presence and importance in bio-hydrogen producing systems with mixed cultures. It was also evident that the reactors with lower substrate concentrations started to suction earlier than others. It had been stated that a drop in substrate concentration can increase the activity of homoacetogenesis [19, 20]. In the reactors that suction, the initial headspace H2 gas percentages decreased towards the end of the incubation period in the range of 6 to 36%; also suggesting the presence of H_2 consuming mechanisms.

In Set-A, where sucrose was used as substrate, the pH values measured at the end of the study was lower than that obtained in Set-B. In addition, suction was not experienced in Set-A. This finding brings the idea that molasses is likely to have intrinsic homoacetogenic microorganisms.

RSM was applied to find out the variable (initial pH, COD and X_0) values at which hydrogen production yield and productivity maximize. By the analysis of the experimental data, the following models were obtained for HY and HP (Equations 4 and 5, respectively). Both of these models (4) and (5), are highly significant with all p values smaller than 0.05. 64.118% of the data can be explained using HY model and 38.738% of the data can be explained by the HP model. The ANOVA results of both models are given in Table 6.

$$\text{HY (mol } H_2 \text{ /g COD)}*10^6 = 3369.96 - 156.81*\text{COD} + 1.89 * \text{COD}^2 \quad (4)$$

$$\text{HP (mL } H_2/L_{reactor}.\text{hour)} *10^3 = 8723.87 - 387.84*\text{COD} + 5.00 *\text{COD}^2 \quad (5)$$

TABLE VI. BOX-BEHNKEN DESIGN WITH THREE INDEPENDENT VARIABLES AND CORRESPONDING EXPERIMENTAL RESULTS OF SET-B

for HY				for HP			
Term	***Coef***	***T***	***P***	***Term***	***Coef***	***T***	***P***
Constant	3669.96	8.346	0.000	Constant	8723.87	1623.34	0.000
COD	-156.81	-4.776	0.000	COD	-387.84	121.20	0.003
COD*COD	1.89	3.5380	0.001	COD*COD	5.00	1.97	0.017
R-Sq = 64.11% R-Sq(pred) = 54.52% R-Sq(adj) = 61.45%				R-Sq = 38.73% R-Sq(pred) = 21.65% R-Sq(adj) = 34.20%			

As seen in Table 6 and Equations 4 and 5, the only parameter that has a significant effect on HY and HY was initial COD concentration in Set-B. Since both models only include the term COD, contour and surface plots for the models cannot be produced. The initial COD concentration of 10 g/L presented the maximum HY and HP among the COD concentrations studied. The maximum HY was calculated as 2.2906 mmol H_2/g COD.

IV. CONCLUSIONS

This study confirmed the high potential of dark fermentative hydrogen production from sucrose and molasses. The optimum conditions, leading to the maximum hydrogen production and yield, were successfully predicted with RSM. Set-A results indicated that HY decreased as the initial COD increased from 10 to 50 g/L, while it increased as the initial pH value increased from 4 to 7. The studied S/X_o values of 4, 12 and 20 g COD/g VSS had no effect on hydrogen production yield. Set-B results showed that the change in HY and HP could not be explained by the combination of all three variables (i.e. initial pH, COD and VSS values) and with the studied ranges. Initial VSS concentration in the reactors had no effect on hydrogen yield and productivity from molasses for the values studied (2.50, 5.0 and 7.5 g/L). The maximum HY and HP values obtained in Set-B, were found to be slightly lower than those found in previous studies in literature. These results were attributed to the homoacetogenic activity and in turn its interference with the HY and HP. This might be due to the substrate type since molasses, for containing potential intrinsic microorganism, might be more suitable to support and trigger the homoacetogenesis than sucrose.

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