Electronic Supplementary Material (ESI) for RSC Advances.

Stimulation of an indigenous thermophillic anaerobic bacterial consortium for Enhanced Oil Recovery

Short Title: Potential of indigenous bacterial consortium for EOR

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Supplementary

Experiment: Optimization of nutrient recipe for enhanced production of metabolites in TERIL63.

Methodology

In response surface methodology (RSM), the component's minimum and the maximum value are taken as the hold value. In order to find the most appropriate combination of these five components, forty six (46) sets of experiments (also known as Run Orders) were generated by the software (Minitab 16) as described in supplementary Table S1.

TABLE S1. Box Behnken design for five variables and experimentally determined actual data by RSM for enhanced production of bacterial metabolites to support the MEOR activity.

Run Order	(Components in g/L)				
	Ammonium chloride	Peptone	Yeast extract	Sodium acetate	Sodium formate
1	2.000	0.5	0.5	0	1
2	1.125	1.0	0.5	1	2
3	1.125	0.5	0.0	0	1
4	2.000	0.5	0.5	1	0
5	0.250	0.5	0.5	2	1
6	1.125	0.0	0.0	1	1
7	1.125	0.5	0.5	1	1
8	0.250	0.0	0.5	1	1
9	2.000	0.0	0.5	1	1
10	0.250	1.0	0.5	1	1
11	0.250	0.5	0.5	1	0

12	1.125	1.0	0.5	2	1
13	1.125	0.5	0.5	1	1
14	0.250	0.5	0.5	1	2
15	1.125	1.0	0.5	1	0
16	1.125	0.5	1.0	1	2
17	1.125	1.0	0.0	1	1
18	2.000	0.5	0.5	2	1
19	0.250	0.5	0.0	1	1
20	1.125	1.0	1.0	1	1
21	0.250	0.5	1.0	1	1
22	1.125	0.5	1.0	0	1
23	1.125	0.5	0.5	2	0
24	1.125	0.5	0.5	1	1
25	2.000	0.5	0.0	1	1
26	1.125	0.5	1.0	1	0
27	1.125	0.5	0.5	1	1
28	1.125	0.5	0.5	1	1
29	2.000	0.5	0.5	1	2
30	1.125	0.0	0.5	1	2
31	1.125	0.0	0.5	0	1
32	1.125	0.0	0.5	1	0
33	0.250	0.5	0.5	0	1
34	2.000	0.5	1.0	1	1
35	1.125	0.5	0.5	2	2
36	1.125	0.5	0.0	1	0

37	1.125	0.5	0.0	1	2
38	1.125	0.5	1.0	2	1
39	1.125	0.5	0.0	2	1
40	1.125	0.0	0.5	2	1
41	1.125	0.0	1.0	1	1
42	2.000	1.0	0.5	1	1
43	1.125	1.0	0.5	0	1
44	1.125	0.5	0.5	1	1
45	1.125	0.5	0.5	0	0
46	1.125	0.5	0.5	0	2

In this study, five key factors were studied at three different coded levels with low, medium and very high value. The relationship and interrelationship of the variables were determined by fitting the second degree polynomial equation to data obtained from 46 experiments using mean values of the triplicates. All these experiments were set for readings after 10, 20 and 30 days. The parameters analysed were gas production and VFAs production. The data was analysed using multiple regressions and a second order polynomial model fitted for predicted optimum levels was expressed in Equation.

Eq. (1): $$Y= \beta_0 + \sum\beta_n X_n + \sum\beta_{nn} X^2_n + \sum\beta_{nm}X_nX_m$$

Where Y is the predicted response, β_0 is the intercept coefficient, β_n is linear coefficient, β_{nn} is quadratic coefficient and β_{nm} is interaction coefficient. Design expert software was used for regression and graphical analysis of the data obtained. The interactive effects of significant variables were further represented in form of contour plots.

Eq. (2): $$Y = \beta_0 + \beta_1 X_1 + \beta_2X_2 +\beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_{11}X_1X_1 + \beta_{22}X_2X_2 +$$

$$\beta_{33}X_3X_3 + \beta_{44}X_4X_4 + \beta_{55}X_5X_5 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{14}X_1X_4 +$$

$$\beta_{15}X_1X_5 + \beta_{23}X_2X_3 + \beta_{24}X_2X_4 + \beta_{25}X_2X_5 + \beta_{34}X_3X_4 + \beta_{35}X_3X_5 + \beta_{45}X_4X_5$$

The response surface and contour plots were generated to understand the interaction of various variables and then used to find the optimum concentration of the medium components affecting the response. The statistical significance of the regression coefficient was determined by Student's t-test. The second order model equation was determined by Fischer's test and the quality of the fit of the polynomial model equation was given by the coefficient of determination R^2.

Results

Experiments were performed in random order to obtain the maximum metabolites produced as actual response. The experimental results were further analysed by using multiple regression and significance of each individual components and their interaction on the production of metabolites. Analysis of variance (ANOVA) was used to analyse the response under different combinations as defined by the Box Behnken design.

TABLE S2. Compositional analysis of formation fluids of Linch, Kalol and Nandasan oil fields.

Parameter	Method	Oil wells				
		L#63	K#529	K#152	K#253	N#60
Heavy metals (mg/L)						
Arsenic (As)	IS 3025 Pt 37:1988	ND	ND	ND	ND	ND
Cadmium (Cd)	APHA 3111 (B)	0.059	0.018	0.033	0.043	0.013
Chromium total (Cr)	APHA 3111 (B)	ND	ND	ND	ND	ND
Copper (Cu)	APHA 3111 (B)	0.177	0.094	0.098	0.247	0.063
Zinc (Zn)	APHA 3111 (B)	0.08	0.136	0.074	0.130	ND
Silver (Ag)	APHA 3111 (B)	0.09	0.12	ND	ND	ND
Nickel (Ni)	APHA 3111 (B)	0.176	0.089	0.125	0.171	0.092
Total Iron (Fe)	APHA 3111 (B)	5.77	0.24	0.227	0.171	0.528
Compositional analysis (mg/L)						
Carbon	IS: 1350/APHA	260	410	520	330	1050
Hydrogen	IS: 1350/APHA	19	30	40	25	78
Nitrogen	IS: 1350/APHA	40	58	80	38	24
Sulphur	IS: 1350/APHA	14.1	25	35	25	7.5
Toxic ions (mg/L)						

Chloride (Cl)	IS 3025 Pt 32:1988	5725	3425	2520	2868.12	1625.18
Fluoride (F)	APHA 4500 (D)	0.40	0.34	0.21	0.30	0.63
Sulphate (SO_4)	IS 3025 Pt 24:1986	10.10	31.57	15.61	27.41	25.68

ND: Not detected or below the detection limit i.e. <0.01

TABLE S3. Regression analysis of central composite design showing model coefficients and significance of regression coefficient for enhanced production of bacterial metabolites for MEOR activity.

Term	Total VFAs (mg/L)				Total Gases (mM)			
	Coefficient	SE coefficient	T-value	*p*-value	Coefficient	SE coefficient	T-value	*p*-value
Constant	970.808	96.67	10.043	0.000	4.21261	0.6607	6.376	0.000
X_1	-5.163	59.20	-0.087	0.931	0.23036	0.4046	0.569	0.574
X_2	34.179	59.20	0.577	0.569	0.36057	0.4046	0.891	0.381
X_3	-53.008	59.20	-0.895	0.379	0.40381	0.4046	0.998	0.328
X_4	599.047	59.20	10.120	0.000	0.58989	0.4046	1.458	0.157
X_5	39.952	59.20	0.675	0.506	-0.17420	0.4046	-0.431	0.670
X_1^2	-42.596	80.15	-0.531	0.600	1.00892	0.5478	1.842	0.077
X_2^2	10.764	80.15	0.134	0.894	-0.34452	0.5478	-0.629	0.535
X_3^2	114.247	80.15	1.425	0.166	-0.23618	0.5478	-0.431	0.670
X_4^2	234.468	80.15	2.925	0.007	1.10422	0.5478	2.016	0.055
X_5^2	-131.229	80.15	-1.637	0.114	0.69546	0.5478	1.270	0.216
$X_1 X_2$	-7.578	119.39	-0.064	0.949	0.35435	0.8092	0.438	0.665

$X_1 X_3$	-20.100	118.39	-0.170	0.865	0.37788	0.8092	0.467	0.645
$X_1 X_4$	67.845	118.39	0.573	0.572	-0.60515	0.8092	-0.748	0.462
$X_1 X_5$	-20.300	118.39	-0.171	0.865	0.87186	0.8092	1.077	0.292
$X_2 X_3$	-35.708	118.39	-0.302	0.765	-0.41549	0.8092	-0.513	0.612
$X_2 X_4$	-57.885	118.39	-0.489	0.629	-0.11124	0.8092	-0.137	0.892
$X_2 X_5$	126.445	118.39	1.068	0.296	0.28904	0.8092	0.357	0.724
X_3X_4	-404.988	118.39	-3.463	0.002	0.11244	0.8092	0.139	0.891
$X_3 X_5$	-37.637	118.39	-0.318	0.753	0.04080	0.8092	0.050	0.960
$X_4 X_5$	38.778	118.39	0.328	0.746	-0.37633	0.8092	-0.465	0.646

X_1 is ammonium chloride (g/L), X_2 is peptone (g/L), X_3 is yeast extract (g/L), X_4 is sodium acetate (g/L), X_5 is sodium formate (g/L).
Total VFAs (mg/L): S = 236.785 PRESS = 5536402
R^2 = 84.45% R^2(pred) = 38.58% R^2(adj) = 72.01%

Total Gases (mM): S = 1.61835 PRESS = 156.658
R^2 = 41.18% R^2 (pred) = 0.00% R^2 (adj) = 0.00%

Total VFAs (mg/L)

$$Y = 970.808 - 5.163X1 + 34.179X2 - 53.008X3 + 599.047X4 + 39.952X5 - 42.596X1X1 + 10.764X2X2 + 114.247X3X3 + 234.468X4X4 - 131.229X5X5 - 7.578X1X2 - 20.100X1X3 + 67.845X1X4 - 20.300X1X5 - 35.708X2X3 - 57.885X2X4 - 126.445X2X5 - 409.985X3X4 - 37.637X3X5 + 38.778X4X5$$

Total gases (mmol)

$$Y = 4.213 + 0.23X1 + 0.36X2 + 0.40X3 + 0.59X4 – 0.17X5 + 1.01X1X1 – 0.34X2X2 - 0.24X3X3 + 1.10X4X4 + 0.695X5X5 + 0.354X1X2 + 0.377X1X3 – 0.605X1X4 + 0.872X1X5 – 0.415X2X3 – 0.111X2X4 + 0.289X2X5 + 0.112X3X4 + 0.04X3X5 – 0.376X4X5$$

In order to determine the maximum metabolites production corresponding to the optimum levels of ammonium chloride, peptone, yeast extracts, sodium acetate, sodium formate, a second order polynomial model was proposed to calculate the optimum levels of these variables. The model explains the role of each variable and their second order interactions in metabolite production for enhanced oil recovery. Statistically significance of the mentioned model equation was determined by Fisher test value, and the proportion of variance explained by the model was given by the multiple co-efficient of determination, R squared value. In first order main effect of X_2, X_4 and X_5 with P –value > 0.05 had significant impact on enhanced metabolite production. It is also suggested that the interaction of X_4 and X_5 was significant for the response (Table S2).

Although it did not show an overall significant interactive effect with any variable, it generated a significant quadratic effect. Table S3 shows the results of the second order response surface model in the form of analysis of variance (ANOVA). Values of Fisher's test, determination coefficient, coefficient of variation and lack of fit, shows that the model is

adequate for prediction within the range of variables employed [1]. Therefore this model can be considered to explain the MCL- metabolites production.

TABLE S4. Analysis of variance (ANOVA) for metabolites production in enhanced oil recovery at coded levels.

	Total VFAs (mg/L)						Total Gases (mM)					
Source	DF	Seq SS	Adj SS	Adj MS	>*F*	>*P*	DF	Seq SS	Adj SS	Adj MS	>*F*	>*P*
Model	20	7611940	7611940	380597	6.79	0.000	20	45.843	45.843	2.2922	0.88	0.61
Linear	5	5831341	5831341	116626	20.8	0.000	5	11.591	11.591	2.3183	0.89	0.50
Square	5	992197	992197	198439	3.54	0.015	5	26.975	26.975	5.3951	2.06	0.10
Interaction	10	788402	788402	78840	1.41	0.234	10	7.277	7.2767	0.7277	0.28	0.98
Residual Error	25	1401675	1401675	56067	-	-	25	65.476	65.476	2.6191	-	-
Lack of Fit	20	1374215	1374215	68711	12.5	0.005	20	24.364	24.364	1.2182	0.15	0.99
Pure Error	5	27459	27459	5492	-	-	5	41.112	41.112	8.2224	-	-
Total	45	9013615	-	-	-	-	45	111.32	-	-	-	-

DF= degree of freedom, SS= sum of squares, MS = mean square

[a] Significance at an alpha level of 0.05.

The plots present the effect of two variables on response at a time, while the other three variables are held at zero level. The interaction between the variables can be inferred from the shape of the contour plots. The circular contour plot of response surface suggests that the interaction is negligible between the corresponding variables. An Elliptical or saddle nature

of the contour plots, on the other hand, indicates the significance of the interactions between the corresponding variables. In the case of elliptical contour plots, the optimum values are obtained at the point of interaction of lines, which are formed by joining the locus [2-3].

Reference

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[2]. Murthy M, Swaminathan T, Rakshit SK, Kosugi Y, Statistical optimization of lipase catalyzed hydrolysis of methyloleate by RSM, Bioproc Biosys Eng 22 (2000) 35-39. doi:10.1007/PL00009097.

[3]. Hong C, Haiyun W, Optimization of volatile fatty acid production with co-substrate of food wastes and dewatered excess sludge using response surface methodology, Bioresour Technol 101 (2010) 5487–5493. doi:10.1016/j.biortech.2010.02.013.

Experiment: Testing the acute oral toxicity of TERIL63 to identify the pathogenicity of microbial consortia.

Results:

Toxic symptoms and mortality were recorded at ½, 1, 2, 3, 4 and 24 hours and later twice a day thereafter up to 21 days to determine their general health. Behaviour and moribund condition, any abnormality observed during this period was recorded and the subsequent progress monitored. The TERIL63 caused No mortality.

Table S5: Mortality data of the TERIL63

Groups	Dose	No. of animal died/ No. of animals treated	Mortality (%)
Males			
1.	Control	00/05	NIL
2.	1 ml/mouse containing 1 x 10^5 CFU	00/05	NIL
Females			
1.	Control	00/05	NIL
2.	1 ml/mouse containing 1 x 10^5 CFU	00/05	NIL

Clinical finding: All the animals appeared normal and showed no clinical signs of intoxication after dosing till and end of the study. No gross abnormalities were observed in the test and control group animals. No live TERIL63 were detected after culturing the tissue-lungs, spleen, kidney, liver, brain, blood, urine and faces.

Table S6: Microbial evaluation of TERIL63 in the tissues of different organs

Groups	Lung	Kidney	Spleen	Liver	Brain	Blood	Urine	Faeces
Males								
1.	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2.	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Females								
1.	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2.	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

-ve = No growth of microbial consortium TERIL63