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Effectiveness of Corn Steep Liquor as a natural low-cost material for the biodegradation of two types of Libyan crude oil

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ABSTRACT

Corn steep liquor (CSL) is a natural low-cost material. This study aimed to evaluate the effectiveness of CSL as a co-substrate to improve crude oil (CO) biodegradation in contaminated seawater. Two types of Libyan light CO, namely, H crude oil (HCO) and L crude oil (LCO) were used in this study. Batch reactors with seawater were used as bioreactors. To develop optimum operating conditions, a central composite design with response surface methodology was used to evaluate the relationship among operating variables, such as HCO and LCO concentrations, CSL dosage, and incubation time. Quadratic models of CO biodegradation (%) proved to be significant with very low probability values (<0.0001). The maximum removal of HCO was 91% using 0.5 % (w/v) CSL and 0.63 % (w/v) HCO. The maximum removal of LCO was 98% using 0.5 % (w/v) CSL and 0.63 % (w/v) LCO. Both bioreactors in an incubation time of 28 d, respectively. We also compared the removal rates of HCO (10.4%) and LCO (10.3%) without the addition of CSL. The obtained optimum conditions were 0.20 % (w/v) CSL for 15 and 10 d at 1% (w/v) of HCO (81%) and LCO (88%). The predicted results of 76% and 84% fitted well with the results of laboratory experiments. Our results reveal that CSL was highly efficient in improving the biodegradation of high concentrations of CO in contaminated seawater.

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INTRODUCTION

Crude oil (CO) hydrocarbon pollution of marine environment is an increasingly serious international concern because of environmental and health reasons. The exploitation of offshore oil resources, use and transportation of petroleum products, waste emission, and frequently occurring oil spill accidents are detrimental to marine ecosystems. Methods using physical skimming and chemical dispersants to solve these pollution problems are both expensive and limited in effectiveness. Thus, opportunities for innovative and comprehensive technologies for the removal of petroleum contaminants have emerged (Bao *et al*., 2012).

Bioremediation is defined as the use of natural microorganisms, plants, or fungi to remove environmental pollutants in water and soil, and aims to completely degrade hydrocarbons into carbon dioxide and water (Gertler *et al*., 2009; Fernández-Luqueño *et al*., 2011).

Strategies used for cleaning oil spills are greatly affected by various factors, such as the type of oil, characteristics of the spill site, and occasional political considerations (Zhu *et al*., 2004; Dave and Ghaly , 2011).

Understanding the characteristics of the oil spill, such as age of oil, weather conditions, surrounding environment, ocean behavior, and impact on marine life, is essential to determine the best oil spill cleanup technique (Dave and Ghaly, 2011).

Bioremediation has proven to be cheaper and more efficient than other techniques because the major constituents of most COs are biodegradable. Bioremediation is simpler and less laborious. Public attitude toward bioremediation is also generally favorable. However, the lack of knowledge about microorganisms and their natural function in the environment can affect the suitability of their use in bioremediation (Thapa *et al*., 2012). Bioremediation is an effective treatment in terms of efficacy, safety on long-term use, cost, and simplicity of administration.

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The interaction between microbes and hydrocarbons has been extensively researched (Bachoon *et al*., 2011; McKew*et al*., 2007; Sei *et al*., 2003). The application of biological treatment technology of CO is a complex process in the marine environment (Hao and Lu, 2009; Nie *et al*., 2010).

The successful application of bioremediation technology in contaminated marine environment requires knowledge regarding on the characteristics of the site and parameters that affect the microbial biodegradation of pollutants (Sabate *et al*., 2004; Macaulay and Rees, 2014).

Biodegradation depends on abiotic or physicochemical factors, which are related to the structure of the pollutant (chemical structure, concentration, and bioavailability) and environmental conditions (oxygen and nutrient availability, pH, temperature, pressure, salinity, and presence of heavy metals) (Barrios, 2011; Jahangeer and Vikram, 2013). Biodegradation is also affected by biotic factors, which depend on the microorganism (microbial population, concentration, and interaction) involved (Obayori *et al*., 2009; Barrios, 2011).

One dynamic factor that affects the environmental cycling process of petroleum is the addition of nutrients. In marine ecosystems, nutrient limitation is generally related to low background levels of nitrogen and phosphorus in seawater (Cappello *et al*., 2007; Thapa *et al*.,2011).

Some of the nutrients, depending on the source and the concentration, may have a direct toxic impact on indigenous microorganisms or may not be bioavailable (Chaineau *et al*., 2005). The use of cheap alternative sources, such as corn steep liquor (CSL), might help circumvent the problem involving nutrients.

According to Kaplan *et al*. (2003) and El-Gendy and Farah (2011), CSL can serve as a good source of nutrients significantly enhances the progress of biodegradation of crude oil hydrocarbon and acts as an emulsifier thereby increasing the amount of bioavailability during metabolic degradation process. Corn steep liquor CSL is a liquid mixture consisting entirely of the water soluble components of corn steeped in water. It has been shown to be very rich in nutrients required by most microorganisms (El-Gendy *et al*., 2013). In addition, CSL has an excellent balance of the majority of essential factors; lactic acid constitutes an easily assumable carbon source, the amino acids and polypeptide constitutes not only sources of nitrogen and carbon, but also sources of buffering agents, and the ashes constitute a source of mineral elements (Shawabkeh *et al*., 2007; Sabean *et al*., 2009).

The target of any bioremediation process is the destruction of the contaminant using cheap available nutrient sources. CSL may be used as a powerful bioremediation tool for the cleanup of environment contaminated with CO (Obayori *et al*.,2009; El-Gendy and Farah, 2011; El- Gendy *et al*., 2013).

Inexpensive and environment friendly options to enhance petroleum hydrocarbon degradation are necessary. Studies on the potential use of organic residues and animal wastes as biostimulating agents are limited. However, few studies have investigated the potential use of crop residues and organic wastes, such as corn residues and sugarcane bagasse, as biostimulating agents (Molina *et al*., 2004).

Amoudo *et al*. (2014); Serbinho *et al*. (2013) and Sousa *et al*. (2012) reported that advances in the utilization of agro-industrial waste materials for the production of biosurfacants have been on rise, as more of these wastes are being identified as appropriate carbon and nitrogen sources. Moreover, increasing environmental awareness necessitated the study of eco-friendly products. Thus, utilization of these byproducts is important from both environmental and economical points of view, can be considered not only as a waste to be treated but also a resource to be recovered, and hence harmonizes industry with environment (Saharan *et al*., 2011).

Response surface methodology (RSM) is typically used to explore the relationships between various explanatory variables and one or more response variables. The key idea behind RSM is to use a set of designed experiments to obtain an optimum response (Bashir *et al*. and ; Mohajeri *et al*., 2010; Montgomery, 2008). RSM is a statistical technique that has been successfully applied in many fields, such as optimization of culture media (Mohan *et al*., and Silva *et al*., 2009; Yönten and Aktaş, 2014, El Mahdi et al., 2014).

In the present study, we elucidated the effectiveness of employing natural low-cost materials to improve the bioremediation of seawater contaminated with different concentrations of CO. The application of varying doses of CSL to remove different CO concentrations is not well documented. Statistical relationships among independent factors, such as CSL dosages, reaction times, and initial concentrations of CO, to improve CO removal were assessed via RSM. RSM, a mathematical and statistical technique that is useful in the optimization of chemical reactions in industrial processes, is commonly used in studies utilizing experimental designs. The primary objectives of the present study are as follows:

i. to investigate the performance of CSL in the removal of different CO concentrations in seawater;
ii. to determine the optimum operational conditions of the proposed method;
iii. to determine the CO removal efficiency using two types of CO with respect to the operational conditions.

MATERIALS AND METHODS

Sampling, microorganisms, and culture conditions:

The polluted water sample used in this study was obtained from a water-in-oil emulsion (70% water

content [wt. /wt.] collected from Nafoora oilfield in Libya.

Strain was isolated and designed as *Pseudomonas sp.* NAF1 and identified according to microscopically and morphological characteristics from CO-contaminated water by a standard culture enrichment technique using basal salt medium (BSM). BSM (pH 7.0) was prepared according to Piddington *et al*. (1995), in which 2.44 g of KH_2PO_4, 5.57 g of $NaHPO_4$. 2.0 g of NH_4Cl, 0.2 g of $MgCl_2.6H_2O$, 0.001 g of $FeCl_3.6H_2O$, and 0.001 g of $CaCl_2.2H_2O$ were dissolved in 1 L of seawater. The mixture was then autoclaved. The strain was stored in 50% (v/v) glycerol at - 80 °C. Each inoculum was grown until the late exponential phase at 30 °C in BSM agar supplemented with 5 g/L glucose (Piddington *et al*., 1995).

To isolate and enumerate total viable cells, BSM and Tryptone Glucose Yeast (TGY) were solidified by 2% agar and plates were used. Each medium was sterilized for 20 min at 120 °C. Microorganisms were tested using spread plate technique and counted as CFU/100 mL, and 3 mL inoculums containing 3.3 X 10^6 cell/mL were added to each bioreactor. The pH was adjusted to neutral at 30 °C (Zahed *et al*., 2010; Ruberto *et al*., 2003). The CSL used in this study was provided by El-Nasr Pharmaceutical Company. The CSL had carbon, nitrogen, and phosphorus content of 6.8%, 1.06%, and 2.14% w/w, respectively. The same conditions in biostimulation were used but without the addition of CSL for the control (simulated natural attenuation). The incubation period was 28 d. All experiments were performed in duplicate, and the average results are reported

Bioremediation experimental design and analysis:

Design Expert software version 6.0.7 was used for the statistical design of the experiments and data analysis. In this study, the central composite design (CCD) and RSM were applied to optimize and assess the relationship among three significant independent variables, namely, (1) L crude oil (LCO) and H crude oil (HCO) concentrations (% w/v), (2) CSL concentration (% w/v), (3) and incubation period (days) (Table 1). The biodegradation of LCO and HCO was considered the dependent factor (response) and performance was evaluated by analyzing the CO removal efficiency. Each independent variable varied over three levels between –1 and +1 at the determined ranges based on a set of preliminary experiments. The total number of experiments obtained for the three factors was 20 (= $2^k + 2k + 6$), where k is the number of factors ($k = 3$). Twenty experiments were enhanced with six replications to assess pure error. Given that only three levels were available for each factor, the appropriate model followed a quadratic pattern, as shown in Eq. (1):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X^2{}_j + \sum_{i} \sum_{<j=2}^{k} \beta_{ij} X_i X_j + e_i \quad (1)$$

where Y is the response, Xi and Xj are the variables, β is the regression coefficient, k is the number of factors studied and optimized in the experiment, and e is the random error.

ANOVA was used for the graphical analyses of data to determine the interaction between the process variables and responses. The quality of the fit of the polynomial model was expressed by the correlation coefficient (R^2). Statistical significance was determined using the F-test in the same program. Model terms were evaluated using the p-value (probability) at 95% confidence level.

Table 1: Coded and actual values of variables.

Symbol	Factors	Coded levels of variables		
		-1.00	0	1.00
A	Crude oil concentration (HCO and LCO) % (w/v)	0.25	0.5	1
B	Corn steep liquor concentration % (w/v)	0.2	0.4	0.8
C	Incubation period (days)	7	17	28

Optimization study:

Two experimental sequences were selected from the first part of our study. LCO (1 % w/v) and HCO (1 % w/v) were used to simulate both light oil spills, respectively. CSL proved to be efficient in the bioremediation of CO compounds. Bioremediation tests performed in the second part of the study were conducted using CSL.

This study was conducted to verify the optimum concentration of CSL as a commercially and readily available co-substrate and nutrient to obtain maximum biodegrading activity in an indigenous microbial population.

Each experimental sequence was designed using CCD. Two bioremediation trials and a single natural attenuation were carried out in Erlenmeyer flask bioreactors (replicate cultures all containing 100 mL of BSM-CO and final concentration of CSL). Approximately 0.20% (w/v) BSM-CO and CSL were incubated in a shaking incubator at 150 rpm under 30 °C for 10 and 15 d, respectively. Flasks without nutrients were placed under the same conditions to evaluate the effect of natural weathering sources. Bacterial inoculum (3 mL) containing 3.3×10^7 cell/mL was added to all bioremediation test reactors. Reactors were shaken continuously on an orbital shaker, and samples were obtained after 10 and 15 d. Cell growth was monitored by the total viable count (cells/mL) on TGY plates.

Non-degraded oil (residual oil) was extracted, and its concentration was determined gravimetrically (Adesodun and Mbagwu, 2008). The control flasks

were also extracted similarly, and degradation was determined by calculating the weight difference.

Analytical study:

Total petroleum hydrocarbons were measured by the Environmental Protection Agency Method–1664 (US-EPA, 1991). Samples were acidified to pH < 2, and serially extracted three times with analytical grade n-hexane (Merck, Darmstadt, Germany) in separatory funnels. The extracts were dried over granular anhydrous sodium sulfate (Merck, Darmstadt, Germany). The solvent was distilled from the extract, and the n-hexane extractable material (HEM) was desiccated and weighed. The HEM was re-dissolved in n-hexane, and silica gel was added to the solution containing re-dissolved HEM to remove polar materials. The solution was filtered to remove the silica gel. The solvent was subsequently distilled, and the silica gel-treated HEM was desiccated and weighed. Quality assurance and control were carried out according to the same procedure. Calibration verification and analysis of blanks were performed daily. Percent recovery (S) was calculated using Eq. (2):

$$S = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}} \quad (2)$$

where n is the number of samples, and x is the percent of recovery in each sample. Matrix spike (MS) was also ested to ensure the accuracy of the analysis.

The relative percent difference between the MS and matrix spike duplicate was computed using Eq. (3):

$$RPD = \frac{| D_1 - D_2 |}{(D_1 - D_2)\ /_2} \quad (3)$$

where D_1 is the concentration of HEM in the sample, and D_2 is the concentration of HEM in the duplicate sample.

Analyses were verified by GC (US-EPA, 1991) using a GC 2000 Series equipped with a flame ionization detector (Fisons Instruments, Milan, Italy). A DB-5 capillary column (J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm ID, film thickness 0.25 µm) was used. The operating conditions were as follows: injector temperature, 300 °C; detector temperature, 300 °C; carrier gas helium, 99.999% (35 mL/s); air flow, 350 mL/s; and make-up gas nitrogen, 30 mL/s. The oven temperature program was 1 min at 60 °C, followed by a temperature increase of 10 °C/min up to 160 °C, 10 min at 160 °C, increase of 4 °C/min up to 300 °C, and finally 10 min at 300 °C. Splitless injection modes were carried out with the splitless time at 0.8 min. The chromatographic data were analyzed using Chrom-Card data system version 2.1 software (Thermo Electron, Rodano, Italy).

RESULTS AND DISCUSSION

The potential biodegradation of CO was assessed based on the development of a visible emulsification activity with a strain of *Pseudomonas sp.* NAF1, which produced rhamnolipids when cultured in BSM using CSL as a co-substrate.

The new isolated organism designated as NAF1 was Gram-negative bacteria, which produced green pigment. The alignment of the 16S rRNA gene sequences of NAF1 with sequences obtained by doing a Blast searching revealed 98% similarity to *P. aeruginosa* PAO1.

Pseudomonas bacteria are also rhamnolipid biosurfactant producers and are involve used in oil decontamination (Aneja , 2004; Müller *et al*., 2012; Khalifeh *et al*., 2013; Xia *et al*., 2014)

It is evident from this investigation that isolated bacterium degraded crude oil and comparison of obtained results and existing statistics with similar studies revealed that isolated *Pseudomonas sp.* NAF1 can be used for bioremediation of crude oil pollutants from the environment. Since growth of the bacterial isolate on crude oil has been associated with the production of biosurfactants, we can conclude that the crude oil metabolizing bacterium is able to secrete surfactants using agro industrial as substrates which further enhance the hydrocarbon degradation. Patel and Desai (1997) have been concluded that *Pseudomonas aeruginosa* GS3 can utilize molasses and CSL as the substrates and produce effective rhamnolipids. Rhamnolipids could be used in the bioremediation of hydrocarbon-contaminated sites and in the petroleum industry; these findings were similar to those of Perfumo *et al*. (2010) and Andrade Silva *et al*., (2014).

The microbial population of CSL which are widely known to produce biosurfactants includes lactic acid bacteria (Vecino *et al*., 2014). El-Gendy *et al*. (2013) also reported that CSL as a powerful and effective source of nutrients for optimizing biodegradation of petroleum contaminants in the aquatic environment.

In recent years, studies have focused on the efficient utilization of agro-industrial residues, such as cassava wastewater (Nitschke and Pastore, 2006), potato peels (Das and Mukherjee, 2007), groundnut oil refinery residue, CSL (Sobrinho *et al*., 2008), molasses (Joshi *et al*., 2008; Saimmai *et al*., 2011), and solid waste dates, SWD (El Mahdi et al., 2014).

de Gusmão *et al*. (2010) studied biosurfactant production by *Candida glabrata* using vegetable fat waste as a substrate, and applied a factorial design and RSM to investigate the effects and interactions of waste, yeast extract, and glucose on the surface tension after 6 d of cultivation. Silva *et al*. (2014) reported that biosurfactants for environmental applications can be produced from low-cost substrates, such as frying oil and CSL.

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Table 2: Experimental matrix and results for HCO and LCO crude oil biodegradation.

Run no.	Factors			H Crude oil removal %			L Crude oil removal %		
	Crude oil Conc. % (w/v)	CSL Conc. % (w/v)	Time (day)	Observed	Predicted	Residual	Observed	Predicted	Residual
1	1.00	0.50	17.50	83.12	86.64	0.198	88.49	94.84	-2.288
2	0.63	0.50	17.50	90.22	90.15	0.024	92.56	92.29	0.073
3	0.63	0.50	7.00	73.93	74.61	-0.299	79.26	78.90	0.130
4	0.63	0.50	17.50	88.43	90.15	0.824	89.64	92.29	-0.726
5	1.00	0.20	7.00	61.36	59.27	0.953	82.68	81.31	0.774
6	0.63	0.50	17.50	89.33	90.15	-0.299	93.15	92.29	0.234
7	0.63	0.50	17.50	90.13	90.15	0.353	92.67	92.29	0.103
8	0.25	0.50	17.50	84.76	86.64	-0.765	95.12	89.92	2.913
9	0.25	0.80	28.00	90.31	94.34	1.354	96.25	98.06	-1.029
10	0.63	0.80	17.50	91.74	97.29	-2.135	96.01	96.53	-0.186
11	1.00	0.20	28.00	86.88	89.43	-1.162	93.34	92.24	0.620
12	0.63	0.50	17.50	90.14	90.15	0.719	91.32	92.29	-0.267
13	0.63	0.50	28.00	91.24	93.40	-0.314	98.01	93.74	0.495
14	0.25	0.20	7.00	55.31	59.27	-1.801	65.46	67.71	-1.274
15	0.63	0.50	17.50	89.36	90.15	-0.288	90.95	92.29	-0.368
16	0.25	0.80	7.00	67.59	72.91	2.131	77.55	79.10	-0.877
17	0.63	0.20	17.50	86.28	83.02	1.255	92.06	89.81	0.812
18	1.00	0.80	7.00	70.77	77.91	-0.974	93.17	91.11	1.159
19	1.00	0.80	28.00	90.92	94.34	-0.189	96.13	94.31	1.025
20	0.25	0.20	28.00	90.14	89.43	0.322	91.93	94.41	-1.406

Table 3a: Analysis of variance for quadratic model terms.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob > F	Remarks
Model	2069.25	5	413.85	48.24	< 0.0001	significant
B	509.22	1	509.22	59.35	< 0.0001	
C	1081.18	1	1081.18	126.01	< 0.0001	
A^2	39.64	1	39.64	4.62	0.0496	
C^2	84.85	1	84.85	9.89	0.0072	
BC	175.59	1	175.59	20.47	0.0005	
Residual	120.12	14	8.58			
Lack of fit	110.99	9	12.33	6.76	0.0244	significant
Pure error	9.13	5	1.83			
Cor total	2189.37	19				

Std. Dev.=1.55, Mean=86.73, C.V.=1.79, PRESS=317.62, R-Squared=0.9893, Adj R-Squared=0.9797, Pred R-Squared= 0.8589, Adeq Precision=38.640.

Table 3b: Analysis of variance for quadratic model terms.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob > F	Remarks
Model	1065.37	9	118.37	6.85	0.0029	significant
A	53.31	1	53.31	3.09	0.1095	
B	125.11	1	125.11	7.24	0.0227	
C	592.06	1	592.06	34.26	0.0002	
A^2	0.66	1	0.66	0.038	0.8488	
B^2	1.07	1	1.07	0.062	0.8083	
C^2	98.52	1	98.52	5.70	0.0381	
AB	1.49	1	1.49	0.086	0.7751	
AC	126.54	1	126.54	7.32	0.0221	
BC	23.63	1	23.63	1.37	0.2693	
Residual	172.81	10	17.28			
Lack of fit	163.43	5	32.69	17.42	0.0035	significant
Pure error	9.38	5	1.88			
Cor total	1238.18	19				

Std. Dev.=3.89, Mean=89.79, C.V.=4.33, PRESS=1096.14, R-Squared=0.8723, Adj R-Squared=0.7574 , Pred R-Squared=0.759, Adeq Precision=11.032

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Biodegradation enhancement:

A total of 20 runs for each LCO and HCO were executed using the CCD experimental design. The interactions among three independent variables were considered for each run to investigate the performance of CO biodegradation. CSL was used to improve the biodegradation efficiency of LCO and HCO in seawater batch bioreactors (Table 2). The removal efficiency ranged from 55% to 91% and 65% to 98% for HCO and LCO, respectively. Similar study recorded that high biodegradation percentage in combined biostimulation and augmentation flasks indicates that CSL can act as a good and sufficient nutrients source. (El-Gendy *et al*., 2013).

The maximum removal in un-optimized conditions for HCO (91%) was obtained at 0.63 % (w/v) HCO, 0.5 % (w/v) CSL, and 28 d incubation time (Figure 1a), whereas the minimum removal (55%) was obtained at 0.25 (w/v) HCO, 0.2 % (w/v) CSL, and a minimum incubation period of 7 d. For LCO, the maximum removal (98%) was observed using 0.5 % (w/v) CSL and 0.63 % (w/v) LCO for 28 d, whereas the minimum removal was observed (65%) using 0.25 % (w/v) LCO and 0.2 % (w/v) CSL for 7 d (Figure 1a and b). These results reveal that the incubation time was the most limiting factor for CO biodegradation. Microorganisms capable of utilizing petroleum hydrocarbons are ubiquitous in marine water, freshwater, and soil ecosystems. Environments with hydrocarbon contamination will have a higher percentage of oil degraders than unpolluted areas (Zhu *et al*., 2004). In the present study, the identified bacterial strain was *Pseudomonas sp.* NAF1. The performance of the strain in CO biodegradation has been previously discussed (Rashedi *et al*., 2006; Selvi and Nithya., 2014). Our results suggest that the addition of CSL facilitated and enhanced the biodegradation process of CO by *Pseudomonas sp.* NAF1.

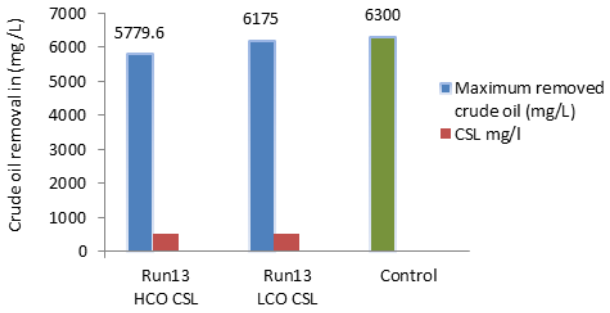

Fig. 1a: Comparison of natural attenuation and maximum bioremediation For LCO and HCO on day 28.

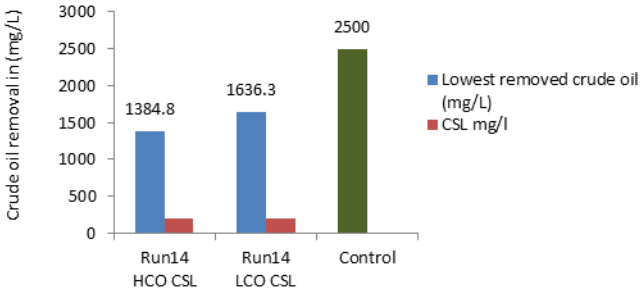

Fig. 1b: Comparison of LCO and HCO lowest bioremediation on day 7.

ANOVA:

Tables 3a and 3b present the ANOVA results of regression parameters of the predicted response surface quadratic models and other statistical parameters for the removal (%) of LCO and HCO. The data in Tables 3a and 3b demonstrate that all models were significant at the 5% confidence level. Given that p values were less than 0.05, the values of the correlation coefficient obtained in the present study for both types of CO removal (R^2 = 0.9893 and 0.8723) were greater than 0.80. For a model with good fit, the minimum correlation coefficient must be 0.80 (Joglekar *et al*., 1987; Nordin *et al*., 2004). The adequate precision (AP) ratio of the models varied between 38.64 and 11.03, which is an adequate signal for the model. AP values higher than 4 are desirable, and confirm that the predicted models can be used to navigate the space defined by CCD.

The regression equation shows that the degradation rate was an empirical function of the test variables in

coded units. Eqs. 4 and 5 provide the models for LCO and HCO removal, respectively.

HCO removal (%) = 15.55 + 45.79 * A + 56.87 * B + 3.697 * C-27.958 * A^2 + 7.354 * B^2 - 0.05091 * C^2 17.27 * A * B - 0.15 * A * C - 1.63 * B * C (4)

LCO removal (%) = 44.40 + 24.07 * A +11.213 * B + 3.04 * C - 0.048 * C^2 - 1.00 * A* C (5)

where A is the CO concentration (mg/L), B is the CSL concentration (mg/L), and C is the time (day).

In these models, the two-level interaction between A and B and second order effect of terms A and C were insignificant, whereas the other terms were significant (insignificant terms were removed from the final equation). Coefficients with one factor show the effect of a particular factor, whereas coefficients with two factors and those with second order terms demonstrate the interaction between the two factors and quadratic effect, respectively. The positive sign in front of the terms indicates a synergistic effect, whereas the negative sign indicates an antagonistic effect (Ahmad *et al*., 2009).

The quality of the fit of the polynomial model of HCO was expressed by the coefficient of determination (R^2), adjusted coefficient (R^2 Adj), and predicted coefficient (R^2 Pred). In this study, R^2 Pred of 0.9893 was in reasonable agreement with R^2 Adj of 0.9797. R^2 and predicted residual sum of squares (PRESS) were 0.9893 and 317.62, respectively. The coefficient of variation (CV) was 4.3.

The quality of the fit of the polynomial model of LCO was expressed by R^2, R^2 Adj, and R^2 Pred. In this study, R^2 Pred of 0.759 was in reasonable agreement with R^2 Adj of 0.757. R^2 and PRESS were 0.9582 and 943.65, respectively. CV was 1.79.

Normal probability plots of the standardized residuals and diagnostics were provided by Design Expert 6.0.7 software to confirm that the selected models provided an adequate approximation of the real system. These plots aided in evaluating the models. Figure 2 (1a and 1b) show the normal probability plots for the standardized removal of LCO and HCO residuals. The normal probability plots predict that if the residuals follow a normal distribution, as shown in Figure 2, then the points will fall along a straight line for each case. However, some scattering is expected even with the normal data. Therefore, the data could be considered to be normally distributed in certain models. Figure 2 (1c and 1d) indicate that the predicted values of the removal efficiencies of LCO and HCO obtained from the model and actual experimental data were in good agreement.

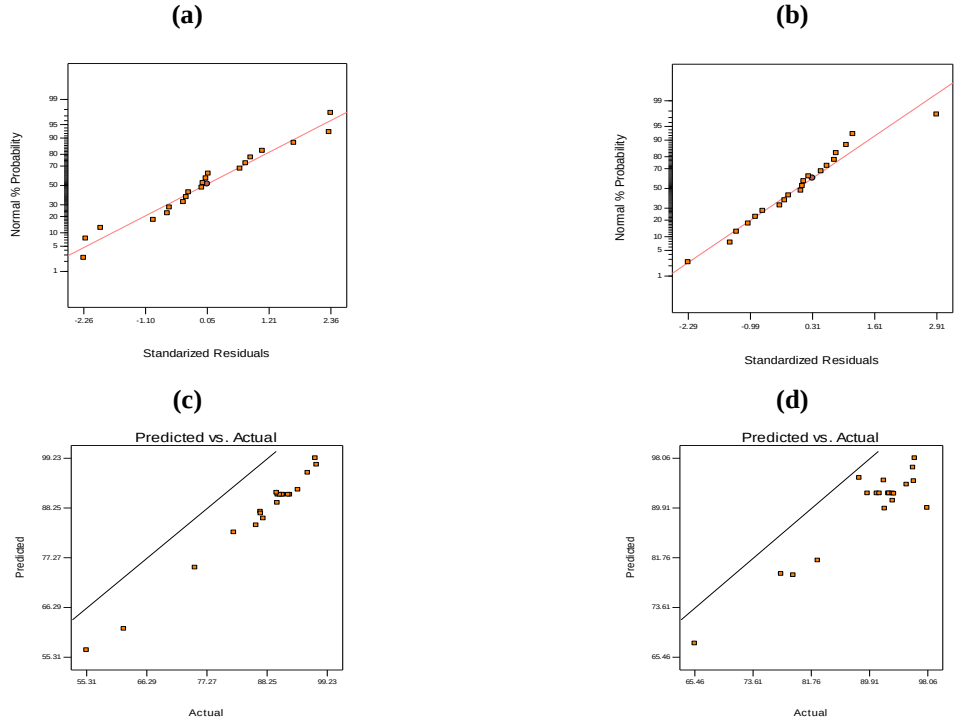

Fig. 2: Design Expert plot. (a & b): Normal probability plot of the standardized residuals, (c &d): predicted values for HCO and LCO removal, respectively.

Treatment efficiency:

To assess the interactive relationships between independent variables and the responses of certain models, 3D surface response plots were obtained using Design Expert 6.0.7 software Figure 3 (2a–2c). The maximum levels of LCO and HCO were 98% and 91%, respectively.

Figure 3 (1a and 2a) present the effects of the interaction between the initial concentrations of LCO and HCO and CSL dosage in CO removal at an

incubation time of 28 d. HCO removal (%) obviously increased with increasing CSL dosage. The maximum removal was obtained at high CSL dosage of 0.5 % (w/v) and initial HCO concentration of 0.63 % (w/v), while no significant effects were observed in LCO removal when both factors were altered. Figure 3 (1b and 2b) present the effects of initial CO concentrations and incubation time on both LCO and HCO removals using 0.5 % (w/v) CSL. For both LCO and HCO, the removal efficiencies improved with increasing incubation time. Figure 3 (1c and 2c) illustrate the cooperative effects between CSL dosage and incubation time at an initial CO concentration of 0.63 % (w/v).

Optimization and verification:

Optimization was carried out to determine the optimum values of HCO and LCO removal efficiency using Design Expert 6.0.7. According to the software optimization step, the desired goal for each operational condition (initial LCO and HCO concentrations, CSL dosage, incubation time, and reaction time) was chosen within the range. The responses (LCO and HCO removal) were defined as the maximum to achieve the highest performance.

The most cost-efficient and environment-friendly conditions for the bioremediation of CO should use the lowest amounts of CSL in the shortest time. Hence, a set of approximate reaction conditions based on the required criteria for maximum oil degradation and minimum nutrient consumption was predicted by a software using numerical optimization with the highest desirability.

The numerical optimization criteria for maximum CO removal were within a range of variables. Using 0.2 and 0.2 % (w/v) CSL for 15 and 10 d, the software predicted 76% and 84% removal for HCO and LCO, respectively with a desirability of 1.00 (Figure 4). Confirmatory experiments were performed at the optimum conditions, and 81% and 88% removal were observed for HCO and LCO, respectively. These results were in reasonable agreement with the model. The removal rates for HCO and LCO without SWD were 5.5% and 14.7%, respectively.

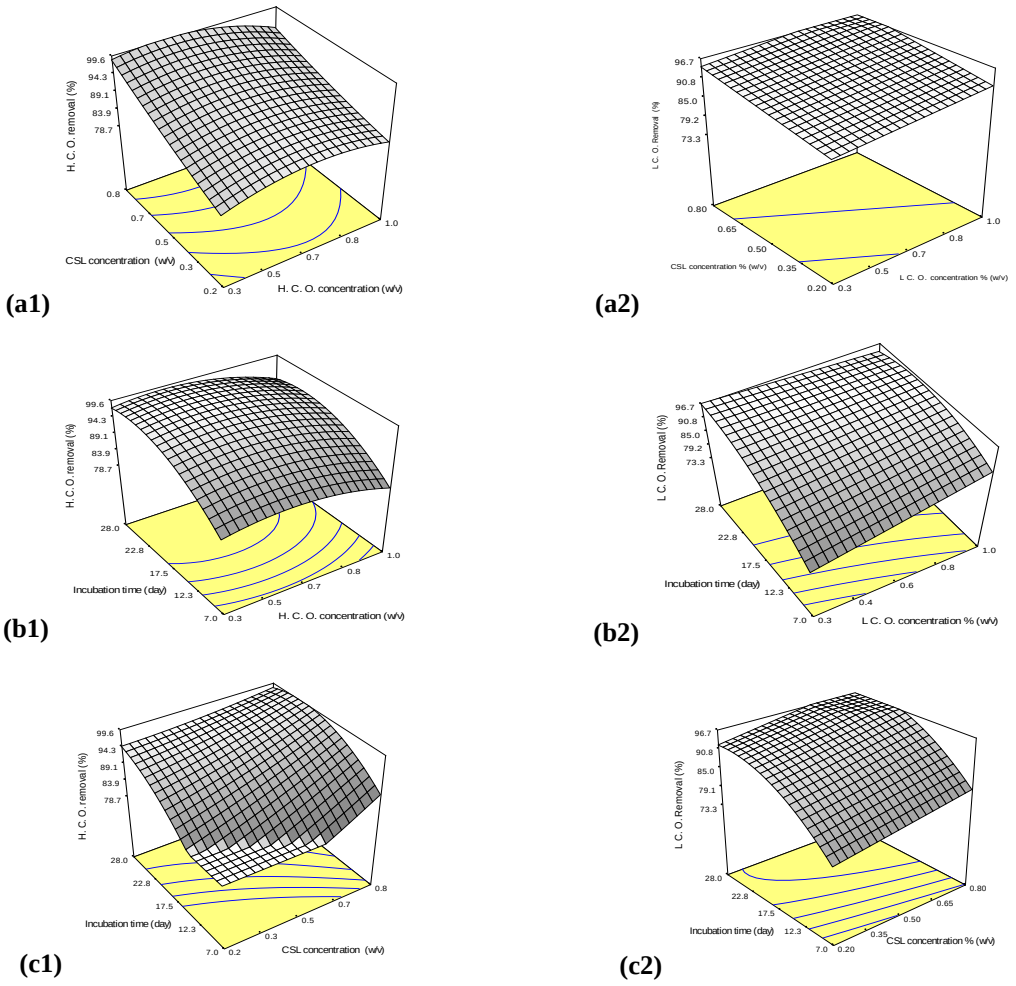

Fig. 3: (a1 – c1): the 3D response surface plots of HCO. bioremediation, (a2 – c2); the 3D response surface plots of LCO bioremediation.

Residuals and errors between predicted and actual values were evaluated for the validation of experiments. Errors were calculated using Eq. (6):

$$Error = \frac{X_{obs} - X_{pre}}{X_{obs}} X\ 100 \quad (6)$$

where X_{Obs} is the observed value, and X_{Pre} is the predicted value.

Residuals ranged between –3 and 3 (Figure 5). Residuals indicate that process optimization by CCD was reliable.

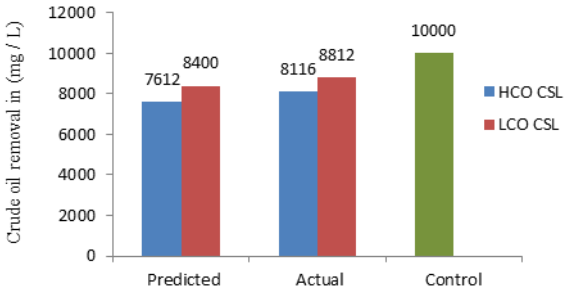

Fig. 4: Comparison between predicted and actual removals on days 15 and 10 for HCO and LCO.

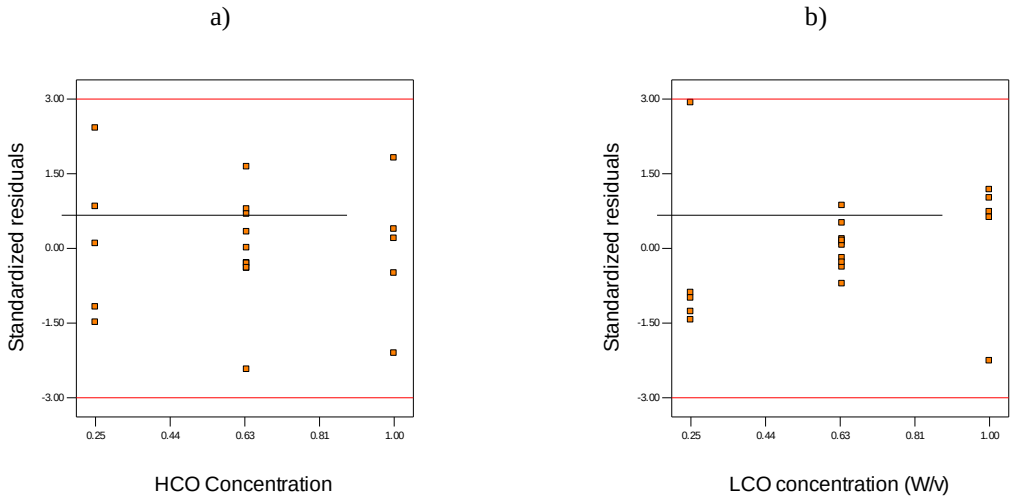

Fig. 5: residual curves: (a) HCO and (b) LCO removal.

Conclusions:

The effectiveness of nutrient CSL supplementation in increasing the biodegradation rate of CO was investigated using RSM. A second-order polynomial mathematical model was generated with multiple regression analysis to describe HCO and LCO bioremediation in artificially contaminated seawater samples. The maximum HCO and LCO removals by natural attenuation and un-optimized bioremediation were 10.4% and 10.3% and 91% and 98% in 28 d, respectively. Numerical optimization was achieved based on desirability functions. Using 0.20 and 0.20 % (w/v) CSL, the software predicted 76% and 84% removal in 15 and 10 d, respectively.

The removals of 81% and 88% were observed experimentally, and in reasonable agreement with the predicted values. This study showed that process variables could be optimized to improve the biodegradation rate. The use of CSL is presented as an attractive option because of its versatility, biodegradability, ecological safety, and environmental acceptance. Its low production cost enhances its use in bioremediation processes.

Greater emphasis is being laid on the procurement of various cheap agro-industrial substrates, including vegetable oils, distillery and dairy wastes, soya molasses, date molasses, animal fat, waste, and starchy waste, as raw materials. These waste products can be used to achieve the benefit of reducing pollutants while producing valuable products. Waste products can also be used as substrates for large-scale production of biosurfactants with advanced technology, which may be investigated in future research.

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