OPTIMISATION OF CONDITIONS FOR MICROBIAL DESULPHURISATION OF CRUDE OIL USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Microbial desulphurisation of crude oil using Aspergillus niger was studied and optimised using a three-variable, three-level Box-Behnken design (BBD) combined with response surface methodology (RSM). A statistical model was developed from the experimental design to determine the effect of spore volume, nitrogen and phosphorus supplementation on the degree of desulphurisation. The fit and significance of the model were tested using analysis of variance (ANOVA). The results obtained revealed that the model was statistically significant ($p<0.0001$) with a low standard deviation (4.82) compared with the mean (63.80) and did not show a significant lack of fit ($R^2=0.98$). The results also showed that the desulphurisation process was positively influenced by nitrogen supplementation but the influence was less significant for phosphorus. The volume of spore was also seen to have a positive influence on the desulphurisation process albeit at intermediate levels and this was sufficient to achieve maximum desulphurisation. Results obtained from RSM revealed that the maximum level of desulphurisation was 80.1% and this was obtained with a spore volume, nitrogen and phosphorus concentrations of 11.53 ml, 0.3 g/l and 0.1 g/l, respectively. Results of replicated experiments conducted at the optimised conditions revealed no difference between predicted results and experimental observations.

***Keywords**: Crude oil, Aspergillus niger, Box-Behnken design, Optimization, Desulphurisation*

1. INTRODUCTION

Crude oil is a naturally occurring mixture consisting of hydrocarbons, sulphur, nitrogen and metals (Yasin *et al.*, 2013). After the hydrocarbons, sulphur (0-6 wt%) constitutes the most abundant component of crude oil and it represents the most important and expensive crude oil component to deal with during refining (Speight, 1999; Van Hamme *et al.*, 2003).

Failure to remove sulphur from crude oil prior to refining results in the production of refined petroleum products containing sulphur compounds. This has the associated problem of the release of oxides of sulphur when the refined products are combusted during use. These oxide gases pose serious environmental risks as they result in the formation of acid rain.

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Furthermore, exposure to oxides of sulphur and acid rain can lead to adverse skin reaction, respiratory effects including bronchoconstriction and increased asthma symptoms (Curtis *et al.*, 2006). In addition, during refining, some intermediate products are taken through further processing steps and these steps usually involves the use of catalysts and if the intermediate products contain sulphur, it could result in the poisoning of the catalyst thus reducing their activity and efficiency. Hydrogen sulphide produced during the process can also result in the corrosion of equipment (Ohshiro *et al*., 1996). Therefore, it is imperative to remove as much sulphur from crude oil in order to produce low sulphur fuels that meet the regulations stating the minimum level of sulphur that refined petroleum products must contain as stipulated by international organisations such as the International Maritime Organisation (IMO) and the European Marine Safety Agency (EMSA) (IMO, 2008; EMSA, 2010; Adegunlola *et al.*, 2012).

Organic sulphur in crude oil is typically removed via hydrodesulphurisation (HDS). HDS is suitable for the removal of organic sulphur compounds (thiols, sulphides, and thiophenes) in the lower-boiling fractions of petroleum in the gasoline range. However, sulphur compounds (benzothiophenes and dibenzothiophenes) in middle-distillate fractions in the diesel and fuel oil range are not readily removed by HDS (Grossman *et al.*, 1999). This limitation as well as the capital intensive nature and chemical limitation of the HDS process has necessitated the need to search for alternative means of removing sulphur from crude oil. Furthermore, the current trend toward stricter regulations on low sulphur fuels is also providing a motivation in this direction.

Microbial desulphurisation has been studied as an alternative to HDS for the removal of organic sulphur from fuels. It involves the use of microorganisms with highly specific metabolic activities, which enable them utilise and transform nearly every known hydrocarbon species present in crude oil (Mohamed *et al.*, 2015). Bacteria with the capacity to desulphurise crude oil via a sulphur selective oxidative pathway that does not remove carbon have been isolated (Wang and Krawiec, 1994; Grossman, 1996; Ohshiro *et al.*, 1996; Labana *et al.*, 2005). The major examples of these bacteria are *Rhodococcus sp.* and *Arthrobacter sulphurous.* Some fungal species (E.g. *Aspergillus flavus*) have also been evaluated for desulphurisation of crude oil (Adegunlola *et al.*, 2010; Adegunlola *et al.*, 2012). In this study, an attempt has been made to utilise *Aspergillus niger* as the microorganism for the desulphurisation process.

Carbon supply is not the only nutritional requirement for the effective metabolic action of microorganisms. Other nutrient supplements such as nitrogen and phosphorus are also essential. Fungal growth may be limited when nutrients are not present in the appropriate proportions (Treseder and Allen, 2002). Moreover, the different nutrients need to be available at their optimum concentrations for the microorganism to function optimally. A search of available literature has shown that no attempt has been made to apply experimental design method with response surface methodology (RSM) to optimise the biodesulphurisation of crude oil using *Aspergillus niger*. Given the reported usefulness and effectiveness of RSM in optimising multivariate processes with its advantages over the traditional one factor at a time method (Montgomery, 2005; Anupama *et al.*, 2010; Fang *et al.*, 2010; Tian *et al.*, 2011; Amenaghawon *et al.*, 2015), this study thus focused on the application of RSM for optimising the microbial desulphurisation of crude oil. Three factors were investigated in this study and these are the concentration of the nutrient supplements ammonium nitrate (NH_4NO_3) and potassium dihydrogen phosphate (KH_2PO_4) as well as the volume of inoculum and this was done using a three variable Box-Behnken design coupled with response surface methodology. The aim was to determine the optimum levels of the factors that will result in maximum desulphurisation.

2. MATERIALS AND METHODS

2.1 Crude Oil

The crude oil used in this study was procured from the Warri Refining and Petrochemical Company Limited, Effurun, Warri, Delta State, Nigeria. The properties of the crude oil sample were as follows: API gravity (35.3), specific gravity (0.85), sulphur (0.15 wt%), viscosity at 40^{o}C (3.28 cSt).

2.2 Microorganism Isolation and Inoculum Preparation

Aspergillus niger, the microorganism used for this study was originally isolated from a crude oil contaminated soil in the Department of Microbiology, University of Benin, Benin City, Edo State, Nigeria. The stock culture was stored on agar slants and harvested by flooding the slants with water and gently scraping its surface to dislodge the spores. The sporulation medium used was potato-dextrose-agar.

2.3 Experimental Design

A three variable Box-Behnken design (BBD) for response surface methodology was used to study, model and optimise the desulphurisation process. The range of the variables that were optimized is shown in Table 1.

Table 1: Experimental range and level of the independent variables

Variables	Units	Symbol	Coded		
			-1	0	1
Spore	ml	X_1	5	10	15
NH_4NO_3	g/l	X_2	0.1	0.2	0.3
KH_2PO_4	g/l	X_3	0.1	0.2	0.3

The experimental design made up of 17 runs was developed using Design Expert 7.0.0 software (Stat-ease Inc.

Minneapolis, USA). The following generalised second order polynomial model (Equation 1) was used to estimate the response of the dependent variable (percent desulphurisation).

$$Y_i = b_o + \sum b_i X_j + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \quad (1)$$

Where Y_i is the response to be predicted, X_i and X_j are the independent variables, b_o is offset term, b_i and b_{ij} are the single and interaction effect coefficients and e_i is the error term. Multiple regression and graphical analysis of the experimental data was carried out using the Design Expert software to obtain a statistical model for describing the desulphurisation of crude oil. Analysis of variance (ANOVA) and coefficient of determination (R^2) were used in the evaluation of the goodness of fit of the model. The optimum values of the variables tested were obtained by numerical optimisation based on the criterion of desirability (Amenaghawon *et al*., 2014).

2.4 Crude Oil Desulphurisation

Biodesulphurisation of crude oil was carried out in 250 ml Erlenmeyer flasks. The desulphurisation of crude oil was effected by growing *Aspergillus niger* cells in a sulphur-free mineral salt medium (SFMSM) with the crude oil acting as a source of sulphur for the *Aspergillus niger* cells. It is important that the microorganism does not consume the carbon of the sulphur containing compounds. In other words, the fungus strain should have the ability to use the sulphur of the crude oil without using its carbon. The consumption of the sulphur present in the crude oil by the *Aspergillus niger* cells resulted in the desulphurisation. In order for the *Aspergillus niger* cells to have proper metabolic function, the SFMSM was supplemented with nutrients such as ammonium nitrate (NH_4NO_3) and potassium dihydrogen phosphate (KH_2PO_4) which served as nitrogen and phosphorus sources respectively. The level of these nutrients as well as the volume of inoculum used were determined according to the experimental design. The volume of crude oil was kept constant at 20 ml. The desulphurisation was allowed to proceed for 4 days after which the residual sulphur content of the crude oil was determined.

2.5 Quantification of Residual Sulphur

To quantify the amount of sulphur left in the crude oil sample at the end of treatment, a conditioning reagent was first prepared by measuring 25 ml of glycerol, 15 ml of concentrated hydrochloric acid, 50 ml of isopropyl alcohol and 37.5 g of sodium chloride into a beaker and then stirred vigorously. This mixture was then made up to 250 ml by adding distilled water (Adeleke *et al*., 2011). An amount (2 ml) of the desulphurised crude oil was measured and added to 10 ml of concentrated hydrochloric acid contained in a Kjedahl digestion flask. Distilled water (20 ml) was then added to the mixture and the content was vigorously agitated to enable hydrolysis. The content of the flask was allowed to stand for 3 hours after which it was filtered using Whatman No.1 filter paper. An amount of the filtrate (5 ml) was dispensed into a test tube and 15 ml of distilled water and 2 ml of the conditioning reagent were added. The test tube was covered and allowed to stand for a few hours. A spatula full of barium chloride ($BaCl_2$) was then added. The optical density of the mixture was read with an ultra violet-visible spectrophotometer (Adegunlola *et al*., 2012).

3. RESULTS AND DISCUSSION

3.1 Statistical Modelling and Analysis

Table 2 shows the result of the 17 experimental runs carried out according to the BBD. Multiple regression analysis was applied to the experimental data to obtain Equation (2) which was used to estimate the response, Y (percent desulphurisation). The values of Y predicted by Equation (2) are also shown in Table 2 along with the experimental data for comparison.

The significance of fit of the model was assessed by performing analysis of variance (ANOVA) and the results are shown in Tables 3 and 4.

$$Y = -118.75 + 30.23X_1 + 384.53X_2 + 119.72X_3 - 19.72X_1X_2 - 3.25X_1X_3 - 301.50X_2X_3 - 1.36X_1^2 - 162.98X_2^2 - 79.73X_3^2 \quad (2)$$

The ANOVA results show that the response model was statistically significant with very low p value as shown in Table 3. The model "lack of fit" p value of 0.5053 indicates that the model did not show lack of fit. Statistical information for ANOVA shows that the model had high coefficient of determination (R^2=0.98) as shown in Table 4. This shows that the model was able to adequately represent the relationship between the chosen factors (spore volume, nitrogen and phosphorus concentration) and the response

(percent desulphurisation). An R^2 value of 0.98 means that the model was able to explain 98% of the variability observed in the response values. The standard deviation was observed to be relatively small compared to the mean (63.80±4.82). The coefficient of variation for the model was obtained to be 7.55. This parameter shows the degree of precision with which the runs were carried out. A value less than 10 is usually recommended (Cao *et al*., 2009). The value obtained show a high reliability and reproducibility of the experiment as recommended by Montgomery (2005). Adequate precision value measures signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 14.68 was obtained in this study which indicates an adequate signal.

Table 2: Experimental design matrix for crude oil desulphurisation

Runs	Factors						Response	
	Coded values			Actual values			Coded values	
	X_1	X_2	X_3	X_1	X_2	X_3	Experiment	Predicted
1	0	1	1	10.00	0.30	0.30	81.25	81.16
2	0	-1	-1	10.00	0.10	0.10	69.69	69.78
3	0	0	0	10.00	0.20	0.20	79.47	80.91
4	0	-1	1	10.00	0.10	0.30	70.75	74.81
5	-1	-1	0	5.00	0.10	0.20	38.73	36.89
6	0	0	0	10.00	0.20	0.20	87.68	80.91
7	1	-1	0	15.00	0.10	0.20	43.72	41.41
8	1	1	0	15.00	0.30	0.20	32.23	34.07
9	0	0	0	10.00	0.20	0.20	76.63	80.91
10	-1	0	1	5.00	0.20	0.30	57.12	54.90
11	0	1	-1	10.00	0.30	0.10	92.25	88.19
12	-1	0	-1	5.00	0.20	0.10	50.89	52.64
13	0	0	0	10.00	0.20	0.20	76.58	80.91
14	1	0	-1	15.00	0.20	0.10	38.47	40.69
15	1	0	1	15.00	0.20	0.30	38.20	36.45
16	0	0	0	10.00	0.20	0.20	84.20	80.91
17	-1	1	0	5.00	0.30	0.20	66.67	68.98

3.2. Response Surface Plots and Numerical Optimisation

Figure 1 depicts the response surface and corresponding contour plot showing the effect of phosphorus and nitrogen supplementation. The results show that nitrogen (NH_4NO_3) supplementation enhanced the desulphurisation process as shown in the increase in the percent desulphurisation when the amount of nitrogen was increased. The trend observed could be explained by the fact that nitrogen supplementation serves to enhance cell growth and production of biomass (Grewal and Kalra, 1995; Ali and Zulkali, 2011). Hence, with growth of cells, the amount of microorganism capable of metabolising the sulphur in the crude oil is thus increased consequently leading to an increase in the rate of desulphurisation.

Table 3: ANOVA Results

Source	Sum of squares	df	Mean squares	F value	p value
Model	6139.34	9	682.15	29.42	< 0.0001
X_1	461.93	1	461.93	19.92	0.0029
X_2	306.41	1	306.41	13.21	0.0083
X_3	1.98	1	1.98	0.085	0.7786
X_1X_2	388.68	1	388.68	16.76	0.0046
X_1X_3	10.56	1	10.56	0.46	0.5214
X_2X_3	36.36	1	36.36	1.57	0.2507
X_1^2	4851.56	1	4851.56	209.21	< 0.0001
X_2^2	11.18	1	11.18	0.48	0.5098
X_3^2	2.68	1	2.68	0.12	0.7440
Residual	162.33	7	23.19		
Lack of Fit	66.53	3	22.18	0.93	0.5053
Pure Error	95.80	4	23.95		
Corrected total	6301.67	16			

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Table 4: Statistical Information for ANOVA

Parameter	Value
R- Squared	0.98
Mean	63.80
Standard deviation	4.82
Coefficient of variation (%)	7.55
Adequate precision	14.68

Phosphorus supplementation also enhanced the rate of desulphurisation as shown in Figure 1. The presence of KH_2PO_4 which serves as the source of phosphorus for *Aspergillus niger* has been reported to enhance microbial growth when its concentration is limited (Grossman *et al.*, 1999; Adegunlola *et al.*, 2012). This is because phosphate is known to be essential for the growth and metabolism of *Aspergillus niger* (Shankaranand and Lonsane, 1994). Comparing the effect of phosphorus and nitrogen supplementation, it is seen that the effect of nitrogen was more significant. This is also corroborated by the much lower p value (p=0.0083) of the model term representing nitrogen as shown in Table 3.

Figure 2 shows the simultaneous effect of spore volume and nitrogen supplementation on the desulphurisation process. The spore volume is an important variable to be considered during crude oil desulphurisation as it determines the population of microorganisms available. The trend observed in Figure 2 shows that crude desulphurisation was maximum at an intermediate level of spore volume. The percent desulphurisation was observed to initially increase from about 70% to 80% when the spore volume was increased from 5 ml to 10 ml. The desulphurisation process was negatively impacted when the spore volume was increased beyond 10 ml. The combined effect of nitrogen supplementation and spore volume on the desulphurisation process can be seen more clearly from the contour plot in Figure 2. The red region shows the region of high percent desulphurisation. This region coincides with the intermediate level of spore volume (i.e. 10 ml).

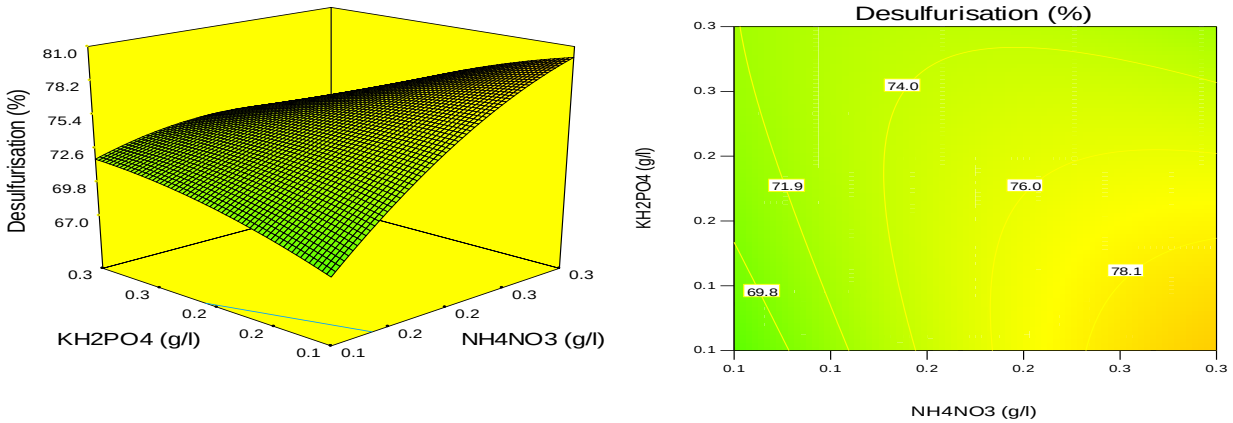

Figure 1: Response surface and contours plots showing the effect of KH_2PO_4 and NH_4NO_3 on crudel oil desulphurisation

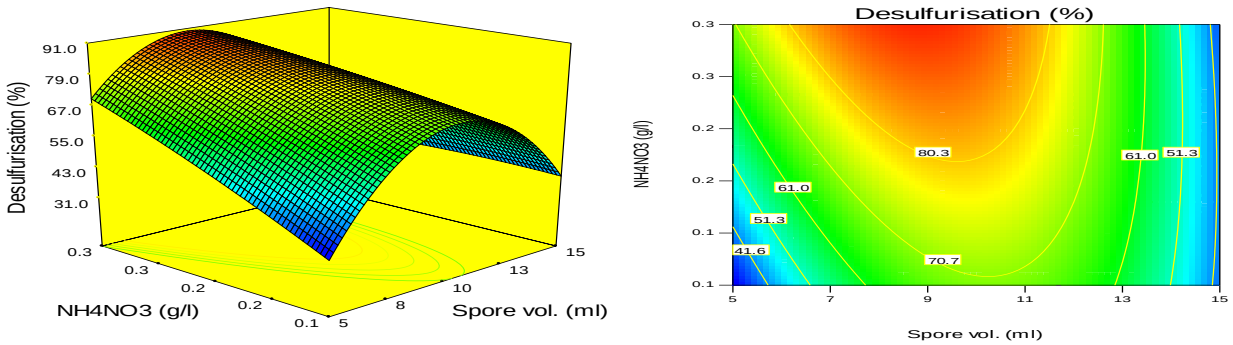

Figure 2: Response surface and contours plots showing the effect of NH_4NO_3 and spore volume on crudel oil desulphurisation

Table 5: Comparison of microbial desulphurisation efficiency with previous work

Reference	Microorganism	Desulphurisation efficiency
This study	*Aspergillus niger*	80.1%
Grossman et al. (1999)	*Rhodococcus* sp.	30.0%
Yu et al. (2006)	*Rhodococcus erythropolis*	47.2%
Li et al. (2007)	*Mycobacterium goodii* X7B	59.0%
Torkamani et al. (2008)	*Stachybotrys* sp.	76.0%
Adegunlola et al. (2012)	*Aspergillus flavus*	94.7%

It can be seen that moving away from this region resulted in a reduced level of desulphurisation, i.e. at lower concentrations of nitrogen. At low values of spore volume, there is the risk of contamination as a result of insufficient microbial cell population and this results in a decline in the growth of cells. However, the decline observed beyond 10 ml of inoculum may be due to the fact that a high inoculum density results in microbial population over crowding which consequently results in increased competition for nutrients as well as rapid consumption of the nutrients (Uyar and Baysal, 2003). Furthermore, Torkamani *et al.* (2008) in their study revealed that as the nutrient medium used in desulphurisation of heavy crude was lacking in nitrogen, the microorganisms utilised the nitrogen present in the hydrocarbon for growth. This clearly reinforces the need for nitrogen supplementation as it largely influences metabolic activities and growth.

The optimum levels of the independent variables and their respective responses were determined from numerical optimisation of the statistical model and the result show that the maximum level of desulphurisation achieved was 80.1% and this was obtained when a spore volume, nitrogen and phosphorus levels of 11.53 ml, 0.3 g/l and 0.1 g/l respectively were used. The efficiency of the desulphurisation process compared favourably with the results of previous researchers as shown in Table 5.

4. CONCLUSION

In this research work, microbial desulphurisation using *Aspergillus niger* was applied to treat sulphur containing crude oil. Design of experiment for response surface methodology has been demonstrated to be useful in optimising the desulphurisation process. A three-level, three-variable Box-Behnken design was used to study the simultaneous effects of spore volume and concentrations of phosphorus and nitrogen on the desulphurisation process. The model developed by RSM to represent the desulphurisation process was statistically significant ($p < 0.0001$) and showed a good fit with the experimental data ($R^2 = 0.98$). The spore volume and concentration of nitrogen were seen to have significant effects on the desulphurisation process (p-values of 0.0029 and 0.0083 respectively) while the phosphorus concentration was observed to have lesser significance (p value of 0.7786). The optimum results obtained from RSM for the spore volume, concentrations of nitrogen and phosphorus were 11.53 ml, 0.3 g/l and 0.1 g/l respectively and the level of desulphurisation obtained was 80.1%.

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