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# Optimization of Humic acid Production using RSM-CCD, its Characterization and Applications on *Vigna mungo*

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**Abstract:** In the present study, the extraction of humic acid was optimized using Response Surface Methodology (RSM) –Central Composite Design (CCD) by altering the concentration of the key constituents such as Lignite, Potassium hydroxide and Sodium hydroxide. Among 20 trials, the maximum humic acid was extracted in anamalgamation of lignite 5g, KOH 4g and NaOH 1g. The humic acid obtained after this process was subjected to evaluate the minerals and characterized by using FESEM-EDAX method. The growth parameters of *Vigna mungo* was studied after applying optimized humic acid.

**Keywords:** Lignite, Humic acid, RSM, CCD, *Vigna mungo, FESEM – EDAX.*

## INTRODUCTION AND EXPERIMENTAL

Humic substances (HS) are complex organic materials found ubiquitously in nature, where they play a lot of environmentally important processes. They are the product of biotic or abiotic degradations of dead plant tissues and animal bodies. Humic acid contains carboxylic, phenolic, alcoholic and carbonyl fractions and are extracted from various sources like forest soil, sediments, coal, peat and lignite. The molecular weight of the compound is between 5,000 and 10,000 Daltons[1]. It contains 33-36% of oxygen and 4% of nitrogen. Many methods have been used to extract the humic acid from brown coal. The extracted product will be alkaline pH, when the cation exchange sites are filled primarily with hydrogen (H) ions. The material is considered as an acid and is called humic acid[1,2,3]. HSs are generally classified into three categories: humic acids (HAs), fulvic acids, and humin. These three humic fractions are structurally similar, but their properties differ, especially with respect to their molecular weight and the number of functional groups present[4].Humic substances help to supply nutrients to plants, makes the soil fertile and productive, increases water holding capacity and seed germination. Humic acid also reduces the other fertilizer requirements, increases aeration of the soil, increase the protein and mineral contents of most crops[5]. Generally humic acid is extracted by alkaline solution (KOH or NaOH) and the extracted product is acidified by HCL. In the present study, humic acid is extracted from ligniteby using KOH and NaOH and optimized by Response Surface methodology-Central composite design (RSM-CCD). The final product was further characterized and applied to *Vigna mungo* to measure humic acid influence on plant growth.

**Evaluation of substrate concentration**

Lignite was weighed in the following concentrations 0.5g, 1.0g, 1.5g, 2.0g, and 2.5g. Further sieved through ≤2mm and thentransferred to 250 ml beaker containing 100 ml of distilled water. 0.56 g (0.1 N) of KOH was added in all the five trials. All the samples were kept for incubation for 15 minutes. Samples were filtered using filter paper and the filtrate was treated with 1 ml of concentrated hydrochloric acid (HCl), the resultant is precipitated. The precipitate has been filtered through Whatmann No. 42 filter paper and weighed as dry weight basis (80ºC for two hours)[6].

**Response surface methodology (RSM)**

Statistical analysis of the model was performed using Stat-ease, Inc. Design expert @ version 7.The significant factors were further subjected to central composite design (CCD). Optimization was carried out using three level (-1, 0, +1) CCD for the production of humic acid and resulted in optimum humic acid production[7,8,9,10]. The interaction of three variables which influence the humic acid production were analyzed and optimized by CCD ($\alpha = 1$) in three levels (Table 1). A total number of 20 experimental runs with different combination of variables (consisting 14 experimental runs and 6 additional runs at the center point) to check the reproducibility. The general form of second order polynomial and its coefficients were analyzed by Design expert. The optimum levels of variables (within the experimental range) for maximum production were determined.

$$\text{Humic acid} = 3.38+0.62A+0.30B+0.21C+0.11AB+0.16AC+0.14BC-0.27A^2-0.24B^2-0.15C^2$$

**Application of Humic acid on *Vigna mungo***

Four pots for both control and treatment were taken for this study. In each pot 10 *Vigna mungo* seeds wereplanted and treated with humic acid once in 15 days. At end of the experiment plant growth parameters such as height of the plant and the number of leaves were recorded and the nutrients like total sugars and total proteins were analyzed[10,11,12,13].

## RESULTS AND DISCUSSION

The objective of the present study is to optimize the combination of lignite, KOH and NaOH for an effective extraction of humic acid from Lignite. The experiment was carried out according to the experimental plan given in Table 1. The experimental and the predicted response of humic acid yield is given in Table 2. Using multiple regression analysis on the experimental data, the following second order polynomial equations were found to explain the humic acid production[14,15].

$$\text{Humic acid} = -2.17101 +1.13517\ \text{Lignite} +0.96830\ \text{KOH} +0.76978\ \text{NaOH} + 0.090000\ \text{Lignite KOH} + 0.52000\ \text{Lignite NaOH} +0.55000\ \text{KOH NaOH} -0.17375\ \text{Lignite}^2 -0.23613\ \text{KOH}^2 - 2.36379\ \text{NaOH}^2$$

The coefficient of determination ($R^2$) for production of humic acid is 0.*976648*. The value of $R^2$ is a measure of total variation of observed values about the mean explained by the fitted model which is often expressed in percentage. Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and the results are shown in Table 3 of humic acid. The F-value is the ratio of the mean square due to regression to the mean square due to error. If the model is a good predictor of the experimental data, the computed F-value is higher than the tabulated F-value.

Contour plots are shown in Fig. 2 for humic acid. The coordinates of the stationary point are called optimum point. The trend of the mutual interactions between slant age and seed age are elliptical.

**Table 1: Experimental plan for optimization of lignite, KOH and NaOH for optimal production of humic acid**

| Compounds | High | Mid | Low |
|---|---|---|---|
| Lignite | 5 | 3.75 | 2.5 |
| KOH | 4 | 3 | 2 |
| NaOH | 1 | 0.75 | 0.5 |

**Table 2 :Actual level of independent variables along with the observed values for the response variable.**

| Std Order | Lignite | | KOH | | NaOH | | Actual Dry weight (in grams) | Predicted Values |
|---|---|---|---|---|---|---|---|---|
| | Actual | Coded | Actual | Coded | Actual | Coded | | |
| 1 | 2.50 | (-1) | 2.00 | (-1) | 0.50 | (-1) | 2.10 | 2.02 |
| 2 | 5.00 | (1) | 2.00 | (-1) | 0.50 | (-1) | 2.10 | 2.7 |
| 3 | 2.50 | (-1) | 4.00 | (1) | 0.50 | (-1) | 1.90 | 2.12 |
| 4 | 5.00 | (1) | 4.00 | (1) | 0.50 | (-1) | 2.80 | 3.25 |
| 5 | 2.50 | (-1) | 2.00 | (-1) | 1.00 | (1) | 3.10 | 1.83 |
| 6 | 5.00 | (1) | 2.00 | (-1) | 1.00 | (1) | 1.50 | 3.16 |
| 7 | 2.50 | (-1) | 4.00 | (1) | 1.00 | (1) | 2.50 | 2.48 |
| 8 | 5.00 | (1) | 4.00 | (1) | 1.00 | (1) | 3.40 | 4.26 |
| 9 | 2.50 | (-1) | 3.00 | (0) | 0.75 | (0) | 3.20 | 1.58 |
| 10 | 5.00 | (1) | 3.00 | (0) | 0.75 | (0) | 3.50 | 3.65 |
| 11 | 3.75 | (0) | 2.00 | (-1) | 0.75 | (0) | 3.40 | 2.21 |
| 12 | 3.75 | (0) | 4.00 | (1) | 0.75 | (0) | 3.40 | 3.22 |
| 13 | 3.75 | (0) | 3.00 | (0) | 0.50 | (-1) | 3.20 | 2.62 |
| 14 | 3.75 | (0) | 3.00 | (0) | 1.00 | (1) | 3.60 | 3.31 |
| 15 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 3.70 | 3.38 |
| 16 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 2.20 | 3.38 |
| 17 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 2.40 | 3.38 |
| 18 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 3.20 | 3.38 |
| 19 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 4.20 | 3.38 |
| 20 | 3.75 | (0) | 3.00 | (0) | 0.75 | (0) | 3.30 | 3.38 |

**Table 3 : Pooled ANOVAimpactstrength.**

| Source | Sum of Squares | DF | Mean Square | F Value | p-value Prob > F | Significant or non Significant |
|---|---|---|---|---|---|---|
| Model | 9.303062 | 9 | 1.033674 | 46.47021 | < 0.0001 | Significant |
| A-Lignite | 5.166568 | 1 | 5.166568 | 232.2701 | < 0.0001 | |
| B-KOH | 1.241807 | 1 | 1.241807 | 55.82711 | < 0.0001 | |
| C-NaOH | 0.579667 | 1 | 0.579667 | 26.0597 | 0.0005 | |
| AB | 0.10125 | 1 | 0.10125 | 4.551833 | 0.0587 | |
| AC | 0.21125 | 1 | 0.21125 | 9.497033 | 0.0116 | |
| BC | 0.15125 | 1 | 0.15125 | 6.799651 | 0.0261 | |
| $A^2$ | 1.062139 | 1 | 1.062139 | 47.74989 | < 0.0001 | |
| $B^2$ | 0.803505 | 1 | 0.803505 | 36.12267 | 0.0001 | |
| $C^2$ | 0.314545 | 1 | 0.314545 | 14.14078 | 0.0037 | |
| Residual | 0.222438 | 10 | 0.022244 | | | |
| Lack of Fit | 0.094105 | 5 | 0.018821 | 0.733282 | 0.6291 | Non-significant |
| Pure Error | 0.128333 | 5 | 0.025667 | | | |
| Cor Total | 9.5255 | 19 | | | | |

$P > 0.05$ is significant

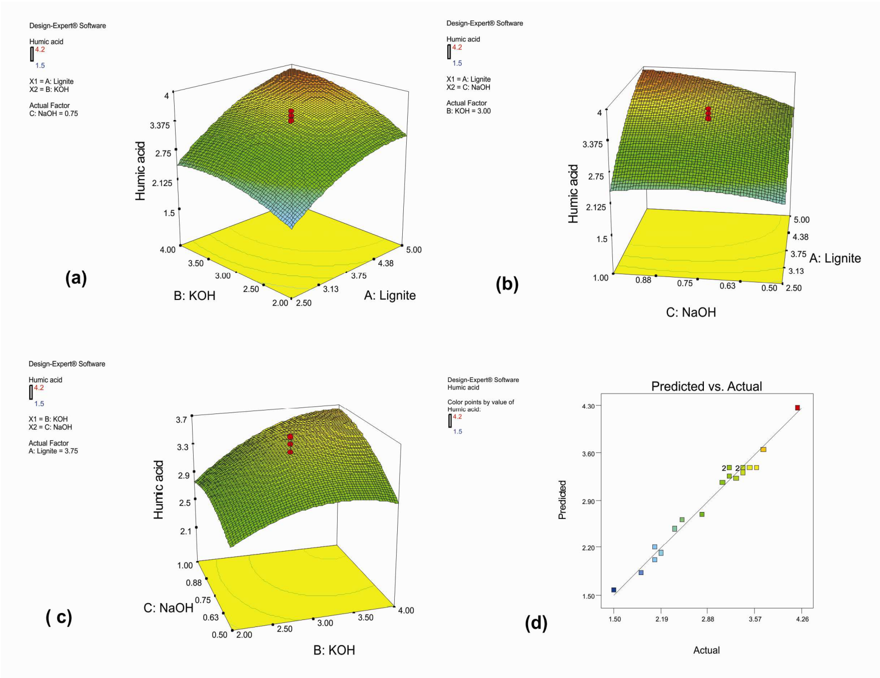


**Fig -1** Surface plot of Humic acid production (a) KOH and Lignite (b) NaOH and Lignite (c) NaOH and KOH and their mutual interaction with the productin of humic acid.

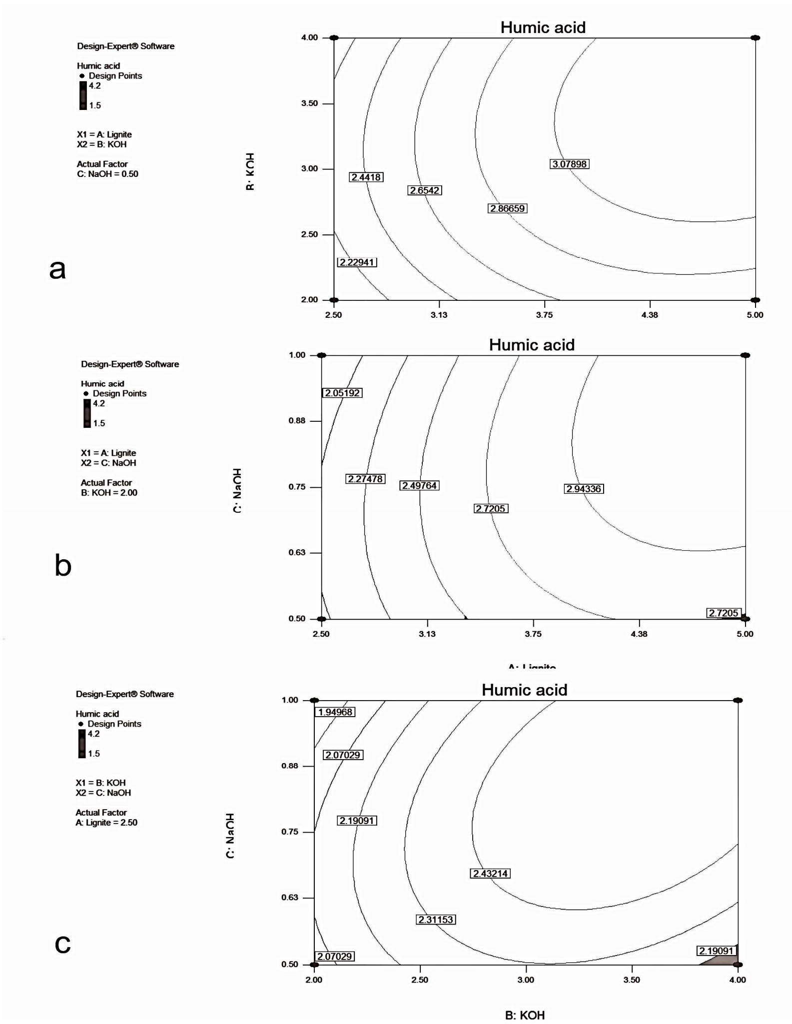
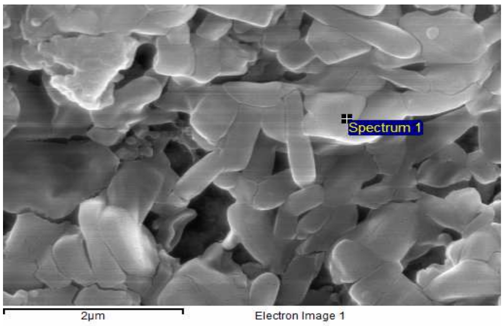


**Fig-2** Contour plot of Humic acid production (a) KOH and Lignite (b) NaOH and Lignite (c) NaOH and KOH and their mutual interaction with the productin of humic acid.

Humic acid can be produced by following a couple of methods i.e. biological and chemical**.** Prakash *et al* *(*2009) reported that *Trichoderma viridi* has the ability to convert lignite into humic acid. Tripathi et al reported that *Aspergillus fumigatus* showed maximum solubilization (22.3%, w/w), and the other fungilike *Fusarium udum*, *Fusarium solani*, *Aspergillus oryzae*, and *Aspergillus sydowii* showed comparatively less solubilization, being in the range of 4.5-16.8% (w/w).

**CHARACTERIZATION OF HUMICACID BY FESEM - EDAX:**



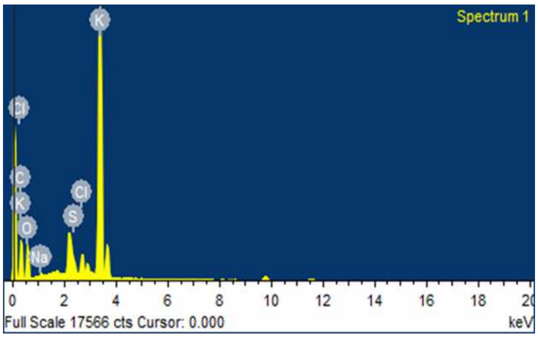




**Fig- 3 & 4** FESEM-EDAX of Humic acid

**FESEM - EDAX:**

In the present study, the optimized humic acid was analyzed for the elemental analysis by performing FESEM-EDAX method. The following standard samples were used i.e., C, O, Na, S, Cl, K and all the elements were normalized. The samples were analyzed for various elemental composition and from the analysis the maximum component observed was Potassium 42.53% (Fig. 3, 4 &Table 5), followed by Oxygen 34.67, Carbon 19.29%, Chlorine 2.72%, Sodium 0.52% and Sulphur 0.28%.Similarly Courtijn and Pourret (2012) reported that elemental composition of the CHA, EDX qualitative measurements indicated the presence of Br, S, Rh, Ba, Ti, Fe, Ni, Mo, Na, Al, Si, Ca, Te, and V. Also he reported that very few of the metallic elements detected can be incorporated into the CHA structure because of the capacity of the CHA to bind many heavy metallic ions through complexation. Other elements such as Si can originate from impurities. S should be incorporated into the CHA structure Simpson (2002). Velasco *et al***.** (2004) analyzed humic acid and the elemental contents from urban compost by fluorescence spectroscopy.

**Table 4 : Regression analysis**

| | | | |
|---|---|---|---|
| **Std. Dev.** | 0.149144 | **R-Squared** | 0.976648 |
| **Mean** | 2.935 | **Adj R-Squared** | 0.955632 |
| **C.V. %** | 5.081551 | **Pred R-Squared** | 0.903717 |
| **PRESS** | 0.917143 | **Adeq Precision** | 25.43212 |

**Table 5: Elements of Humic acid**

| Element | Weight% | Atomic% |
|---|---|---|
| C K | 19.29 | 32.33 |
| O K | 34.67 | 43.61 |
| Na K | 0.52 | 0.45 |
| S K | 0.28 | 0.17 |
| Cl K | 2.72 | 1.54 |
| K K | 42.53 | 21.89 |

**Table 6:** Effect of Humic acid on *Vigna mungo* and its nutrients content

| Treatment | Height cm | No of leaves | Total proteins (µg) | Total sugars(µg) |
|---|---|---|---|---|
| Humic acid | 27 | 18 | 110 | 130 |
| Control | 18 | 15 | 75 | 90 |

**Effect of Humic acid on *Vigna mungo:***

In the present study, 0.5% of humic acid treated for 15 days. After 45 days the number of leaves growth, height of the plants, total sugars and total protein were evaluated by Anthrone and Lowry's method (Table 6). Control plants were separately maintained. The humic acid helps in nourishing the plants with essential nutrients like organic carbon and nitrogen and the role of fulvic acid is to transport nutrients and thus improve the growth of the plant[13]. *Trichoderma viridi*has effectively converted humic acid from Lignite and similar effect was obtained on sorghum and *Stevia rebaudiana*and the same author also reported that 60% of potassium humate enhances the mushroom growth and 0.2% of humic acid enhancing the growth of *Spirulina plantensis.* Gutierrez-Miceli *et al.* [12] developed liquid formulation using vermicomposting leachate having high concentration of humic acid which enhanced the growth of *Sorghum bicolor* (L.) Moench.

## CONCLUSION:

The report reveals that the following combination 5 g of KOH and 1g of NaOH have enhanced the extraction of humic acid from 5g of Lignite. This extracted humic acid contains all essential nutrients that are useful in improvingthe growth rate of *Vigna mungo.*

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