International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2014): 5.611

Optimization of Physiochemical Conditions for the Production of Catechol-2, 3-dioxygenase from Phenol Degrading Bacteria under Submerged Fermentation

Mukesh Kumar Singh[1], Bhawana[2], Ajay Kumar[3]

[1]GO Organic Biotech, Veena Bhawan, Chaudhary Tola, mahendru, Patna -6 Bihar

[2]Department of Virology, RMRIMS, Patna 7, Bihar

[3]GO Organic Biotech, Veena Bhawan, Chaudhary Tola, mahendru, Patna -6 Bihar

Abstract: ***Phenol and its components are extremely toxic and can easily be found in different industrial sewage such as oil refinery, petrochemical industry and mines, especially collier and chemical factories and one of most important pollutant of the environment (especially, water). Many bacteria are capable of utilising phenol via Catechol pathway to yield energy therefore our, efforts have been done to find the most efficient microbe which can be used for the bioremediation of phenol. In this study Phenol degrading bacteria were isolated by using Bushnell Haas Agar. Among the isolated bacteria, the efficient organism is identified by using phenol degradation assay. Strain BP1 was found to be most efficient in phenol degradation.) was the most potent phenol degrading bacteria showing complete degradation of 600mg/liter of phenol within 24hours. The isolate showed maximum growth in 600mg/liter of phenol and was found to tolerate and degrade even as high as 1000mg/liter of phenol. Thereafter strain BP1 is biochemically characterized and optimized the physiochemical conditions for the production of catechol-2,3-dioxygenase of Strain BP1 under submerged fermentation. We use the Response Surface Methodology (RSM) to optimize the culture condition for the production of phenol degrading enzyme catechol-2,3-dioxygenase to elucidate the optimum level of the most significant parameters for catechol-2,3-dioxygenase production, with minimum effort and time.***

Keywords: Phenol, catechol-2, 3-dioxygenase, Bacteria, Response Surface Methodology

1. Introduction

In the ecosystem, continuous and repeated exposure to xenobiotic compounds provides selective advantage for the evolution of degradative pathway in microorganisms. However, because of the enhancement of microbial degradation of many chemicals, the efficacy of several aromatic compounds including phenolic compounds and pesticides has been reduced [1]. In particular, loss of insecticidal activities has been reported in soils that have received continuous applications, resulting in the enhanced degradation of these compounds by soil microorganisms [2]. Isolation of phenolic compound degrading microorganisms is important to determine the mechanism of process of microbial metabolism, mechanism of enzyme/gene evolution and use of microbes for the detoxification of polluted environment. Many microorganisms that utilize phenol as a sole source of energy have been isolated [3, 4, and 5]. Characteristics of microorganisms such as their small size, ubiquitous distribution, high specificity, surface area, potentially rapid growth rate and unrivalled enzymatic and nutritional versatility cast them as recycling agents. Moreover, the diversity of inorganic and organic materials present on Earth match diversity of habitats whose physiochemical characteristics span wide ranges of pH, temperature, salinity, oxygen tension, redox potential, water potential, etc. This distribution of resources between environments gave origin to a selective evolutionary diversification of microorganisms, resulting in an evolved microbial world capable of exploiting all the naturally occurring metabolic resources on Earth [6]. Biodegradation is a metabolic process that involves the complete breakdown of an organic compound altered by microbial activity. The alteration may occur by intra- or extracellular enzymatic attack that is essential for growth of the microorganisms. The attacked substances are used as a source of carbon, energy, nitrogen, or other nutrients or as final electron acceptor. The rate of biodegradation depends on environmental factors, numbers and types of microorganisms present, and the chemical structure of the target compound [6]

In this study we have isolated the phenol degrading bacteria from oil polluted soil samples and also optimized the physiochemical conditions for the production of catechol-2, 3-dioxygenase from most efficient phenol degrading bacteria under submerged fermentation. This is a novel approach for physiochemical optimization for fermentation condition of phenol degrading bacteria isolated from soil.

2. Material and Methods

Isolation of Bacterial strains and colony characterization
Samples were collected from various places which were suspected to be contaminated with hydrocarbons or other xenobiotic compounds like pesticides etc. following were the sample sources. Sample 1 (S1)- soil collected from a field beside the soya factory in Patna., Sample 2 (S2)- soil from auto market (Patna) Sample 3 (S3)- oil spilled in automobile

market (Patna) Sample 4 (S4)- soil from pesticide treated fields (Samastipur) Sample 5 (S5)- soil taken from alcohol industry (Mokama, Patna) Sample 6 (S6)- soil collected from behind chemistry dept. (Patna university, Patna) Sample 7 (D)- waste effluent collected from RCF (Rashtriya chemical and fertilizer plant), Baurini, Begusarai 1 gram of the soil sample was diluted with 9ml of distilled water, centrifuged and 1ml of the supernatant was inoculated in 100ml of Sterile Bushnell haas broth (with 200mg of phenol per litre) in 250ml flask. The flasks were incubated at 30^0 Celsius for one week at 120 rpm for first enrichment. After one week 10ml from these flasks were inoculated in fresh sterile liquid media and kept under same conditions for second enrichment. Similarly third enrichment was also done and loop full from these flasks were streaked on sterile Bushnell haas agar plates to obtain pure isolated colonies. These plates were kept at 30^0 Celsius for one week. After one week all the plates showed growth of colonies with various colony characteristics. The single isolated colonies were again restreaked on sterile fresh media plates. Sample 1 showed the presence of eight different bacterial colonies, four in sample 2, no colonies were obtained with sample 3 where as three different colonies were obtained from sample 4. Sample 5 showed the presence of single colony with star shaped appearance, sample 6 showed the presence of three different colonies. Sample 7 showed the presence of five different colonies. These colonies were again streaked on sterile Bushnell has solid media containing 800mg phenol/litre. Plates were kept at 30^0 Celsius for one week and it was observed that very few isolates were successful to tolerate 800mg phenol/litre of media. These isolates were selected for further studies. These isolates were named as follows- BP1, BP2, BP3, BP4, BP5a , BP5b. Gram staining of the isolates were performed along with observing colony characteristics. These isolates were then streaked on fresh sterile BHM plates and used for further tests.

Assay of phenol elimination by isolated bacteria

For evaluating phenol elimination with degrading bacteria, Gibes method was used. In this method, gibes indicator or 2,4- dichloroquinone-4-chloroimide was used, which reacted with phenol and produced a blue colour compound. For assay, after centrifuging media, 150 µl of media supernatant (pH = 8) was mixed with 30 µl of NAHCO3 (pH = 8). Then, 20 µl of gibes indicator (1 mg/l) was added to the mixture, vortexed and kept for 15 - 45 min at room temperature with thermo mixer and finally, the mixture absorbance was read at 630 nm. A Catechol 2,3 dioxygenase producing bacteria was isolated from the soil sample of auto market patna and named it BP1 strain. BP1 was gram +ve with cocci shaped which could utilize lactose and urea but was –ve to citrate. The strain was subcultured at interval of 4-5 weeks and routinely maintained on Bushnell's Hass medium.

Catechol 2,3 dioxygenase enzyme assay

To attain required cell biomass for enzymatic assay bacterial cells were harvested by centrifuging at 10,000 g for 10 min at 4^oC. Cell pellet was washed twice with saline and resuspended in 10 mM Tris-Hcl buffer (pH 8). This cell suspension was homogenized using sonicator for 10 min. debris was removed by centrifugation at 20,000 g at 4°C for 20 min. The supernatant was used for enzyme assay. Reaction mixtures (3 ml) in 50 mm KH2PO4:K2HPO4 buffer pH 7.2 containing 1 mmol catechol were equilibrated at 55°C before adding the cell extract (100 ml). The increase in absorbance at 375 nm caused by the formations of the reaction product 2-hydroxymuconic semialdehyde was monitored [7]. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 mmol 2-hydroxymuconic aldehyde per minute at 55°C.

Residual phenol determination:

Put 20 µl samples, a Gallic acid calibration standard or blank into 1 cm, 2ml plastic or glass cuvette. Add 1.58 ml water followed by 100 µl folin ciocalteau reagent. Mix thoroughly by pipeting or inverting and incubate to 8 minute. Add 300 µl sodium carbonate solutions, mix and incubate 2 hours at room temperature. Measure sample absorbance at 765 nm.

Submerged fermentation and RSM Experimental Design and Statistical Analysis:

To achieve enough amounts of cells, isolates were grown in nutrient agar supplemented with phenol which act as carbon and energy source. Then inoculum from nutrient agar plate was transferred to freshly prepared 100 ml minimal salts medium. All growth experiments were run in 250 ml Erlenmeyer flasks containing 100 ml of medium at 30 degree Celsius on a rotary shaker at different speeds according to experiment. A Box- Behnken [8] factorial design was used in the optimization of culture conditions for the production of catechol 2, 3 dioxygenase enzyme. Four factors and five level face centred cube design requiring a total of 29 experiments were adopted in this study. The independent variables studied were pH (X1), Agitation speed (X2, rpm), Incubation time (X3, hr) and Phenol (X4, mg). The response (dependent variable) was Catechol 2, 3 Dioxygenase activities (U per ml). Each independent variable was studied at three coded levels (-1, 0, +1). The minimum and maximum level of each independent variable and the experimental design with respect to their coded and uncoded levels are presented in Table 3. The relation between the coded values and actual values were described as in the following equation

$$Xi = \frac{xi - x0}{\Delta xi}$$

Where Xi is the independent variables coded value, xi is the independent variables actual value, x0 is the independent variable actual value on the center point and Δxi is the step change value. The second order model used to fit the response to the independent variables is shown in equation

$$Y = \beta_0 + \Sigma\beta i\, xi + \Sigma\beta ii\, x^2 i + \Sigma\beta ij\, xixj$$

Where Y is the response (enzyme activity); β_0 βi, βii and βij are regression coefficient for intercept, linear, quadratic and interaction terms, respectively and xi and xj are independent variables. A second order regression analysis of the data was carried out to get empirical model that define response in terms of the independent variables. Analysis of variance (ANOVA) was performed in coded level of variables to study the effects of independent variables to study the effects of independent variables. The 3-D graph were generated to understand the effect of selected variables individually and in

combination to determine their optimum level for maximal production of catechol 2, 3 dioxygenase.

3. Results and Discussion

Screening of most efficient phenol degrading bacteria

Absorbance reading of phenol assay performed to estimate the residual phenol left after pure isolates were inoculated in sterile Bushnell hass liquid media containing 600mg of phenol/liter of the media. The readings were taken after 24hours of incubation at 600nm. Strain BP1 (Code Name) was the most potent phenol degrading bacteria showing complete degradation of 600mg/liter of phenol within 24hours. The isolate showed maximum growth in 600mg/liter of phenol and was found to tolerate and degrade even as high as 1000mg/liter of phenol. Strain BP1 was gram +ve with cocci shaped which could utilize lactose and urea but was –ve to citrate.

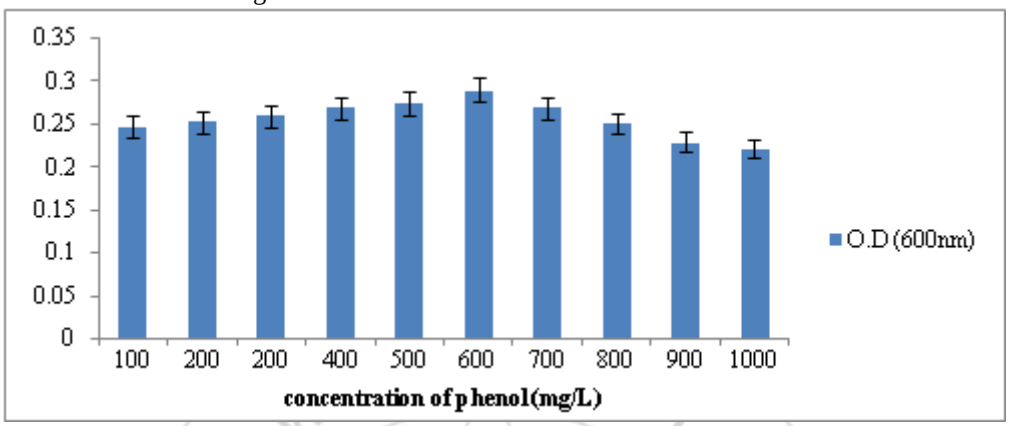
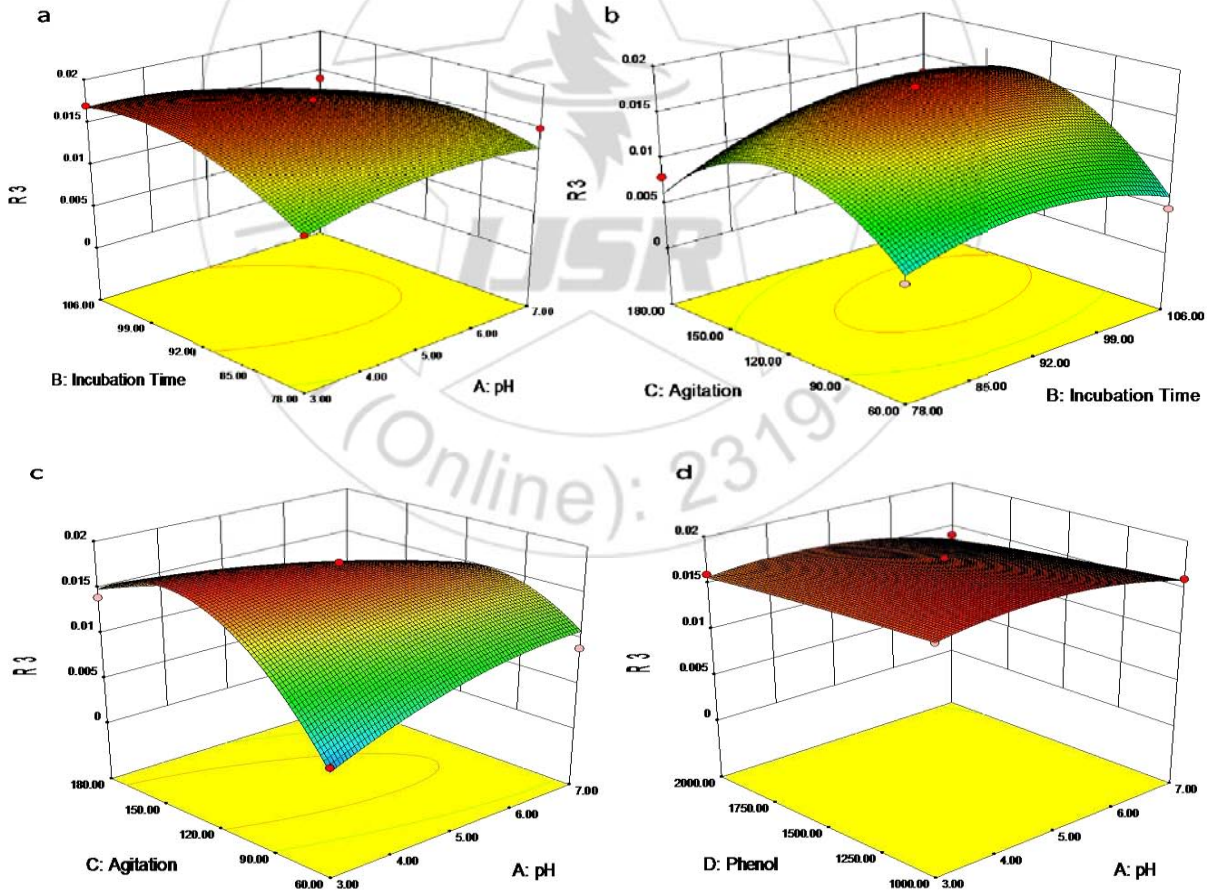


Figure 1: Growth of strain BP1 at different concentration of phenol

Physical optimization of culture condition using RSM



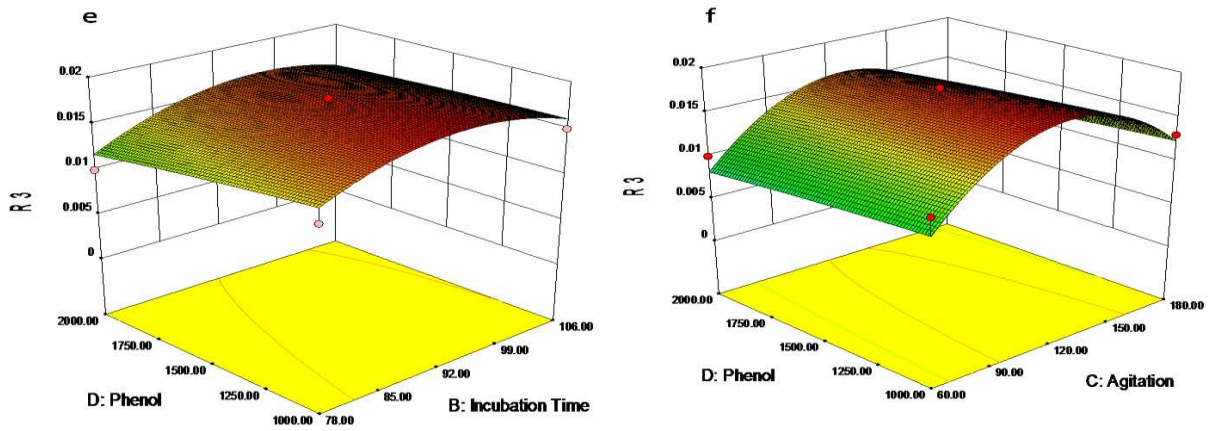
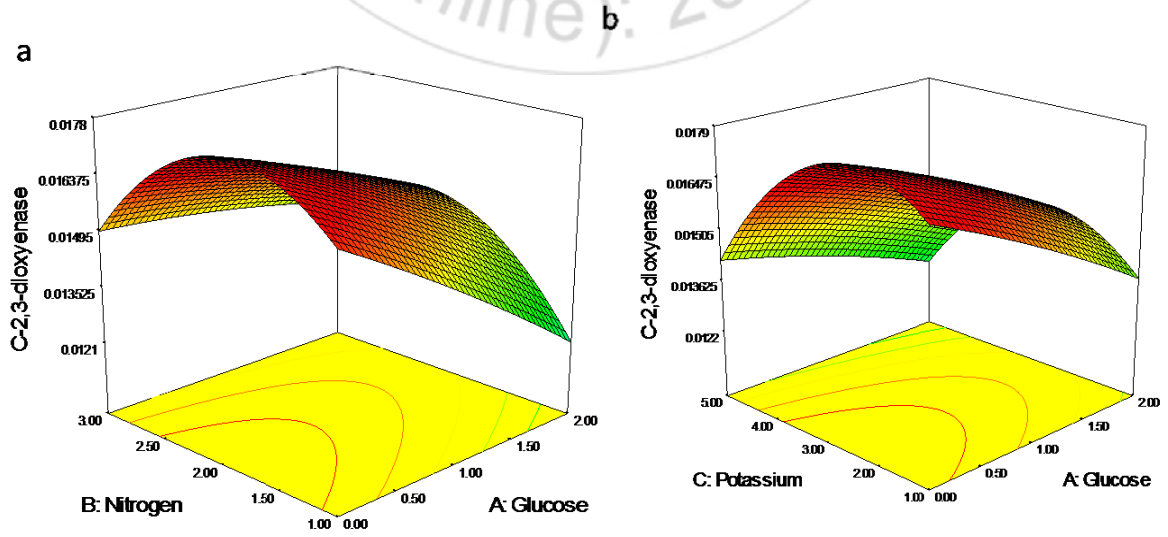


Figure 2: (a) Effect of pH and incubation time on the production of Catechol 2, 3 dioxygenase. Other variable was held at zero level (coded), (b) effect of pH and agitation on the production of catechol 2, 3 dioxygenase. Other variable was held at zero level (coded). (C) Effect of pH and Phenol on the production of Catechol 2,3 dioxygenase. Other variable was held at zero level (coded), (d) effect agitation and incubation time on the production of catechol 2, 3 dioxygenase. Other variable was held at zero level . (e) Effect of Phenol and incubation time on the production of Catechol 2,3 dioxygenase. Other variable was held at zero level (coded), (f) effect of Phenol and agitation on the production of catechol 2,3 dioxygenase. Other variable was held at zero level (coded).

Box-Behnken [8], factorial design was used in optimization of cultural conditions for saccharification of glucose. 29 experiments were adopted in the study. The independent variables studied were pH, incubation time, agitation speed and Phenol whereas the dependent variable was Catechol 2, 3 dioxygenase. Each variable was studied at three coded levels (-1, 0,+1)The three-dimensional (3-D) response surfaces (Fig. 2 a-f) were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum value of each factor for maximum Catechol 2,3 dioxygenase production . .The three-dimensional plots (Fig. 2(a)) show that the increase in incubation time and pH cause an increase in the Catechol 2,3 dioxygenase production to optimum values of 93 hr and 5 pH respectively. The three-dimensional plots (Fig. 2(b)) show that the increase in agitation speed and pH cause an increase in the Catchol 2,3 dioxygenase production to optimum values of 125 rpm and 5.5 pH respectively, whereas further increase in pH and agitation speed decreases the enzyme production. The three-dimensional plots (Fig. 2(c)) show that the increase in pH and Phenol cause an increase in the Catechol 2,3 dioxygenase production to optimum values of 5 pH and 1500 mg/l respectively. The three-dimensional plots (Fig. 2(d)) show that the increase in incubation time and agitation speed cause an increase in the Catechol 2,3 dioxygenase production to optimum values of 92 hr and 120 rpm respectively.further increase in incubation time and agitation speed decreases the enzyme production. The three-dimensional plots (Fig. 2(e)) show that the increase in Phenol and incubation time cause an increase in the Catchol 2,3 dioxygenase production to optimum values of 1500 mg/l and 92 hr respectively, whereas further increase phenol and incubation time decreases the enzyme production. The three-dimensional plots (Fig. 2(f)) show that the increase in agitation speed cause an increase in the Catechol 2,3 dioxygenase production to optimum values of 120 rpm . whereas further increase in agitation speed decreases the enzyme production.

Chemical optimization of culture condition using RSM



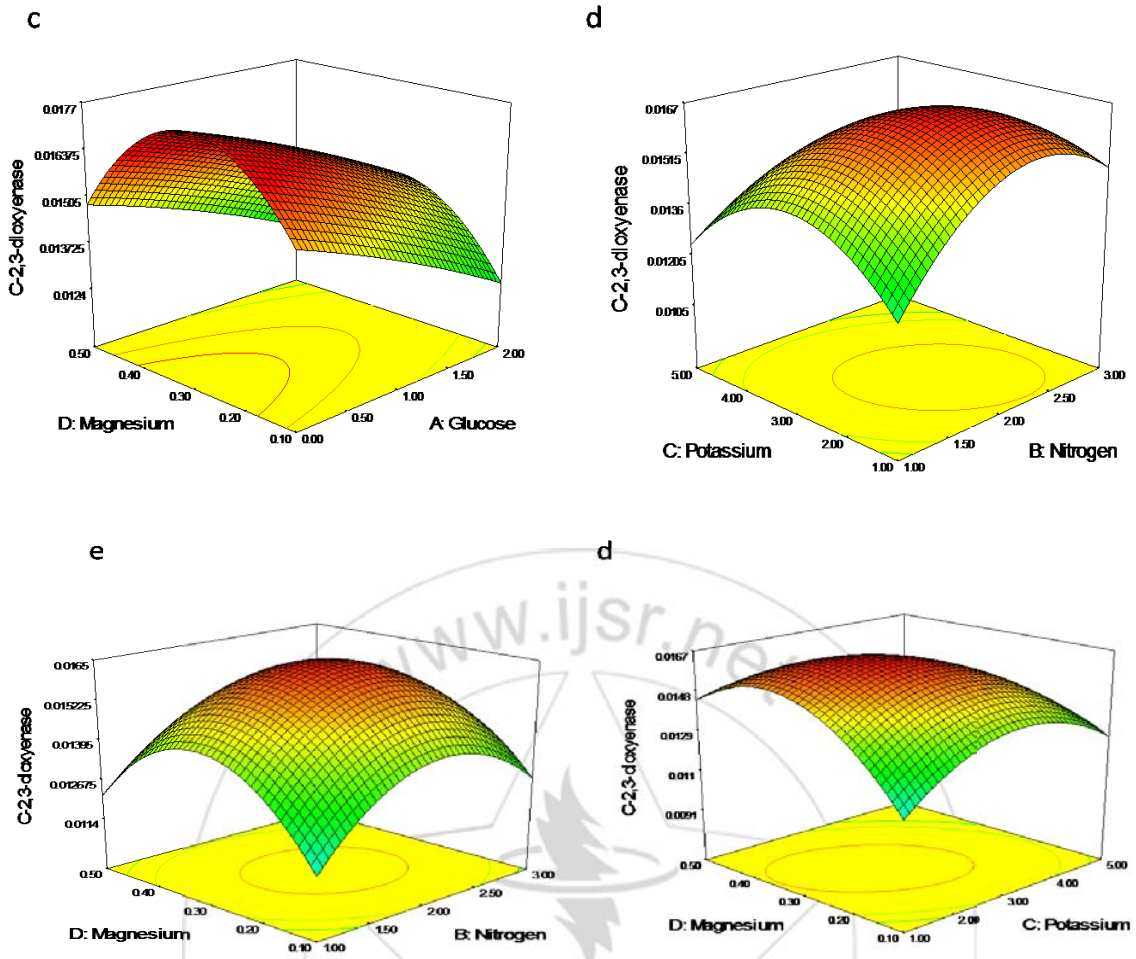


Figure 3: (a) Effect of nitrogen and glucose on the production of Catechol 2,3 dioxygenase. Other variable was held at zero level (coded), (b) effect of potassium and glucose on the production of catechol 2,3 dioxygenase. Other variable was held at zero level (coded). (C) Effect of magnesium and glucose on the production of Catechol 2,3 dioxygenase. Other variable was held at zero level (coded), (d) effect of potassium and nitrogen on the production of catechol 2,3 dioxygenase. Other variable was held at zero level (coded). (e) Effect of magnesium and nitrogen on the production of Catechol 2,3 dioxygenase. Other variable was held at zero level (coded), (f) effect of magnesium and potassium on the production of catechol 2,3 dioxygenase. Other variable was held at zero level (coded).

Box-Behnken [8] factorial design was used in optimization of cultural conditions for Catechol 2,3 dioxygenase productions.29 experiments were adopted in the study. The variables studied were glucose, nitrogen potassium and magnesium. Each variable was studied at three coded levels (-1,0,+1) The three-dimensional (3-D) response surfaces (Fig. 3a-f) were plotted on the basis of the model equation to investigate the interaction among variables and to determine the optimum concentration of each factor for maximum catechol 2,3 dioxygenase production . The three-dimensional plots (Fig. 3(a)) show that the increase in nitrogen increases and glucose decreases enzyme production with optimum value of 2g/l of nitrogen. where further increase in nitrogen decreases the enzyme production. The three-dimensional plots (Fig. 3(b)) show that the increase in potassium increases and glucose cause decreases in the enzyme production to optimum values of 3g/l of potassium. further increase in potassium decreases the enzyme production. The three-dimensional plots (Fig. 3(c)) show that the increase in magnesium increases and glucose decreases the enzyme production to optimum value of 0.30 g/l. where further increase in magnesium decreases the enzyme production. The three-dimensional plots (Fig. 3(d)) show that the increase in potassium and nitrogen cause an increase in the enzyme production to optimum values of 3 g/l and 2.2g/l respectively, where further increase in nitrogen and potassium decreases the enzyme production. The three-dimensional plots (Fig. 3(e)) show that the increase in magnesium and nitrogen cause an increase in the enzyme production to optimum values of 0.30g/l and 2.2g/l respectively. further increase in magnesium and nitrogen decreases the enzyme production. The three-dimensional plots (Fig. 3(f)) show that the increase in magnesium and Potassium cause an increase in the enzyme production to optimum values of 0.30g/l and 3.5g/l respectively, whereas further increase in magnesium and potassium decreases the enzyme production.

Till date several efforts have been made to isolate new and efficient microbe having the ability to degrade phenol and its compounds in an efficient way. The primary purpose of this project was to isolate efficient bacterial strains for

bioremediation of phenol. The phenol degradation ability of isolate BP1 was checked up to 1000mg/l concentration. The isolate was able to degrade the given amount of phenol within 24hours, which is very much desired for the microbe to be used for bioremediation of such compounds. Different methods have been used for the elimination of phenol, but the use of bacteria can be one of the cheaper, efficient and secure methods. Bacteria with rapid reproduction in the presence of phenol and its compound have shown extraordinary ability in phenol elimination. From here we conclude the Response Surface Methodology which was performed to optimize the culture condition for the production of phenol degrading enzyme by optimization tool has helped to locate the optimum level of the most significant parameters for phenol degrading enzyme production, with minimum effort and time.

References

[1] Rani, N.L. and Lalitha Kumari, D. Degradation of methyl parathion by *Pseudomonas putida*. Can. J. Microbiol, 1994; 40:1000-1006
[2] Chaudhary, G., Ali, A. and Wheeler, W. Isolation of a methyl parathion-degrading *Pseudomonas sp.*,that possesses DNA homologous to the opd gene from a *Flavobacterium sp.* App. Environ. Microbiol, 1988; 54(2):288-293.
[3] Sethunathan, N. and Pathak, M.D. Increased biological hydrolysis of diazinon after repeated application in rice paddies. J. Agri. Food Chem, 1972; 20: 586-589.
[4] Rangaswamy, V. and Venkateswarlu, K. "Activities of amylase and invertase as influenced by the application of monocrotophos, quinalphos, cypermethrin and fenvalerate to groundnut soil," Chemo, 1992**;** 25: 525–530.
[5] Siddavatam, D., Khajamohiddin, S., Manavathi, B., Pakala, S.B. and Merrick, M. Transposon-like organization of the plasmid-borne organophosphate degradation (opd) gene cluster found in *Flavobacterium sp.* Appl. Environ. Microbiol, 2003; 69: 2533–2539
[6] Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D. and Walter, M.V. Manual of Environmental Microbiology. Washington, D.C. Amer. Soci. Microbiol, 1997.
[7] M. Gary et al.(1981). Inhibition of Catechol 2,3-Dioxygenase from Pseudomonas putida by 3-Chlorocatechol.Applied and Environmental Microbiology.41,1159-1165
[8] Box G.P. and Behnken D.W. (1960). Some new three levels design for study of quadratic variable Technometrics. 2, 456-475.

Author Profile

Mukesh Kumar Singh received his B.Sc. and M.Sc. degrees in Zoology and biochemistry respectively from Patna university Patna in 2010 and 2012, respectively. He is now working in GO Organic biotech as research associate (RA)

Bhawana received her B.Sc. and M.Sc. degrees in Biotechnology from Vinoba Bhave University, Hazaribag in 2011 and 2013, respectively. He is now working in RMRIMS, Patna as Project Staff.

Ajay Kumar received his B.Sc. degree in Zoology from Patna University, Patna in 2010 and M.Sc. degrees in Biotechnology from GJUS&T, Hisar, in 2012. He is now working in GO Organic biotech as Senior Scientist.