International Journal of Current Advanced Research
ISSN: O: 2319-6475, ISSN: P: 2319-6505, Impact Factor: SJIF: 5.995
Available Online at www.journalijcar.org
Volume 6; Issue 10; October 2017; Page No. 7082-7091
DOI: http://dx.doi.org/10.24327/ijcar.2017.7091.1077

Research Article

OPTIMIZATION THE DEGRADATION OF CHLOROXYLENOL BY FREE AND IMMOBILIZED KLEBSIELLA PNEUMONIAE D2

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A R T I C L E I N F O

Article History:

Received 14th July, 2017
Received in revised form 19th August, 2017 Accepted 12th September, 2017
Published online 28th October, 2017

Key words:

Choroxylenol, degradation, optimization, immobilization

A B S T R A C T

Two samples from phenol contaminated soil and waste water were collected for the isolation of bacteria degrading chloroxylenol. Out of eleven isolates, isolate D2 was the most promising showing a degradation efficiency of 19.9%. The selected isolate was identified using 16S rDNA analysis as Klebsiella pneumoniae D2. Statistical designs were applied to optimize the medium composition and cultural conditions in favor of increasing the degradation efficiency of K. pneumoniae D2.The Plackett-Burman design was applied to determine the significant factors affecting chloroxylenol degradation. The degradation efficiency increased to 30.56%.Box-Behnken design was adopted to further investigate the mutual interactions between the variables and to identify their optimal values that would generate maximum chloroxylenol degradation. Under the optimized medium composition and culture conditions, K.pneumoniae D2 degraded 55.7% chloroxylenol after 24 hrs. Bacterial cells were adsorbed on different solid supports. The results showed that the degradation efficiency increased up to 88.3% on using 20 cubes of polyurethane foam.The degradation efficiency decreased by 10% on reusing adsorbed cells of K.pneumoniae D2 on polyurethane foam, up to 10 cycles. Immobilization accelerated the degradation. The time was reduced to 9 hrs reaching a degradation of 88.3% compared to free cells.

INTRODUCTION

Organic pollutants represent a potential group of chemicals that can be seriously hazardous to human health (Nair *et al*., 2008; Liu *et al*., 2009). It is the active ingredient in Dettol, which is a household disinfectant solution that is widely used in the United Kingdom and in a number of common wealth countries. Chloroxylenol is a very effective antimicrobial agent against many Gram+ and Gram– bacteria, fungi, and viruses (Goddard and McCue, 2001) that cause infections since it disrupts the proton gradient of the cell membrane necessary for the bacteria to produce adenosine triphosphate (ATP). The ATP deficiency results in cell death due to starvation (Wilson and Mowad, 2007). Chloroxylenol also oxidizes the structure of the cell and impedes the nutrients from passing through the cell wall, causing a loss of normal enzyme, so it has unique antiseptic properties Based on physical and chemical properties, chloroxylenol released to the environment will partition between the atmosphere, water and soils. It has low volatility and low water solubility, but will readily sorbs to soils and sediments (Toxnet, 2014).

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Statistical planned experiments are better to minimize the error, to determine the effect of parameters and to achieve results in an economical manner (Abdel-Fattah*et al*., 2005). Statistical design methods are considered as a better methodology for maximum the degradation of many of phenolic compounds (Ghanem *et al*., 2009). Many designs have been used to improve the efficiency of degradation (Zhou *et al*., 2011; Singh*et al*., 2017). Plackett-Burman design provides a fast and effective way to identify the important factors among a large number of variables, thereby, saving time and maintaining convincing information on each parameter (Abdel-Fattah *et al*., 2005). Response surface methodology (RSM), which includes factorial design and regression analyses, helps in evaluating the important factors, building models to study the interactions between the variables or desirable responses (Ghanem *et al*., 2013).

Immobilization of cells onto inert surfaces could offer a higher surface area to facilitate growth of biomass and degradation rate (Davis and Westlake, 1978). Of great interest to bioremediation is the potential of immobilizing microorganisms onto polyurethane foams (PUF), alginate and other matrices to degradehydrocarbons and toxic wastes (Oh *et al*., 2000; Diaz *et al*., 2002). Immobilized microorganisms could degrade these compounds at a higher initial concentration and for a longer period. In addition, these cells

were also protected from harmful effects of toxic wastes. In some of these studies, immobilized cells could be reused and stored for long periods without losing their degrading abilities. Chloroxylenol is a bactericidal, but many molds and bacteria such as *P.aeruginosa* are highly resistant (Ihejirika *et al*., 2014). However, Chloroxylenol degradation using the fungus *Aspergillus niger* had found rapid rates of biodegradation, with 100% removal within 6 days (Ghanem *et al*., 2013). Surprisingly, its mechanism of action has been little studied despite its widespread use over many years and there is no report focusing on biodegradation of chloroxylenol by *Klebsiella pneumoniae*D2.

The main objective of this study is to isolate a local bacteria capable of degrading chloroxylenol and maximize the degradation by statistical designs and immobilization of cells.

MATERIALS AND METHODS

Materials

Microorganisms

The bacterium used in the present study was locally isolated from phenol-contaminated waste water (sewage sludge). It was identified by 16S rDNA sequencing as *Klebsiella pneumoniae*D2 strain.

Media

Modified Minimal synthetic medium (MMSM) used in this work contained in g/l (Glucose, 2.0; $(NH_4)_2SO_4$, 0.5; $(NH_4)NO_3$, 1.0; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1.0; KH_2PO_4, 0.5; NaCl, 0.5; $CaCl_2$, 0.02)(**Ghanem *et al*., 2009).]**. Nutrient agar medium contained in g/l (Peptone, 5; Yeast extract, 5; Sodium chloride, 3) was used for subculture of bacterial strain.The pH of the medium was adjusted to 7.0 with 1N NaOH or HCl prior to sterilization. Sterilization was carried out by autoclaving for 20 min at 121°C. Filter sterilized chloroxylenol was added to the medium after sterilization.

Methods

Collection of samples

Samples from phenol contaminated soil and waste water were collected in sterile bags and transferred immediately to the lab in an ice box.

Preparation of chloroxylenol stock solution

Chloroxylenol (40 mg) was dissolved in 10 ml (5 ml ethanol and 5 ml distilled water) then sterilized by the use of a bacterial filter and kept in a sterile bottle.

Enrichment and isolation of chloroxylenol degrading bacteria

Enrichment has been applied for the isolation of bacterial strains capable of degrading the phenolic compound chloroxylenol (Nagamani *et al*., 2009).Two flasks (250 ml), each containing 100 ml of sterilized MMSM, were supplemented with 2 µg/ml chloroxylenol as the sole carbon source and inoculated by 0.5 gm of soil or 0.5 ml of waste water (sewage) samples. The flasks were incubated in an orbital shaker (160 rpm) at 30 ^oC until growth appeared. The resulted growth was used to inoculate sterilized MMSM supplemented with 4 µg/ml chloroxylenol and enrichment proceeded until growth was observed in presence of 8 µg/ml chloroxylenol. One ml of each sample was plated on solid MMSM supplemented with the same concentration of the chloroxylenol. Eleven colonies were chosen from the plates based on morphological differences. They were purified and subcultured on slants of nutrient agar medium. Cultures were incubated at 30 °C for 24 hrs, stored in the refrigerator at 4 °C. All bacterial isolates were tested for their degradation efficiency of chloroxylenol using the method mentioned later. Only one isolate was selected as the most potent chloroxylenol degrader for further studies.

Phylogenetic analysis

The selected bacterial strain was subjected to phylogenetic analysis using 16S rDNA gene sequence (Das *et al*., 2014).

DNA extraction

Genomic DNA was isolated using the DNA extraction kit (fermentas) and monitored using electrophoresis in 1% agarose gel.

Amplification of 16S rDNA gene

The PCR reaction mixture contained 5 µl of template DNA, 2 µl of reverse primer(5'TACGGYTACCTTGTTACGACT T3'), 2 µl of forward primer (5'AGAGTTTGATCMTGG CTCAG3'), 2 µl of dNTP ,4 µl of $MgCl_2$ (25 mM), 5 µl of PCR buffer (10x) and 1µl Taq polymerase. Distilled water was added to 50µl final volume. The cycling programmer was 95 °C for 5 min, 35 cycles at 95°C for 30 sec, and 72 °C for 2 min, at the end the reaction was incubated at 72°C for 10 min. The 16S rDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16S rDNA region. The PCR mixture consists of 30 picomoles of each primer, 10 ng of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94 ^oC for 1 min, 55 ^oC for 1 min and 72 ^oC for 2 min. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis **(Ausubel *et al*., 1999)** and the remainderwas purified using QIAquick PCR purification reagents (Qiagen). DNA sequences were obtained using an ABI PRISM 377 DNA Sequencer and ABI PRISM BigDye Terminator Cycle Sequencing (Perkin Elmer). The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gel electrophoresis

Gel electrophoresis was carried out using 1 % agarose gel prepared in TAE buffer, the DNA samples were loaded on the gel after mixing with the loading dye. The extracted DNA and PCR products were visualized by UV transilluminator after staining the gel with ethidium bromide (10 mg/ml) for 20 min.

DNA sequencing

PCR products were sequenced using the DNA sequencing facility offered by the U.S.B American Company through SIGMA-Egypt using the above mentioned forward and reverse primers. Partial 16S rDNA gene sequences were analyzed by comparison with the 16S rDNA genes in the GenBank database. The nearest relatives of the sequenced PCR product was obtained by BLAST searches. A phylogenetic tree was built up using Mega 4 program. The 16S rDNA sequence was deposited in GenBank.

Bacterial growth determination

Bacterial growth was determined by measuring the turbidity (optical density) of the culture at λ= 600 nm using a spectrophotometer (OPTIMA SP 300).

Measurement of chloroxylenol

For quantitative estimation of phenol and other phenolic compounds (chloroxylenol), the method described by Martin (1949) and Farag and Abd-Elnaby, (2014) was used throughout the work. This method is based on rapid condensation with 4-aminoantipyrine followed by oxidation with potassium ferricyanide under alkaline conditions to give a red-coloured product. The developed red color was measured immediately at 492 nm.

Plackett-Burman experimental design

Plackett-Burman design is one of the so called "screening designs ". Such designs are traditionally used for identifying important factors from many potential factors**.** In the analysis of this design, usually only main effects are estimated. The Plackett-Burman experimental design, a fractional factorial design (Plackettand Burman, 1946; Yu *et al*., 1997; Amara and Salem, 2010). Was used in this research to reflect the relative importance of various environmental factors on chloroxylenol degradation in liquid cultures.

In this study the selected 11 independent variables among the components of basal medium were investigated. The selected variables included nutritional factors such as glucose, $(NH_4)_2SO_4$, $(NH_4)NO_3$, $MgSO_4.7H_2O$, K_2HPO_4. KH_2PO_4, NaCl, $CaCl_2$ and chloroxylenol concentration, in addition to inoculum size and culture volume.

For each variable, a high (+) and low (-) level were tested. Trial no. 12 represents the basal control. All trials were performed in duplicates and the averages of chloroxylenol degradation observation results were recorded. From main effect results, a near optimized medium was predicted which should give maximum degradation of the desired compound.
The main effect of each variable was determined with the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) /N$$

Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the chloroxylenol degradation percentages in trails where the independent variable (xi) was present in high and low concentrations, respectively, and N is the number of trails divided by 2. The main effect with a positive sign indicates that high concentration of this variable is nearer to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired sample were calculated for determination of variable significance.

Box-Behnken design

In order to describe the nature of response surface in the experimental region and to elucidate the optimal concentrations of the most significant independent variables, a Box-Behnken design (Box and Behnken, 1960; Ghanem *et al*., 2013). was applied, which is a response surface methodology. Factors of highest confidence levels namely; glucose (A), $MgSO_4.7H_2O$ (B), and culture volume(C) were tested in three levels (low, basal, and high) coded (-1, 0, and +1). Accordingly, fifteen treatment combinations were executed.

For predicting the optimalpoint, the following second order polynomial model was fitted to correlate relationshipbetween independent variables and response:

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_{12}+ \beta_{13}X_{13}+\beta_{23}X_{23}+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2$$

Where, Y is the dependent variable (chloroxylenol degradation %), X1, X2 and X3 are the levels of the independent variables; β_0 is regression coefficient at the center point; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are the second order interaction coefficients; and β_{11}, β_{22} and β_{33} are quadratic coefficients. The values of the coefficients were calculated using Microcal Origin 4.1 software and the optimum concentrations were predicted using Microsoft Excel 2000. The quality of the fit of the polynomial model equation was expressed by coefficient of determination, R^2. The optimal value of chloroxylenol degradation was estimated using the solver function of Microsoft Excel tool. Three-dimensional graphical representations were also constructed using Statistica 6.1 software, in order to reflect the effects as well as the interactions of independent variables on the objective.

Immobilization by adsorption

Different solid supports namely pumice, luffa pulp (LP), polyurethane foam (PF), charcoal, ceramic and porcelain were investigated for their ability to adsorb the bacterial cells. The used supports were in the form of particles (about 0.5 cm diameter) in the case of pumice, charcoal, porcelain and ceramic or in the form of small cut cubic pieces of about 0.5 cm length, in the case of LP and PF (Farag *et al*., 2015). The adsorption process was carried out in an orbital shaker (180 rpm). One ml of cell suspension ($O.D_{600}$=0.728) was inoculated into flasks contained from 20 to 35 particles or pieces of the tested solid support and 50 ml of optimized mineral medium. The flasks were then shaked at about 180 rpm. Cell adsorption was monitored at short time intervals by measuring the decrease in the turbidity (OD) of the cell suspension at 600 nm. At the end of the operation, the medium was removed from the flasks leaving the supports loaded with bacterial cells. The desired mineral medium which containing chloroxylenol (as a sole carbon source) was then added and cultivation was proceeded as usual (Agarry and Aremu, 2012).

RESULTS AND DISCUSSION

Selection for the most potent bacterial strain

Chloroxylenol metabolizing bacteria were isolated from phenol contaminated soil and waste water samples in MMSM supplemented with chloroxylenol in upgraded concentrations (2-8 µg/ml) as a sole carbon source. After incubation for 24 hrs, eleven colonies were selected purified, and grown in liquid MMSM supplemented with 8µg/ml chloroxylenol in an orbital shaker (180 rpm) at 30^{o}C. After degradation, the fermentation media were centrifuged at 7000xg for 20 min in a cooling centrifuge and supernatants were used to measure the residual chloroxylenol concentration quantitatively by the spectrophotometric method using 4-aminoantipyrine as color indicator with maximum absorbance of 492nm according to the method of Martin (1994). All experiments were performed in triplicates and the average of the three independent experiments was taken as the result.

The percentage of biodegradation percent for each isolate was investigated as shown in Fig.1 which ranged from 9.6% to 19.9% depending on bacterial species. Isolate D2 showed the highest percentage of biodegradation (19.9%), whereas the lowest percentage (9.6%) was obtained with isolate D10. Bacterial isolate D2 was selected as a most potent chloroxylenol degrader isolate for further studies.

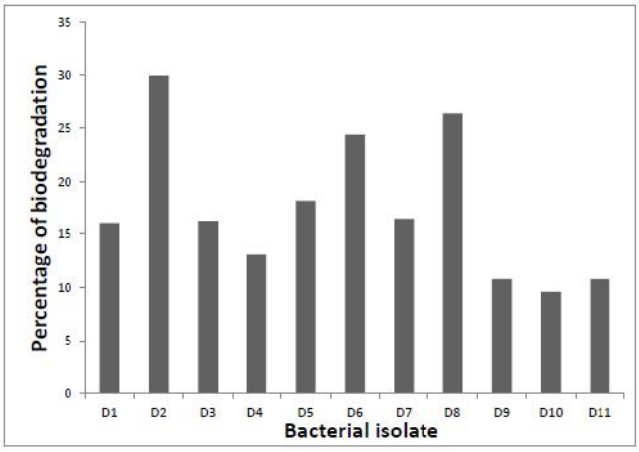

Fig 1 Percentage of chloroxylenol degradation by different bacterial isolates. Bacteria were grown in minimal medium supplemented with 8 µg/ml of chloroxylenol and incubated at 30 °C under shaking condition for 24 hrs.

Identification of isolate D2 using phylogenetic analysis

Partial sequencing of the 1500 bp of 16S rDNA of the bacterial strain was compared with previously database using Blast analysis. The 16S rDNA sequence showed 99% sequence homology to several species of the genus *Klebsiella pneumoniae.* Hence, the strain was identified as *Klebsiella pneumoniae* D2. The 16S rDNA sequence was submitted to the GenBank database under accession number KP859509.1. Bacterial sequences were aligned using MEGA 4 program and a phylogenetic tree (Fig.2) was constructed showing the relative relationship between identified *Klebsiella pneumoniae* D2 and other bacterial species.

Previous studies showed that *Klebsiella* species are capable for phenol degradation. In an early study,(Heesche-Wagner *et al*., 1999).] characterized a *Klebsiella oxytoca* strain that grew on phenol as the only source of carbon and energy. *Klebsiella* sp. strain ATCC13883T capable of degrading carbofuran phenol (2, 3-dihydro-2, 2 dimethyl benzofuran- 7-ol) was also reported. The degradation rates of 20 and 30 mM carbofuran phenol by free and immobilized cells in batch and semi-continuous shaken cultures were compared (Kadakol *et al*., 2011).Several bacterial strains belonging to the species of *Pseudomonas*, *Bacillus*, *Klebsiella*, *Ochrobactrum*, *Rhizobium*, etc. were reported for phenol degradation (Ma *et al*., 2010).Many other genera of gram-negative and also gram-positive bacteria are described as degraders of phenolic compounds, but not limited to, *Acinetobacter*, *Agrobacterium*, *Burkholderia*, *Ralstonia*, *Rhodococcus* (Koutny *et al*., 2003).*Klebsiella oxytoca* C302 was previously reported to be a bacterial degrader of benzoate, catechol, 4-hydroxybenzoate, and protocatechuate (Kim *et al*., 2000). The rate of biodegradation of phenol by *Klebsiellaoxytoca* strain was studied in the nutrient broth and M9 minimal medium. It was found that *K.oxytoca* degraded phenol at elevated phenol concentration where 75% of initial phenol concentration of 100 ppm will degrade within 72 hrs (Shawabkeh *et al*., 2007). Apart from this, many other genera have also been reported for phenol degradation such as *Acinetobacter*, *Agrabacterium*, *Burkholderia*, *Ralstonia* and *Rhodococcus* (Singh *et al*., 2013).

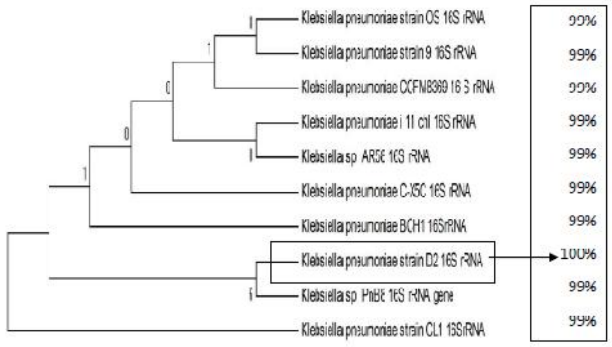

Fig 2 Phylogenetic tree based on 16S rDNA gene sequencing, showing the phylogenetic relationship of bacterial isolate D2 within representative species of the genus *Klebsiella* and reference sequences extracted from the GenBank Database.

Optimization of chloroxylenol degradation by K.pneumoniae D2 using statistical designs

Optimization of culture conditions for maximum degradation was planned via two approaches; The first dealt with evaluating the different medium constituents using the Plackett-Burman design and the second was to optimize the most important factors that significantly affected the degradation process using the Box-Behnken design.

Evaluating the significance of medium constituents using the Plackett-Burman design

The Plackett-Burman design (Plackett and Burman, 1946) provided a fast and effective way to identify the important factors among a large number of variables by saving time and maintaining important information on each parameter. It was applied in this study to reflect the relative importance of different medium constituents with respect to degradation of chloroxylenol compound.

The chosen levels of 11 variables are presented in Table 1. All experiments were performed in duplicates and the results (average of the observations) were presented also in Table2. The results indicate a wide variation in chloroxylenol degradation ranging from 4.7% to 30.56%, in the 12 trials. The variation suggests that the optimization process was important for improving the degradation efficiency of chloroxylenol. The results reveal that the levels of factors at trial (3) were the best achieving a degradation of 30.56%.

The main effect of each variable upon chloroxylenol degradation was estimated as the difference between both averages of measurements at the high level (+1) and the low level (-1) of that factor. The main effect results of each medium component are presented graphically in (Fig.3). Main effect analysis revealed that, seven out of the nine variables ($(NH_4)_2SO_4$, $MgSO_4.7H_2O$, NaCl, K_2HPO_4, culture volume, $CaCl_2$ and chloroxylenol) included in this study were found to have a positive influence on chloroxylenol degradation, indicating that the higher concentrations of these variables are ideal for enhancing chloxylenol degradation , whereas glucose, KH_2PO_4, inoculum size and $(NH_4)NO_3$ had negative effect towards degradation process, indicating that lower concentrationsof these factors in experimental range were favourable for increasing chloroxylenol reduction (Fig.3).

Table 1 Screening of factors affecting chloroxylenol degradation by *K.pneumoniae*D2 and their levels in the Plackett-Burman experiment design

Factors	Symbols	Levels (g/l)		
		High level 1	Basal level 0	Low level -1
Glucose	G	3.0	2.0	1.0
$(NH_4)_2SO_4$	AS	0.75	0.5	0.25
$(NH_4)NO_3$	AN	1.5	1.0	0.5
$MgSO_4.7H_2O$	Mg	0.75	0.5	0.25
KH_2PO_4	KMP	0.75	0.5	0.25
K_2HPO_4	KDP	1.5	1.0	0.5
NaCl	Na	0.75	0.5	0.25
$CaCl_2$	Ca	0.03	0.02	0.01
Inoculum size (ml/flask)	I.S	1.5	1.0	0.5
Culture volume (ml/flask)	CV	150	100	50
Chloroxylenol con (µg/ml)	Ch	12.0	8.0	4.0

Table 2`Placket-Burman experimental design matrix with coded and levels of independent variables affecting chloroxylenol degradation by *K.pneumonia* D2 with degradation % as response

Trials	*Independent variables*											*Degradation (%)*
	G	*AS*	*AN*	*Mg*	*KMP*	*KDP*	*Na*	*Ca*	*I.S*	*C.V*	*Ch*	
1	+	+	-	+	+	+	-	-	-	+	-	9.85
2	+	-	+	+	+	-	-	-	+	-	+	4.70
3	-	+	+	+	-	-	-	+	-	+	+	30.56
4	+	+	+	-	-	-	+	-	+	+	-	10.17
5	+	+	-	-	-	+	-	+	+	-	+	9.00
6	+	-	-	-	+	-	+	+	-	+	+	15.30
7	-	-	-	+	-	+	+	-	+	+	+	30.00
8	-	-	+	-	+	+	-	+	+	+	-	12.80
9	-	+	-	+	+	-	+	+	+	-	-	18.90
10	+	-	+	+	-	+	+	+	-	-	-	14.40
11	-	+	+	-	+	+	+	-	-	-	+	17.59
12	-	-	-	-	-	-	-	-	-	-	-	9.70

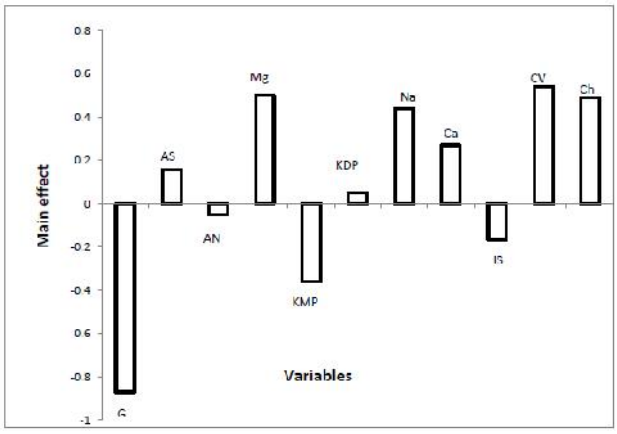

Fig 3 The main effect of the medium constituents on degradation of chloroxylenol compound according to the Placket-Burman experimental results

Variables with the confidence levels greater than 90% were considered as significant. Glucose was considered the most significant factor (95% confidence level), followed by culture volume at 77%, and $MgSO_4.7H_2O$ at 70%. The confidence levels of other variables were below 70%; hence, their individual effects were negligible.

The range of the examined levels of variables (Table 3), which based on t-value showed that negative level of glucose, positive level of $MgSO_4.7H_2O$ and culture volume are significant at 95%, 70% and 77% respectively. $(NH_4)No_3$, Inoculum size, KH_2PO_4 and glucose had negative effect on chloroxylenol degradation, while $MgSO_4.7H_2O$, chloroxylenol concentration, K_2HPO_4, NaCl, $CaCl_2$, $(NH_4)_2SO_4$ and culture volume had a positive effect. The necessary statistical analysis shown in Table 3. It was apparent that the most significant three factors for chloroxylenol degradation were glucose (1 g/l), culture volume (150 ml), and $MgSO_4.7H_2O$ (0.75 g/l). The data collected after 24 hrs showed that maximum phenol removal efficiency of 30.56 % was accessible at 0.1% of glucose concentration. This might be due to the fact that glucose acts as a growth substrate in presence of phenol due to its simple structure as compared to phenol. But it was decreased to 4.7% with increasing glucose concentration to 0.3%. Glucose addition up to a specific low concentration could improve the degradation rate, but impeded the degradation process at higher concentrations. The predicted medium composition and culture conditions to be near optimum, which resulted from application of Plackett-Burman statistical design was (g/l): $(NH_4)_2SO_4$, 0.75; $(NH_4)NO_3$, 1.5; $MgSO_4.7H_2O$, 0.75; K_2HPO_4, 0.5; KH_2PO_4, 0.25; NaCl, 0.25; pH7; inoculums size, 0.5ml; chloroxylenol, 12 µg/ml ; $CaCl_2$, 0.03 ; glucose, 1; volume culture/250 ml Erlenmyer flask, 150 ml and agitation, 180 rpm at 30 ^oC for 24 hrs.

Glucose supports growth and the addition of this conventional carbon source substantially increases cell density (Loh and Wang, 1998). Similarly, the addition of non-toxic compounds may stimulate the viability of cells and enhance degradation (Topp and Hanson, 1988).

The presence of glucose in the culture medium increased the tolerance of the organisms to high phenol concentrations by providing a good source readily metabolisable carbon to support cell growth. Hence, it was concluded that glucose on minimal salts medium supported phenol degradation (Lakshmi and Sridevi, 2009). However, with the glucose concentration further increased, the degradation rate of phenol decreased and dropped below the rate achieved in the absence of glucose. That may be a result of catabolite repression by glucose. The presence of glucose can inhibit the utilization of the target substrate (Basha *et al*., 2010). Therefore, the presence of low concentration of glucose is beneficial for the phenol biodegradation (Zhang *et al*., 2013).

Magnesium sulphate (hydrated) at its higher level (0.75g/l) proved to be a significant factor. Magnesium (principal inorganic cation in cells and constitutes approximately 1 % of the dry weight of the microbial cell) plays a role as a cofactor for many enzymatic reactions or cell wall components; it stimulate enzyme reactions associated with a synthesis of cell materials (Cameotra and Singh, 2008).

Aerobic microorganisms utilize oxygen primarily as the terminal electron acceptor for aerobic respiration. Also, molecular oxygen is required as a co-substrate for the microbial degradation of wide variety of organic chemicals; including hydrocarbons and aromatic ring compounds (Basha *et al*., 2010).

Chloroxylenol degradation by immobilized cells of K.pneumoniae D2

Immobilized cells have higher activity, higher cell density and longer stability than free cells. Hence, the immobilized cells have the potential to degrade toxic chemicals at higher concentration as compared to the freely suspended cells (Wang *et al*., 2014). Immobilization of microorganisms protects the microorganisms from being damaged as well as maintains continuous cell growth and phenol degradation. These advantages have encouraged researchers to investigate the application of immobilized cells in the biodegradation of toxic compounds, such as phenol, pyridine, dibenzothiophene, and quinoline (Li *et al*., 2005). Immobilization of microbial cells on inert supports was widely used in bioprocess wastewater

Table 3 Statistical analysis of Plackett-Burman design

	Ch		Mg		KMP		KDP		G		Na		Ca		A.S		A.N		I.S		C.V	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Variable	4.7	9.85	9.85	10.17	9.85	30.56	9.85	4.7	9.85	30.56	10.17	9.85	30.56	9.85	9.85	4.7	4.7	9.85	4.7	9.85	9.85	4.7
	30.56	10.17	4.7	9	4.7	10.17	9	30.56	4.7	30	15.3	4.7	9	4.7	30.56	15.3	30.5	9	10.17	30.56	30.56	9
	9	12.8	30.56	15.3	15.3	9	30	10.17	10.17	12.8	30	30.56	15.3	10.17	10.17	30	10.17	15.3	9	15.3	10.17	18.9
	15.3	18.9	30	12.8	12.8	30	12.8	15.3	9	18.9	18.9	9	12.8	30	9	12.8	12.8	30	30	14.4	15.3	14.4
	30	14.4	18.9	17.59	18.9	14.4	14.4	18.9	15.3	17.5	14.4	12.8	18.9	17.59	18.9	14.4	14.4	18.9	12.8	17.59	30	17.59
	17.59	9.7	14.4	9.7	17.59	9.7	17.59	9.7	14.4	9.7	17.59	9.7	14.4	9.7	17.59	9.7	17.59	9.7	18.9	9.7	12.8	9.7
Mean	17.85	12.47	17.9	12.42	14.19	17.14	15.44	14.89	10.4	19.91	17.56	12.77	16.66	14.67	16.01	14.3	14.86	15.46	14.26	16.07	18.11	12.21
Main effect	5.38		5.48		-4.95		0.55		-9.51		4.79		2.99		1.71		-0.6		-1.81		5.9	
t- value (at 95 % significance level tα = 2.2)	1.17		1.19		- 0.83		0.11		- 2.4		1.03		0.62		0.34		- 0.12		-0.37		1.306	
Significance level			70%						95%												77%	

Optimization of chloroxylenol degradation applying the Box-Behnken design

The present strategy of medium improvement is to determine the optimal level of each key independent variable, as identified by the Plackett- Burman experiment. The Box-Behnken design, a response surface methodology is widely applied and reported in many studies [Box and Behnken, 1960; Hank *et al*., 2010).

The most significant variables (glucose (X1), $MgSO_4.7H_2O$ (X2) and culture volume (X3)) were included in the model. Each factor was examined at three different levels (-, 0 and +) or (low, basal, and high). Table 4 represents the fractional factorial design of Box-Behnken, which comprised 15 different trials. All trials were performed in duplicates and the average observations were used.

The results were presented in the form of surface plot (Fig.4A,B,C). The figures show that when $MgSO_4.7H_2O$ level increases and glucose level decreases, the percentage of degradation increases, at a low level of culture volume. The optimum levels of three components were estimated using the solver function of the Microsoft Excel truly and found to be 0.5 g/l glucose, 1.125 g/l $MgSO_4.7H_2O$, and 112.5 ml culture volume. For predicting the optimal point, within experimental constrains, a second-order polynomial function was fitted to the experimental results (non-linear optimization algorithm).

$$Y=19.46-4.43X_1-2.08125X_2+5.726X_3+0.6125X_1X_2+0.7725X_1X_3+0.525X_2X_3+13.49X_1^2+9.999X_2^2+5.859X_3$$

The observed degradation value was 55.7 %, when the predicted was 60 %. Therefore, this high degree of accuracy confirmed the validity of the model under optimum conditions as follows (g/l): (glucose, 0.5;$MgSO_4.7H_2O$, 1.125; $(NH_4)_2SO_4$, 0.75; $(NH_4)NO_3$, 1.5; K_2HPO_4, 0.5; KH_2PO_4, 0.25; NaCl, 0.25; pH7; inoculums size, 0.5ml; chloroxylenol, 9 µg/ml; $CaCl_2$, 0.03; culture volume, 112.5 ml and agitation, 180 rpm at 30^oC for 24 hrs). The statistical analysis was represented in Table 5.

Table 4 Observations of the Box-Behnken experiment

Trial	Factor 1 Glucose (X1)	Factor 2 $MgSO_4.7H_2O$ (X2)	Factor 3 Culture volume (X3)	Chloroxylenol degradation %
1	0.5	0.75	112.5	46.40
2	1.5	0.75	112.5	44.40
3	0.5	0.75	187.5	40.30
4	1.5	0.75	187.5	40.70
5	0.5	0.375	150	38.99
6	1.5	0.375	150	20.40
7	0.5	1.125	150	55.70
8	1.5	1.125	150	40.30
9	1	0.375	112.5	35.30
10	1	0.375	187.5	30.80
11	1	1.125	112.5	38.80
12	1	1.125	187.5	36.40
13	1	0.75	150	20.20
14	1	0.75	150	18.50
15	1	0.75	150	19.50

Table 5 Statistical analysis of Box-Behnken design showing coefficient values, t- and p-values for each variable Glucose(X1), Culture volume (X2) and Magnesium sulphate (X3)

Term	Estimate	Std Error	t Ratio	Prob>|t|	Confidence level (%)
Intercept	19.46	3.90	4.986915	0.004151	99.60
X1	-4.43	2.39	-1.85323	0.123037	87.60
X2	-2.08125	2.39	-0.87066	0.423762	57.60
X3	5.72625	2.39	2.395494	0.061965	93.80
X1X2	0.6125	3.38	0.181182	0.86334	13.60
X1X3	0.7725	3.38	0.228512	0.8283	17.17
X2X3	0.525	3.38	0.155299	0.88266	11.74
X1*X1	13.49	3.51	3.835793	0.012176	98.70
X2*X2	9.999167	3.51	2.841793	0.03617	96.30
X3*X3	5.859167	3.51	1.665193	0.156755	84.30

Confidence level (%)= 1-p-value multiply by 10

treatment. The system could provide several advantages over freely suspended cells such as simple reuse of the biomass, production of high cell concentration, protection of cells against phenol toxicity, easier liquid-solid separation and minimal clogging in continuous-flow systems (Couto *et al*., 2004).

Immobilization by cell adsorption

For successful immobilization, the support must be conducive to cell viability and has a proper permeability to allow sufficient diffusion and transport of oxygen, essential nutrients, metabolic waste and secretory products across the polymer network (Stolarzewicz *et al*., 2011).

Accordingly, it seemed worthy to test the possibility of adsorbing *K.pneumoniae* D2 cells on different supports to enhance chloroxylenol degradation.

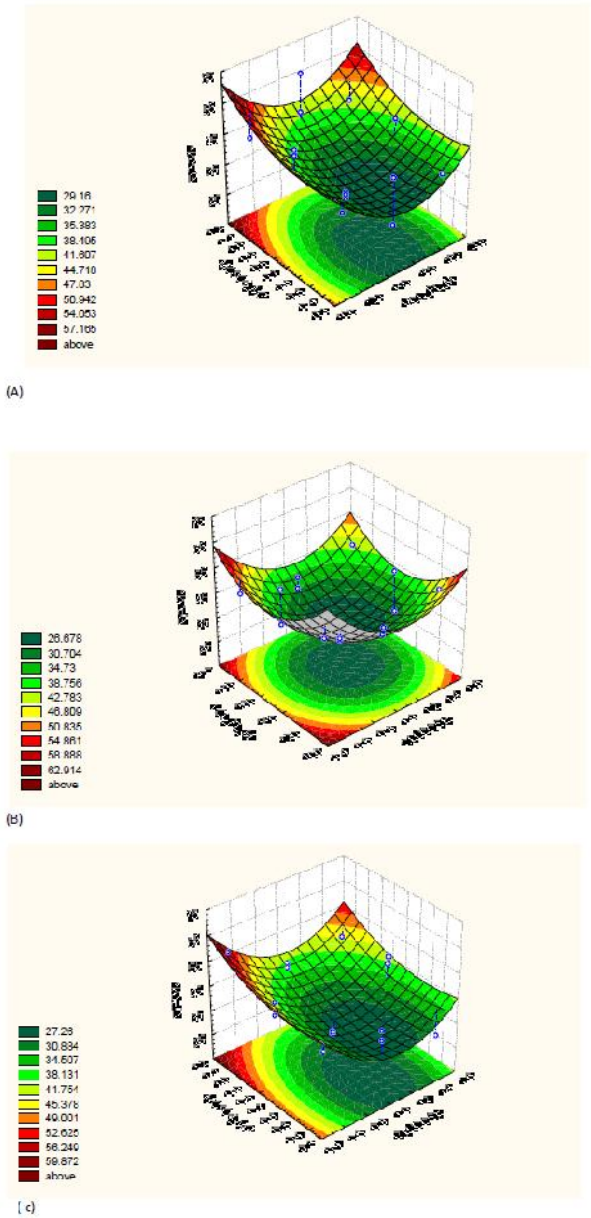

Fig 4 (A) The respose of degaradation of chloroxylenol compound (%) as a function of culture volume (ml) and $MgSO_4.7H_2O$ (g/l) (A), as a function of glucose (g/l) and culture volume (ml) (B) and as a function of glucose (g/l) and $MgSO_4.7H_2O$ (g/l) (C) based on the Box-Behnken experimental results.

The support materials used were Pumice, Porcelain, Ceramic, Luffa pulp and Polyurethane foam.

Data of this experiment shown in Fig. 5 revealed that *K.pneumoniae* D2 cellsimmobilized on Polyurethane foam pieces were the most active in degrading chloroxylenol showing the highest percentage of degradation (88.3%) after 24h of incubation. This value was 32.6% higher than that obtained with free cells. Pumice cubes showed also a good degradation, but lower than that obtained with polyurethane foam, recording 75% of degradation after 24 hrs. The degradation percentage (60.9%) obtained with Luffa as an adsorbing material was slightly higher than that of free cells, but lower than values obtained with polyurethane foam or pumice. On the other hand, cells adsorbed on porcelain or ceramic showed the least efficient in degradation percentages and the values recorded were lower than that of free cells.

Excellent support material is characterized by its ability to trap high number of bacteria. Several studies showed that natural support such as pumice, modified rice straw and luffa are a good biosorbent for some pollutants in water, such as oil-spills (Sun *et al*., 2008) and heavy metal ions (Rocha *et al*., 2009). Bioceramic and sponge were used as carriers due to their inert and highly porous nature (Misson and Razali, 2007).

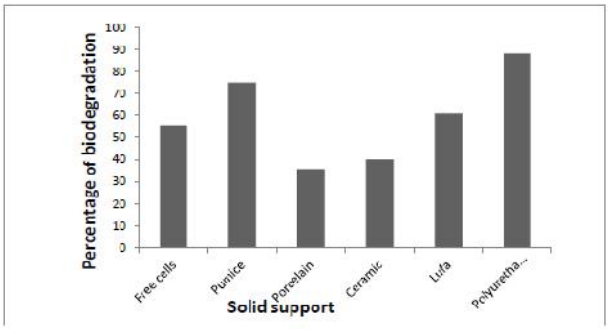

Fig 5 Chloroxylenol degradation percentage by *K.pneumoniae* D2 adsorbed on different solid supports.

The degradation rate of carbofuran phenol by immobilized cells of *Klebsiella* sp. strain ATCC13883T in various matrices: polyurethane foam (PUF), alginate, polyacrylamide, agar and alginate-clay, powdered activated charcoal (PAC) was previously studied (Kadakol *et al*., 2011). The data obtained from immobilized cells in all the matrices with batch culture suggested that the rate of degradation of carbofuran phenol, even at higher concentration (30 mM) was much higher than that of freely suspended cells.

The luffa pulp was used as carrier material for immobilizing various microorganisms for the purpose of either adsorption or degradation of various xenobiotics (Ortiz-Hernández *et al*., 2013). Their potentiality as carriers for cell immobilization was very high due to their random lattice of small cross sections coupled with very high porosity.

The biodegradation potential of pure cultures of *Pseudomonasaeruginosa* and *Pseudomonasfluorescens* immobilized on polyurethane foam was studied in batch culture using synthetic phenol in water (Agarry and Aremu, 2012). *Rhodococcus* sp. F92 was efficiently immobilized onto polyurethane foam and the immobilized cells were able to degrade a variety of petroleum products such as ALC, ASC, diesel and oil slops. The results suggest the potential of using PUF-immobilized *Rhodococcus* sp. F92 to bioremediate

petroleum hydrocarbons in an open marine environment (Quek *et al*., 2006). *Bacillusflexus* strain XJU-4, capable of degrading 3-nitrobenzoate, was immobilized in various matrices, namely polyurethane foam (PUF), polyacrylamide, sodium alginate (SA), sodium alginate-polyvinyl alcohol (SA-PVA) and agar (Mulla *et al*., 2012).

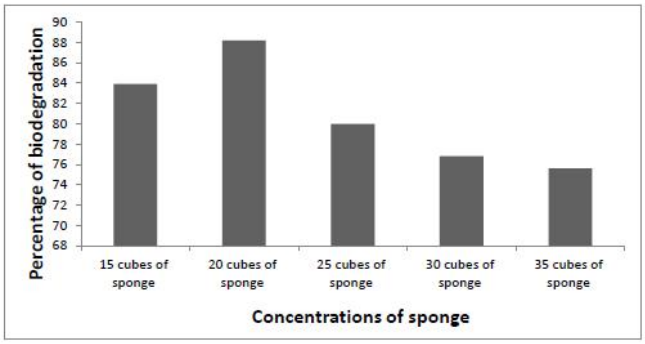

Fig 6 Degresation percentage of chloroxylenol by K. pneumonia D2 cells immobiliced in different amount of polyurethane foam

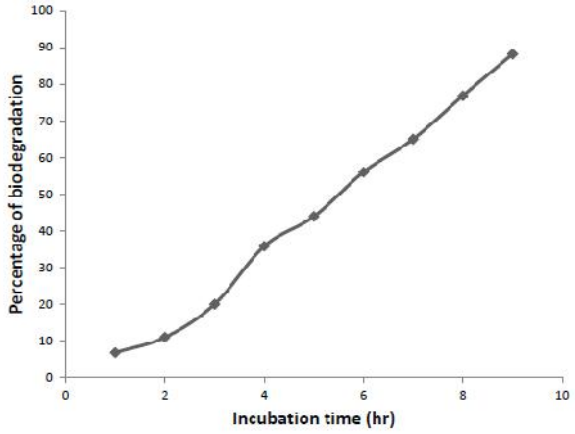

Fig 7 Time course of chloroxylenol degredation by K. pneumonia D2 cells under optimized conditions.

Reuse of adsorbed K.pneumoniae D2

In this experiment the optimum biodegradation medium supplemented initially with 8 µg/ml of chloroxylenol was inoculated with 20 cubes of polyurethane foam as an adsorbant for *K.pneumoniae* D2 cells .The reuse of the polyurethane foam cubes was carried out by removing the medium after complete degradation of chloroxylenol compound. The polyurethane foam cubes were washed with sterile distilled water and then another 100 ml medium containing 8µg/ml of chloroxylenol compound was added to the polyurethane foam cubes, and a new cycle was run. This process was repeated several times all over the experiment. The results (the data not shown) indicated that percentage of biodegradation was only decreased by 10% after the adsorbed *K.pneumoniae* D2 cells were reused for 10 cycles (incubated for 9 hrs each time)

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How to cite this article:

Ghanem, K.M *et al* (2017) 'Optimization The Degradation of Chloroxylenol By Free And Immobilized Klebsiella Pneumoniae D2', *International Journal of Current Advanced Research*, 06(10), pp. 7082-7091.
DOI: http://dx.doi.org/10.24327/ijcar.2017.7091.1077

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