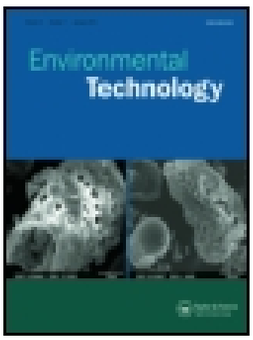

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A novel thermally stable heteropolysaccharide based bioflocculant from hydrocarbonoclastic strain *Kocuria rosea* BU22S and its application in dye removal

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Abstract

A new bioflocculant named pKr produced by hydrocarbonoclastic strain *Kocuria rosea* BU22S (KC152976) was investigated. First, quantitative analysis of hydrocarbon was carried out in order to study the degradation rate of crude oil by BU22S. Gas Chromatography - Flame Ionization Detector (GC-FID) analysis confirmed the highly potential of the strain BU22S in the degradation of n-alkanes. Second, Plackett-Burman experimental design and response-surface methodology were carried out to optimize pKr production. Glucose, peptone and incubation time were found to be the most significant factors affecting bioflocculant production. Maximum pKr production was about 4.72 ±0.02 g/L achieved with 15.61 g/L glucose, 6.45 g/L peptone and 3 days incubation time. Chemical analysis of pKr indicated that it contained 71.62% polysaccharides, 16.36% uronic acid and 2.83% proteins. Thin Layer Chromatography (TLC) analysis showed that polysaccharides fraction consisted of galactose and xylose. Fourier Transform InfraRed (FT-IR) analysis revealed the presence of many functional groups hydroxyl, carboxyl, methoxyl, acetyl and amide that likely contribute to flocculation. *K. rosea* pKr showed high flocculant potential using kaolin clay at different pH (2-11), temperature (0-100°C), and cations concentrations. The bioflocculant was particularly effective in flocculating soluble anionic dyes Reactive Blue 4 and Acid Yellow with a decolourization efficiency of 76.4% and 72.6%, respectively. The outstanding flocculating performances suggest that pKr could be useful for bioremediation applications.

Key words: *Kocuria rosea* BU22S; crude oil degradation; bioflocculant; Response-surface methodology; flocculation activity; dye removal

Introduction

Chemical flocculants are components that stimulate flocculation by aggregation of colloids and other suspended particles. They are extensively applied in potable water and industrial wastewater treatment. They have many advantages of being effective in terms of flocculating efficiency and availability. In contrast, they are implicated in various human health problems, they have been reported to be neurotoxic and carcinogenic [1-5,9]. In fact a clear link was demonstrated between aluminum in drinking water and human neurological disorders [1,5,6]. These chemical polymers are also known to be neither biodegradable nor ecofriendly [6]. Hence, because of their detrimental nature, more attention has been given to the use of bioflocculants produced by microorganisms. Therefore, bioflocculants are considered as potentially promising alternative to conventional chemical polymers because of their efficiency, biodegradability, non-toxicity, and non-secondary pollution [7, 8]. Several bioflocculant-producing microorganisms (bacteria, fungi and yeast) have been recently reproted and their bioflocculants have been characterized. They are mostly composed of polysaccharides, proteins, glycoproteins, nucleic acids and lipids. The flocculant produced by *Rhodococcus erythropolis* [9] is predominantly protein in nature. *Bacillus mucilaginosus* [10], *Proteus mirabilis* [11] and *Bacillus toyonensis* [12] were shown to produce glycoproteins biofluccculants, whereas those of *Paenibacillus elgii* [13], *Serratia ficaria* [14] and *Klebsiella mobilis* [15] are mainly polysaccharides. Some of these biomacromolecules, polysaccharide based-bioflocculant draw particular attention especially with regard to wastewater treatment. They have a unique structure and some functional properties. They contain ionizable functional groups which enable them to be effective not only in removing suspended solids, heavy metals, dyes, pathogens but also in reducing the turbidity of different types of industrial wastewater effluents [16]. In fact, polysaccharide-based flocculants have been investigated in the treatment of several industrial effluents such as brewery wastewater [14, 17], dying wastewater [13], swine wastewater [9], textile effluents [18] pulp and paper mill effluent [14], poultry wastewater [19], and dairy woolen wastewater [20].

Despite these promising features of bioflocculant, several limiting aspects are hampering their large-scale production and industrial application. Particularly; their low production yield and restrained flocculating efficiency [21-22]. Consequently, it has become imperative to screen and

identify new bioflocculant-producing strains and investigate strategies for the optimization of fermentation conditions to improve bioflocculant production [23-24].

Actinobacteria are known as good sources of secondary metabolites of economic importance [25, 26]. Among these, *Kocuria rosea* was screened in previous studies for their potential use in numerous biotechnological and industrial applications such as the production of proteolytic enzymes, keratin-hydrolysing proteinases, antibiotics, and biosurfactant [27-30]. However, *K. rosea* remains underexplored and yet hold tremendous promise as source of novel bioflocculant-producing organisms. In the current study, we have evaluated a marine *Actinobacteria* isolated from hydrocarbon-polluted sediments [28] for crude oil degradation and bioflocculant production. Furthermore, production yield optimization was attempted through manipulation of physicochemical parameters and subsequently, the bioflocculant was characterized through compositional analysis and flocculation activities. In addition, a series of experiments were carried out to study the flocculation activities towards kaolin clay and dyeing solutions.

1. Materials and methods

1.1. Bacterial isolate and hydrocarbons biodegradation

1.1.1. Bacterial strain

The hydrocarbonoclastic bacterium *Kocuria rosea* strain BU22S (Genebank accession number of 16S rRNA sequence; KC152976) was isolated from hydrocarbon contaminated sediments from a refinery harbor of the Bizerte coast in Northern Tunisia. Isolation was performed on mineral medium supplemented with 1% crude oil as the sole carbon source. Stain BU22S showed peculiar characteristics of biosurfactant and emulsification activity [28].

1.1.2. Growth conditions and hydrocabon analysis

Kocuria rosea strain BU22S was tested for the ability to grow in the presence of different hydrocarbons as sole carbon source. Mineral solid medium ONR7a [31] hydrocarbons were inoculated with 100 µl of strains cultures (OD_{600}= 0.5). Incubation was performed at 30°C for 7days. For hydrocarbon analysis, culture was prepared by inoculating 100 µl of microbial cells into 50 mL of ONR7a liquid mineral medium [31] supplemented with 1% of crude oil. Incubation was performed at 30°C for 21 days. The composition of Total Extracted and Resolved Hydrocarbons and their derivates (TERHCs) were analyzed by high-resolution GC-FID (DANI Master GC Fast Gas Chromatograph System, DANI Instruments S.p.A.). Index selected for this study were: *n*-C17/Pristane (*n*C17/Pr), *n*-C18/Phytane (*n*C18/Ph) in order to evaluate the relative biodegradation of

n-alkanes. The degradation of TERCHs was expressed as the percentage of TERCHs degraded compared to negative abiotic control.

2.2. Production and characterization of bioflocculant

2.2.1. Inoculum preparation

K. rosea strain BU22S was grown and maintained on a medium containing, (g/l) 10g glucose, 2g NaCl, 0.25g KH_2PO_4, 5g peptone and 0.2g $MgSO_4$. The pH was adjusted to 8.0. *K. rosea* was grown on this medium at 30°C for 24 hours.

2.2.2. Culture conditions for pKr production

For the production of the bioflocculant, strain BU22S was cultivated in 250 ml flasks containing 100 ml of culture medium. The independent variables such as: glucose, peptone, KH_2PO_4, NaCl, pH, agitation, incubation time and incubation temperature, were varied according to the design of experiments as presented in Table 1.

2.2.3. Optimization of pKr production medium by response surface methodology (RSM)

Plakett-Burman (PB) design was used in the present report to identify the components that significantly affected bioflocculant production. It is a successful tool used in several preliminary studies in which the principal objective was to identify those components that can be fixed or excluded in a further optimization process [32]. The PB design was based on the first order polynomial model: (equation 1)

$$Y = b_0 + \sum b_i X_i \qquad (1)$$

where Y is the estimated target function (i.e., pKr yield) and b_i are the regression coefficients. The contrast coefficient, noted b_0, was calculated as the difference between the average of measurements made at the high (+) and the low (-) levels of the factors. From the regression analysis of the variables, the significant levels at 95% level ($p \leq 0.05$) were considered to have greater impact on pKr production. Seven variables including glucose, peptone, KH_2PO_4, inoculum size, pH, temperature and incubation time were examined in a 16-run trial. The choice of these variables was based on previous literature works [33, 34] and preliminary experiments. Each variable was presented in two levels, high and low, with actual levels shown in Table 1. Response value was measured in terms of pKr yield. NemrodW, 9901 software was used to design and analyze the data throughout the experiments.

Three factors, glucose concentration (X_1), peptone concentration (X_2) and incubation time (X_3) were selected from PB design that significantly affected pKr production and were further optimized

by RSM. A Box-Behnken statistical design with 3 factors and 3 levels (Table 2) was applied to elucidate the interactions of these variables on the bioflocculant production. The experimental designs are shown in Table 3. The relationship between the response (pKr yield) and the three quantitative variables was fitted by a second-order model in the form of quadratic polynomial equation (2):

$$Y = b_0 + b_1X_1 + b_2 X_2 + b_3 X_3 + b_{11} X_1^2 + b_{22} X_2^2 + b_{33} X_3^2 + b_{12} X_1X_2 + b_{13} X_1X_3 + b_{23} X_2X_3 \quad (2)$$

Where Y is the pKr production, b_0 is the model constant, X_1, X_2 and X_3 are the independent variables, b_1, b_2 and b_3 are the linear coefficients, b_{12}, b_{13}, and b_{23} are the cross-product coefficients, and b_{11}, b_{22} and b_{33} are the quadratic coefficients.

2.2.4. Quantifying pKr production and flocculating activity

To obtain the purified bioflocculant the fermentation broth was centrifuged to remove the cells by centrifugal separation (12.000 r/min, 20 min). Two volumes of cold ethanol were then added to the supernatant and left overnight at 4°C. The precipitate was dissolved in deionized water (1/10 of initial volume), dialyzed against deionized water and lyophilized to obtain the bioflocculant. The yield was determined on dry weight basis.

The flocculating activity of pKr was measured according to the method reported by Kurane et *al.* [35] in which Kaolin clay was chosen as the suspended solid. First, 93 mL kaolin suspension (4.0 g/L), 5.0 mL $CaCl_2$ (1%, m/V) and 2.0 mL liquid bioflocculant (were mixed and vigorously stirred (180 r/min) for 2 min and then slowly stirred (80 r/min) for 5 min, and allowed to stand for 5 min. The supernatant absorbance was measured by a spectrophotometer at 550 nm (Analytic Jena SPEKOL 2000). The fermentation supernatant was replaced with a culture medium at the same concentration in the control experiment. The flocculating activity (*FA*, %) was calculated according equation (3) :

$$FA = (A_0 - A)/A_0 \text{ x}100\% \quad (3)$$

Where *FA* is the flocculating activity, A_0 and A were the absorbance variables at 550 nm of the control and the sample supernatant, respectively

2.2.5. Characteristics of pKr obtained from the lyophilized material

Total sugar content of pKr was determined by the phenol-sulfuric acid method using glucose as the standard solution [36]. Protein content was measured by the Bradford method using bovine serum albumin as the standard solution [37]. The content of uronic acid was determined by the sulfuric acid-carbazole method [38] and with D-glucuronic acid as the standard. The analysis of

monosaccharides in pKr was carried out by Thin Layer Chromatography (TLC). The purified pKr (20 mg) was hydrolyzed with 1.5 ml of 2 N trifluoroacetic acid by heating at 110^{o}C for 3 h. The resulting hydrolysate was diluted with distilled water and neutralized with 1 N NaOH solution after cooling at room temperature. The solution was filtered by a membrane filter (0.45 μm) before analysis. To determine the monosaccharide component of the hydrolysate,TLC was performed on silica gel with methyl-ethyl-cetone/acetic acid/methanol [3:1:1(v/v/v)] as a developing solvent system. Developed spots were visualized by immersing the plate in potassium permanganate (3%)/ anhydrous sodium carbonate (4%) [2:1 (v/v)] solution for about 20 seconds following heating of the plate. Glucose, galactose, xylose, maltose, lactose and saccharose were used as standards.

Fourier transform infrared (FT-IR) spectroscopy of pKr sample was obtained over a wave number range of 400-4000 cm^{-1} to determine the functional groups (Bruker Vertex 70 FTIR spectrometer).

2.2.6. Flocculating properties of pKr

To obtain the optimal concentration of the bioflocculant the effects of pKr concentrations was studied according to Tang et *al.*[39]. pKr was dissolved in ultrapure water to get a 0.066 mg/ml concentration (stock solution). Ten different concentrations were prepared from the stock solution (0.065mg/L, 0.13mg/L, 0.24mg/L, 0.65mg/L, 1.2mg/L, 2.5mg/L, 6mg/L, 11mg/L, 18.8mg/L, 33mg/L) and mixed into Kaolin suspension (4g/L) to test the dosage impact.

The effects of solution pH and temperature on flocculating activity were examined by measuring the flocculating activity of the reaction mixture containing the optimal concentration of pKr at specified ranges of pH (2-11); to investigate the thermal stability, a pKr and kaolin suspension mixture was kept for 30 min in a water bath with various temperatures from 0 to 100°C. Furthermore, effect of various cations on the flocculant activity of pKr was determined using the method described above, except that the $CaCl_2$ solution was replaced by various metal salt solutions. Solutions of KCl, NaCl, $MgCl_2$, $CaCl_2$, $FeSO_4$, $FeCl_3$ and $AlCl_3$ concentration is 0.09M were used as cation sources. All the experiments were conducted in triplicate.

2.2.7. Decolourization of dyeing solutions

The decolourization of dyeing solution was determined by the method described by Li et *al.* [13]. Four dyes Acid Yellow 17 (AY17), Reactive Blue 4 (RB4), Basic Red (BR) and Basic Blue 3 (BB3) were used in this study. Three milliliter pKr solution (500 mg/L) was added into 100 ml dye solution (100 mg/L), and agitated at 150 r/min for 12 hr. After centrifugation at 4000 rpm for 5 min, the supernatant was measured with a spectrophotometer (Analytic Jena SPEKOL 2000). All wavelengths were determined experimentally as the wave with maximal absorbance (400 nm, 595 nm, 524nm and

654 for AY17, RB 4, BR and BB3, respectively). The residual dye concentration was calculated according to the calibration curve for each dye measured, the percentage of dye removal was determined according to equation 4:

$$DA = (C_0 - C_e)/C_0 \times 100\% \quad (4)$$

where *DA* was the decolourization activity, C_0 was the initial dye concentration and C_e was the residual dye concentration of the supernatant.

2. Results and discussion

2.1. Hydrocarbon degradation

Kocuria rosea strain BU22S was selected for this studies basing on its high rate production of biosurfactant and its emulsification activity [28]. Its phylogenetic position inferred from the 16S rRNA gene sequence is shown in Fig. 1. We tested the ability of the bacterium to grow in presence of different hydrocarbons (Table 4). The results showed that BU22S was able to use different hydrocarbon as only carbon source in solid media. After incubation of 21 days at 28°C, the increase of turbidity of the culture by comparison to the negative control was considered as an indication of the ability of the strain to degrade the crude oil. The data obtained using GC -FID analysis showed a highest rate degradation of ~91% and 85% of n-alkanes and crude oil respectively (Fig. 2). Degradation of almost all n-alkanes (rate of degradation > of 90%) was observed. Shorter *n*-alkanes of C12~C15 were almost completely degraded by BU22S. n-alkanes with a medium length (C16-C18) were highly degraded with a rate of degradation > ~ 70% than long chains (C19-C40) due to their low solubility which inhibits their degradation by bacteria. However, *Kocuria*, was not classified as Marine Obligate Hydrocarbonoclastic Bacteria (OMHCB), but it is heterotrophic bacteria that can be considered as a potential candidates for application in bioremediation process based on its ability to produce biosurfactant and emulsification activity which facilitate hydrocarbons degradation. Capability of *Kocuria* genus to use hydrocarbons as the only sources of energy and organic carbon was described in other studies [40, 41]. BU22S may find great application in bioremediation of hydrocarbon contaminated environments.

2.2. Screening of signal factors for bioflocculant production using PB design

The relative significance of seven variables was investigated using PB design (Table 1). The first-order model equation for predicted bioflocculant production with the factors regardless of their significance was as follows:

$$Y = 0.964 + 0.178\,X_1 + 0.098\,X_2 + 0.014\,X_3 - 0.031\,X_4 + 0.051\,X_5 + 0.036\,X_6 + 0.129\,X_7 \quad (5)$$

where *Y* was the predicted bioflocculant production, and X_1, X_2, X_3, X_4, X_5, X_6, X_7 were coded values of glucose, peptone, KH_2PO_4, inoculum size, temperature and incubation time, respectively. The analysis of variance (ANOVA) showed that determinant coefficient R^2 of the first-order model and the p-values were 0.924 and 0.0004, which means the model is significant and 92.40 of the total variations could be explained by the model. Among these variables, glucose, peptone, and incubation time had significant influence on bioflocculant production according to their *P*-values (< 0.05, significant at 95% level). Glucose, peptone and incubation time showed positive effect on bioflocculant production within the tested range, indicating that bioflocculant production increased with the increased levels of these three factors (Fig. 3)**.** As a result, glucose, peptone, and incubation time were selected to optimize the production of the bioflocculant using RSM.

2.3. Response surface optimization of pKr production

The experiments were planned to obtain a quadratic model consisting of 12 runs and 5 center points. The range and levels of three independent variables are shown in Table 2, and the Box-Behnken design matrix together with the experimental and predicted bioflocculant data are shown in Table 3. After analyzing the experimental results through multiple regressions, the relationship between pKr production and test variables glucose, peptone and incubation time was related by the following second-order polynomial equation (6):

$$pKr = 3.248 + 0.400\,(\text{glucose}) - 0.020\,(\text{peptone}) + 0.330\,(\text{incubation time}) - 0.340\,(\text{glucose})^2 - 0.375\,(\text{peptone})^2 - 0.450\,(\text{incubation time})^2 - 0.488\,(\text{glucose})(\text{incubation time}) + 0.212\,(\text{glucose})(\text{incubation time}) + 0.328\,(\text{peptone})(\text{incubation time}) \quad (6)$$

The analysis of variance for the fitted model (Table 5) showed that the regression sum of squares was statistically significant at the level 99.9% and the lack of fit was not significant. Consequently, the model represents well the measured data. The R^2 and adjusted determination coefficient Adj R^2 values were 0.960 and 0.908, respectively. Indicating a high degree of correlation between the observed and predicted values for the production of pKr. From the R^2 value, it was concluded that only 4 % of the variation for pKr production could not be explained by the model.

The linear coefficients (X_1 and X_3), the quadratic term coefficients (X_2^2 and X_3^2), and the interaction terms (X_1X_2 and X_2X_3) had highly significant effects on pKr production (P <0.001), followed by the quadratic term coefficients X_1^2 with significant effect (P < 0.05). Among the significant equation terms, glucose (X_1) showed the most direct proportional relationship with pKr production. Three-dimensional response and two-dimensional contour plots are the graphical representations of the

equation 4 (Fig. 4). These plots provide a method to visualize the relationship between pKr production and the experimental level of each variable and facilitated the location of the optimum experimental conditions. According to many studies [42-44] a circular contour plot indicates that the interactions between the corresponding variables are negligible whereas an elliptical or saddle plots illustrated greater significance of interaction.

The response surface plot and contour plot in Fig. 4A1.A2 shows the effects of the glucose and peptone on pKr yield and their interaction when incubation time was fixed at zero level (3 days). At a higher concentration of glucose and lower concentration of peptone pKr production increased whereas at a higher concentration of glucose and peptone pKr production decreased, which suggested that high concentration of peptone suppressed the biosynthesis of pKr. An elliptic contour plot in Fig. 4A1 was observed, indicating a significant interaction between glucose and peptone for bioflocculant production.

Fig. 4B1.B2 graphed the effects of glucose and incubation time on pKr yield when peptone was fixed at zero level. As bioflocculant is highly synthesized during late exponential growth or in the stationary phase, decrease in incubation time may affect negatively the production. Higher incubation time lower the production of pKr due to the production of certain enzymes, such as saccharases, may act upon polysaccharides, and deteriorating the production formation.

By analyzing the response surface plots and contour representation, the optimal values of tested variables for the highest pKr production (4.72±0.02 g/L) were glucose concentration 15.61 g/L, peptone concentration 6.45 g/L and incubation time 3 days, which was about 3.32-fold increase compared with using the original medium (1.42 g/L).

2.4. Verification of optimum conditions

The model predicted that the maximum bioflocculant production was (4.72 ± 0.02 g/L). To validate the adequacy of the model equation, three additional verification experiments were carried out under above-mentioned conditions. The mean pKr production was 4.66 ± 0.04 g/L that agreed well with the predicted value and indicating the validity of the model. The concentrations of the carbon and nitrogen source of optimum conditions were much lower than the result of Raza et *al.*(48.5 g/L and 10 g/L versus 15.61 g/L and 6.45 g/L) for much yield of bioflocculant (3.44 g/L versus 4.66 g/L) [45].

2.5. Characteristics of pKr

2.5.1. Chemical composition analysis of pKr

Chemical analysis showed that the purified bioflocculant was composed of 71.62 % total sugar content, 2.83% total protein content, and 16.36% uronic acid. TLC analysis (Fig. 5) revealed that the

polysaccharide fraction of pKr was consisted of two monosaccharides galactose and xylose. This finding differed from the polysaccharides produced by bacterial strains. Prior to this study, very few bacterial bioflocculants have been reported to have xylose in their structure. In fact, xylose is rather common in fungal glycans [13, 46].

2.5.2. Functional groups analysis of pKr

Infrared spectrometer (Vertex 70 ATR Bruker Diamant) was used to demonstrate the physical structures and functional groups of pKr. As shown in Fig. 5, the intense absorption peak at 3375 cm^{-1} was characteristic of OH stretching from hydroxyl group, and absorbed water molecules. The absorbance peaks in the 2954-2852 cm^{-1} region were due to the stretching vibration of CH, CH_2 and CH_3 [47]. Presence of these groups is confirmed by bands at 1377 cm^{-1}[48]. The absorption located at 1741 and 1643 cm^{-1} was assigned to the C=O stretching of the acetyl group in pKr [49]. The absorption in the 1643 cm^{-1} region of the spectra can be assigned to the COO^- and C=C groups [50] and a week peak at 1547 cm^{-1} could be attributed to NH bending of amides II of osamines, as confirmed by the low amount of protein (2.8%). The absence of a doublet at 1250-1230 cm^{-1} indicated that no sulfate groups were present in this bioflocculant. The bands within the 1119 -1153 cm^{-1} region were attributed to the vibration of C-O-C bond [49]. The absorption peak at 1074 cm^{-1} is related to methoxyl groups, typical group of sugar derivatives [51, 52]. The1000–1125 range is characteristic of uronic acid, O-acetyl ester linkage bond [53]. The small absorption band at 876 cm^{-1} could be associated with β-glycosidic linkages between the sugar monomers [55]. The peaks at 697 and 507 cm^{-1} are the absorption peaks for the aromatic CH bending vibration [54].

In summary, the infrared spectrum confirmed the presence of characteristic peaks for carbohydrates and amides: carboxyl, hydroxyl, methoxyl and amino groups, it can be inferred that pKr is a β type heteropolysaccharide containing some proteins. The bioflocculant participates in the flocculation mainly through available hydroxyl, carbonyl, acetyl and carboxyl groups which induces very high binding capacity.

2.6. Effect of dosage, pH, temperature and metal ions on the flocculating activity of pKr

The effects of pKr dosage, pH, temperature and metal ions on flocculating activity were shown in Fig. 6.

2.6.1. Effect of pKr dosage

The effect of bioflocculant dosage (Fig. 6A) showed that flocculation efficiency of pKr increased from 47.3% to 89.2% with the addition of bioflocculant dose at the range of 0.065 mg/L to 1.2 mg/L. Flocculation decreased to 61.3% with further dose addition to 33 mg/L, indicating that 1.2 mg/L pKr provides optimum flocculation efficiency for Kaolin particles. Fig. 6A also showed that more or less

dose of pKr would deteriorate flocculation. When the pKr is insufficiency it caused inadequate bioflocculant molecules to absorb the suspended kaolin particles, the bridging phenomena cannot effectively form. Unfavorably, more dose of pKr inhibited flocs from forming due to the stronger repulsive forces between them [2, 56, 57]. The relationship between dosage and flocculating rate of pKr was similar to that of the bioflocculants produced by other pure strains [56, 58]. Comparatively, pKr showed lower optimal concentration (1.2 mg/L) which could be attributed to a possible high molecular weight of the bioflocculant. In fact, flocculant with high-molecular-weight involves more adsorption points and stronger bridging leading to high flocculation activity. These findings are economically desirable.

2.6.2. pH stability of pKr

The flocculating properties of the purified bioflocculant were influenced by the system conditions such as pH and temperature. Fig. 6B shows that pKr was quite stable at wide range of pH between 2 and 8 and more than 85% flocculation was achieved at this range. The optimal activity of 89.6% was observed at pH 7.0. While with pH higher than 8.0 the flocculation rate decreased. According to Yang et *al.* this may be due to the bioflocculant shows different electric states at different pH and that will affect the flocculation ability [59]. The favorable pH range varies for the bioflocculants produced by different strains. For example, the flocculating activity was over 80.0% in the range of 4.0-8.0 for *Klebsiella sp.* [60], and the optimal pH range was 5.0 to 9.0 for *Bacillus mojavensis* 32A [61], also the flocculating activity was higher than 92.0% in the range of 3.0-8.0 for *Bacillus sp.* AEMREG7 [62]. Finding out the optimum pH range is a basic step during the flocculation via bioflocculants. Our results demonstrate that pKr is suitable to be applied in acidic (pH2) and neutral matrixes.

2.6.3. Thermo-stability of pKr

The thermal stability of the bioflocculant depends on its activity ingredients. Many finding indicated that the bioflocculants with sugars backbone in the structure were thermostable, while those made of protein or peptide were generally sensitive to heat [9]. After being heated in a water bath for 30 min, the flocculation performance of pKr was high and stable at temperature range from 30 to 100°C, within which, the minimum flocculating rate was 85.5% (Fig. 6C). The polysaccharide-backbone composition of pKr was assumed to explain the excellent thermal stability of the bioflocculant produced by *K. rosea*. The thermal stability may be due to the presence of hydroxyl group involved in the formation of hydrogen bonds in pKr structure [63]. However, pKr was less stable than the

bioflocculant produced by *Aspergillus flavus* which retained high flocculating activity above 90% over a temperature range of 10–100°C [58].

2.6.4. Effects of metal ions

The addition of metal ions to kaolin suspensions during the bioflocculation process is required to induce effective flocculation by cation-dependent bioflocculants, such as bioflocculants produced by *Halomonas sp*. [64], *Micrococcus sp.* [8]. Commonly, cations are applied to neutralize the negative charges of cation-dependent bioflocculants and kaolin particles, thereby increasing the adsorption of bioflocculant onto kaolin particles. The effects of cations on the flocculating activity of pKr are similar to the previous studies by Pu et *al.* on the bioflocculant produced by two strains of *Rhizopus sp* [65]. It can be seen from Fig. 6D that divalent cations (Mg^{2+}, Ca^{2+} and Fe^{2+}) were more effective than monovalent ($Na^{+}$and K^{+}) and trivalent cations (Fe^{3+} and Al^{3+}). The role of bivalent cations is to increase the initial adsorption of pKr on kaolin by decreasing the negative charge on both bioflocculant and particle [66-68]. However, the flocculating rate decreased by approximately 15% to 17% during the addition of monovalent cations respectively. During the experiment, it was found that the flocculating rate was negatively affected by trivalent cations (Fe^{3+} and Al^{3+}). The flocculation efficiency was decreased by 25%. These trivalent cations possibly alter the surface charge of kaolin particles and cover the adsorb sites [14]. The competition of the positively charged particles and less adsorb sites induce the low flocculating activity, explaining the flocculation activity reduction in the presence of Al^{3+} and Fe^{3+} [69]. These findings are consistent with previous studies where several bioflocculants have been shown to be cation-dependent [8, 14, 67]. Interactions of pKr with different cations are very crucial for its application as a bioflocculant material. Indeed future studies should evaluate the best combination of pKr and cations which flocculate suspended particles in real wastewater.

2.7. Decolourization experiment of the pKr

In the flocculation experiments, two anionic and two cationic dyes were used. The results showed that depending on the dye used, pKr exhibited different decolourization activity. The bioflocculant had moderate anionic dyes removal ability, with the decolourization rates for RB 4 and AY17 being 76.4% and 72.6%, respectively ; lower rates were observed when used with cationic dyes for BR and BB3 being 23.4% and 11.2%, respectively. These results suggest that pKr was more effective for anionic dyes than cationic dyes. Similarly, the bioflocculant produced by *A. parasiticus* was effective for the removal of anionic dyes [68]. Conversely, the bioflocculant produced by *P. elgii* possesses functional groups that have the ability to decolourize cationic dyes in wastewater. It has a removal

rate of 65% for methylene blue and 72% for Red X-GRL. Lower removal efficiencies (< 50%) were obtained when it was used to treat anionic and neutral dyes [13]. As reported by Deng et *al.* the decolourization ability is related to the size of the dye molecule: bigger molecules adsorbed on the bioflocculant may prevent others from being adsorbed. However, in this study, the removal of the dyes is not consistent with the size (RB4 >AY17 >BR> BB3) [70]. This phenomenon may be attributed to the structure and the complex multi-point adsorption between the dye and pKr molecules during the flocculation process. In fact molecules of RB4 contained anthraquinone structure with hydroxyl groups which participate in binding the dye to pKr molecules and could explain the obtained decolourization rate.

3. Conclusions

The Actinobacterial strain *K. rosea* BU22S was isolated from an hydrocarbon-polluted sediment and found to degrade crude oil and produce the bioflocculant named pKr. The maximum production of about 4.72 ±0.02 g/L was obtained in the optimized medium with glucose at 15.61 g/L, peptone at 6.45 g/L, and incubation time of 3 days. This bioflocculant was found to have good thermal stability, require additional cations and was shown to be effective for the removal of some anionic dyes. It was also suitable to be applied in acidic and neutral circumstances. The main active fractions of pKr were found to be polysaccharides consisting of galactose, xylose and glucuronic acid. Multiple functional groups present within pKr contributed to its high flocculation efficiency. Although further studies are required to investigate the link between the ability of hydrocarbon degradation and the pKr production, results from this study suggest the potential use of *K. rosea* in crude oil degradation. Further, pKr bioflocculant constitute a good candidate as useful material for biotechnological processes mainly for environmental bioremediation.

Acknowledgements

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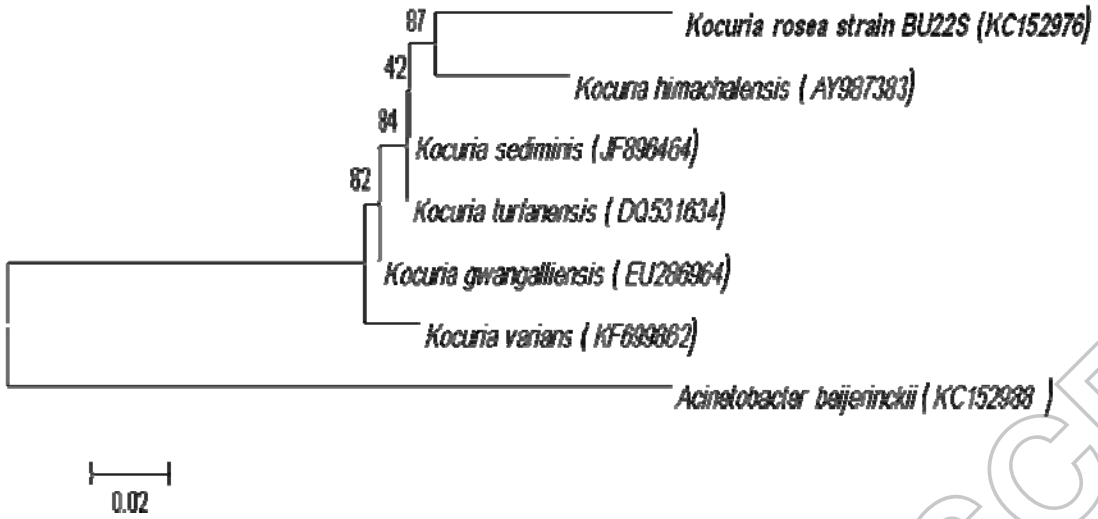

Figure 1. Phylogenetic analysis of 16S rRNA gene sequence of bacterial isolate *Kocuria rosea strain BU22S based on*16S rDNA partial sequences. Phylogenetic dendrogram was evaluated by performing bootstrap analysis of 1000 data sets using MEGA 6.06. 16S rRNA sequence accession numbers of the reference strains are indicated in parentheses.

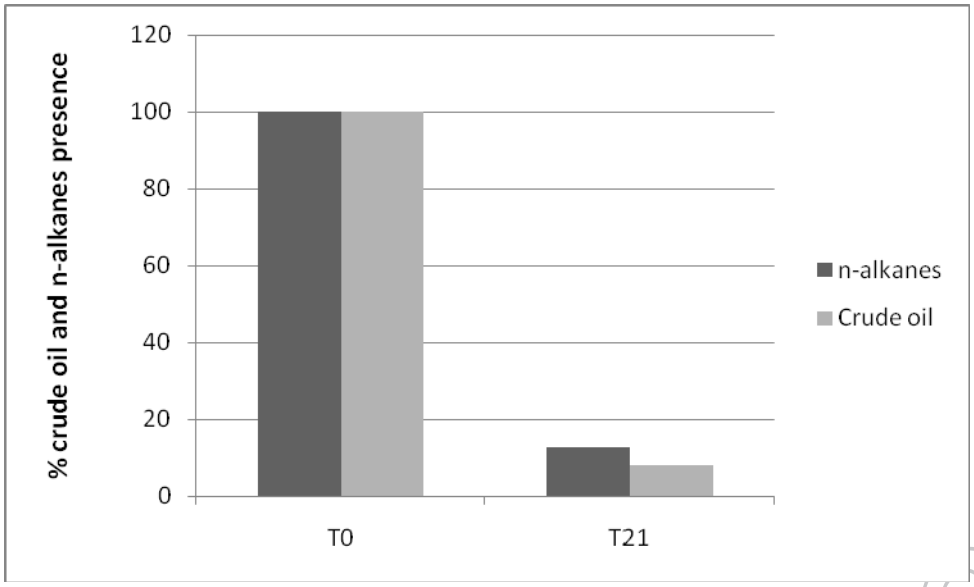

Figure 2. Relative percentage of n-alkanes C12-C40 (dark grey bars) and crude oil (white grey bars) present in cultures after 21 days (T21) of incubation, data expressed as the percentages compared to negative abiotic control (T0).

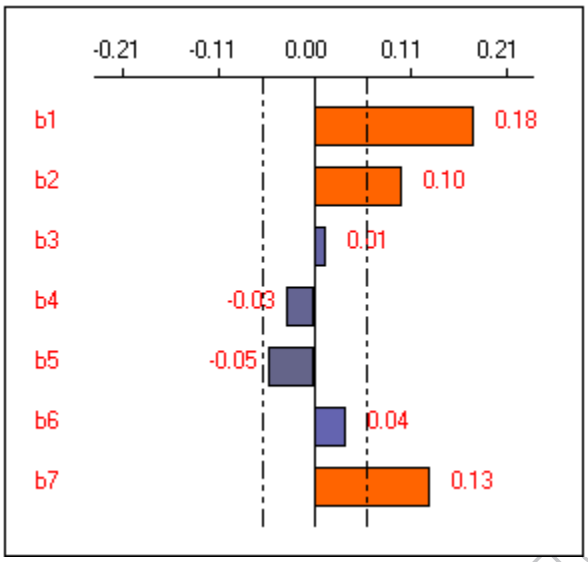

Figure 3. Pareto chart of the standard effects of the tested seven factors to pKr production. Glucose, peptone, and incubation time were determined to be significant.

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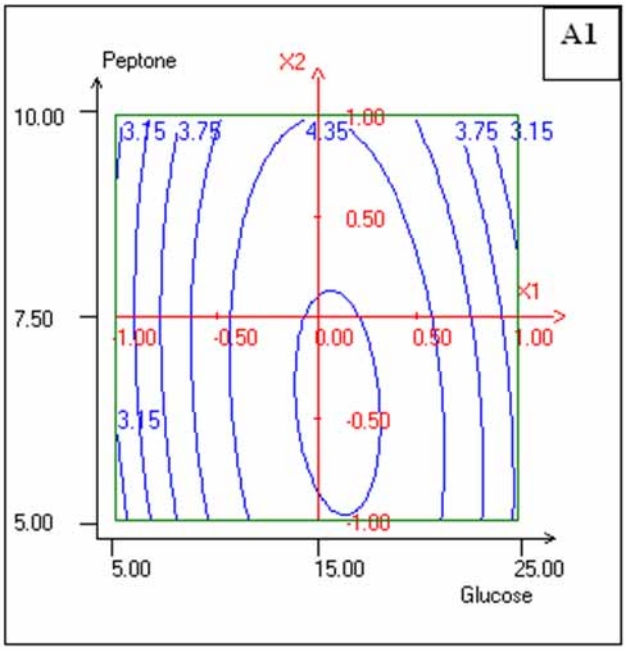
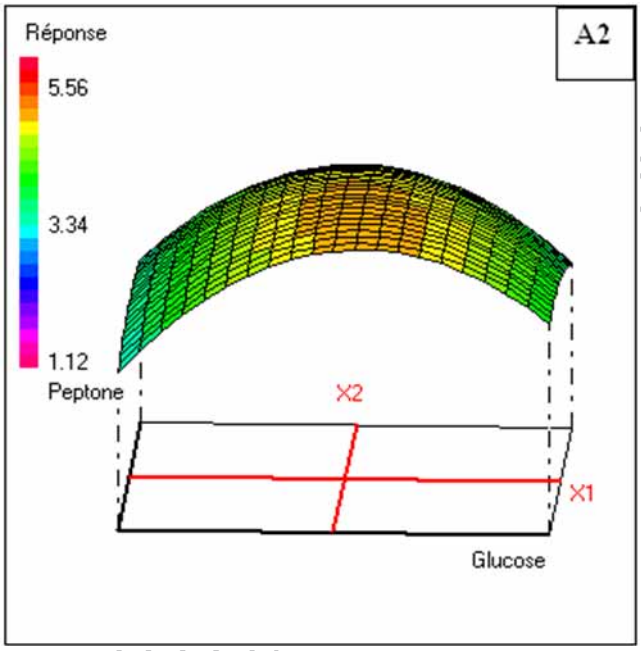
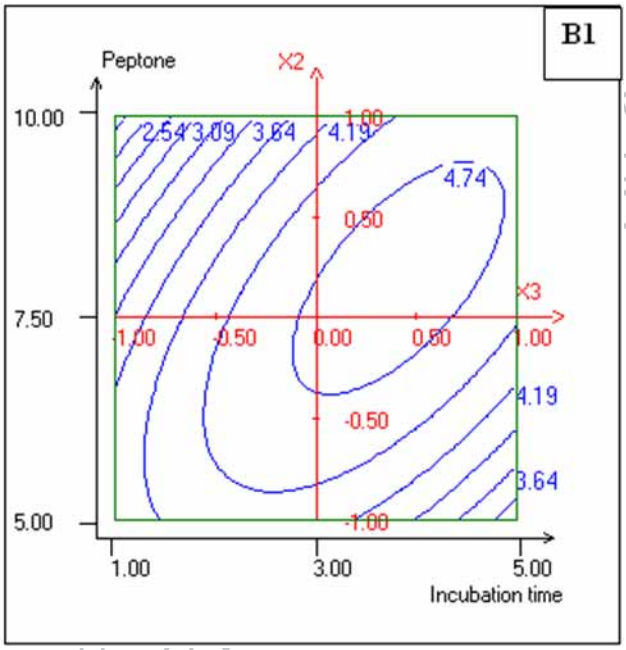
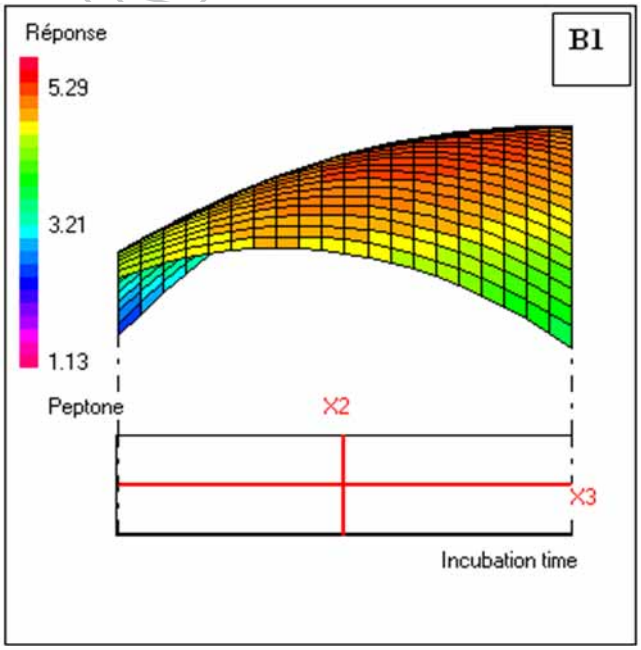
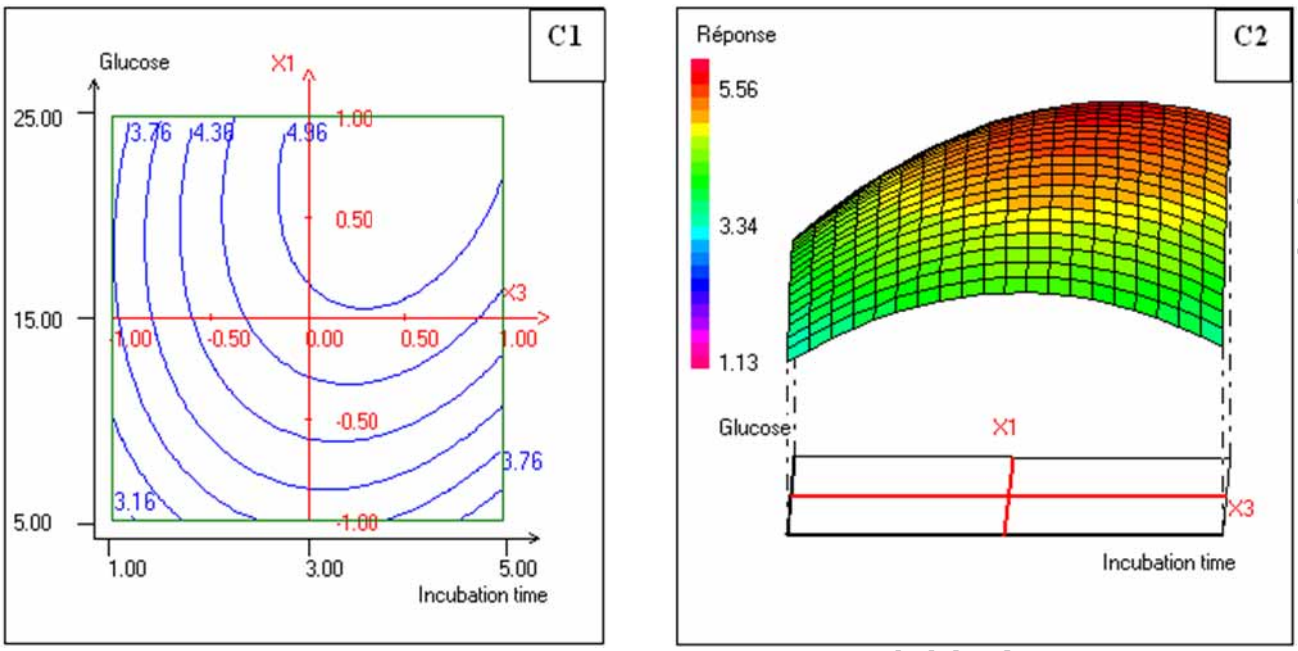










1 **Figure 4.** The 2D-contour plots and 3D-response surface of pKr yield (g/L) versus the tested
2 variables (g/L): glucose and peptone (A1and A2); peptone and incubation time (B1 and B2); glucose
3 and incubation time (C1 and C2).
4
5

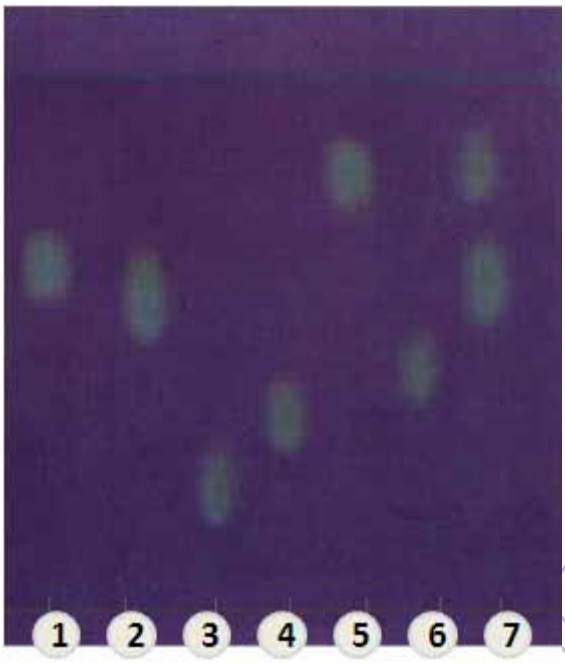


Figure. 5. Thin layer chromatography analysis of monosaccharide's composition of pKr.

Lane 1: glucose ; lane 2: galactose ; lane 3: lactose; lane 4 :maltose; lane 5 : xylose; lane 6 : saccharose. lane 7: pKr

Development system : potassium permanganate (3%)/ anhydrous sodium carbonate (4%) [2:1 (v/v)]

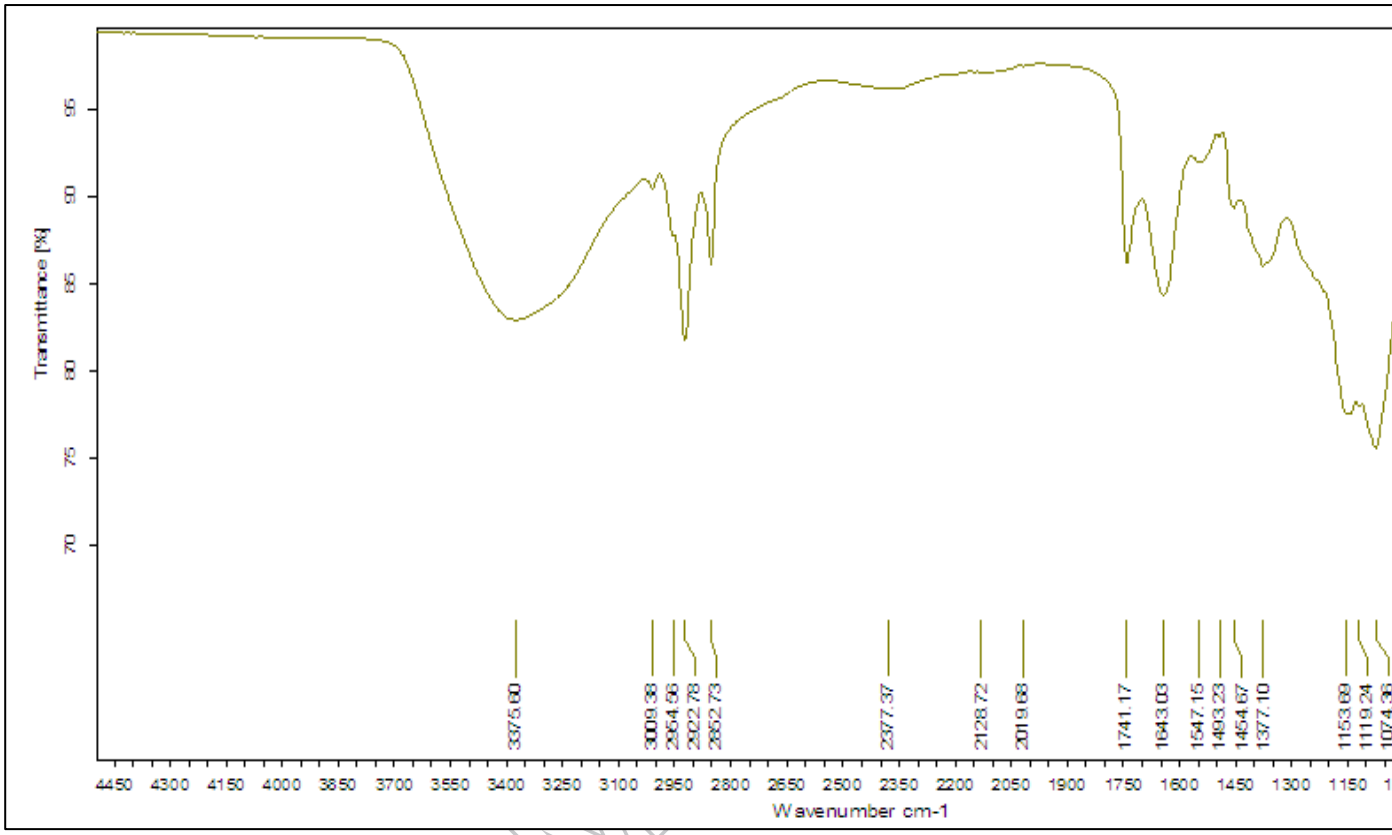
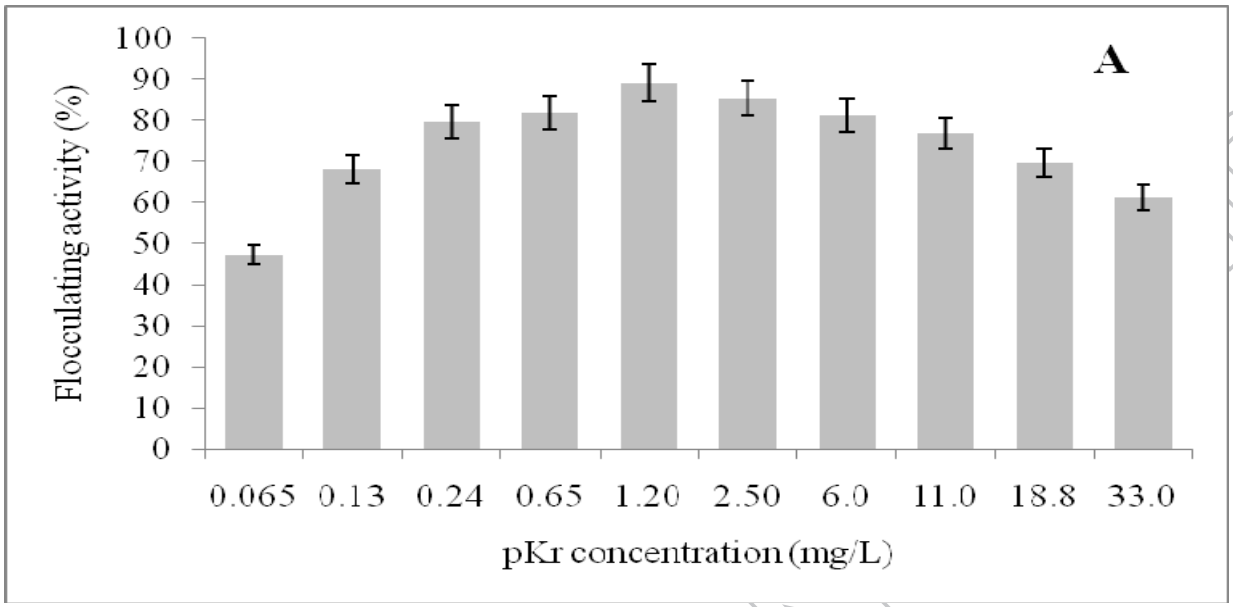
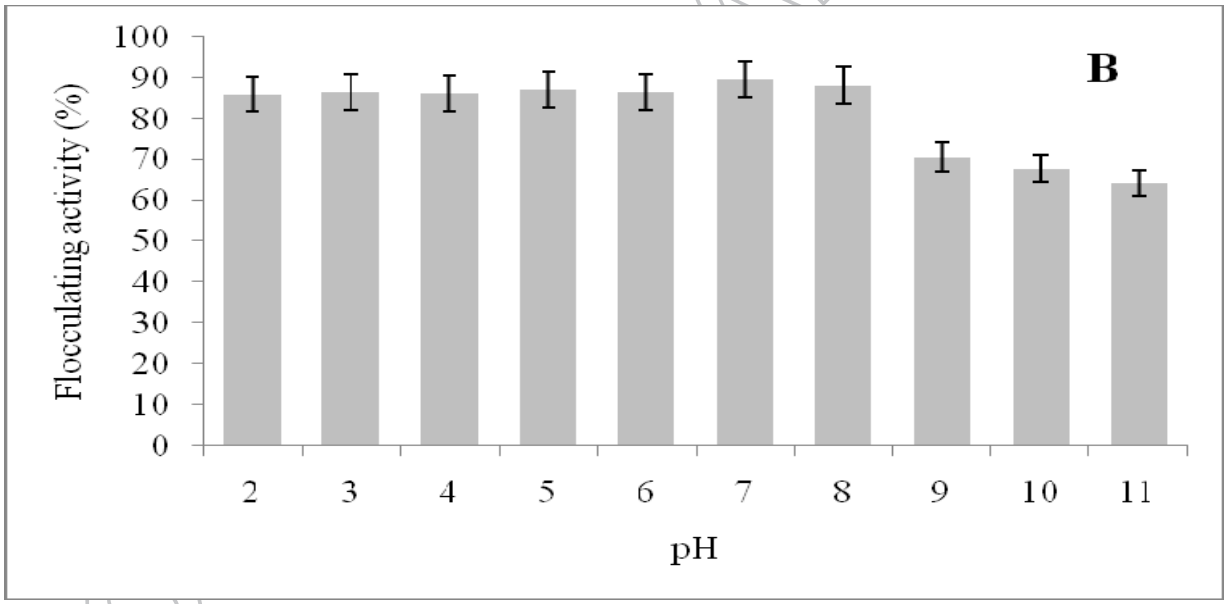
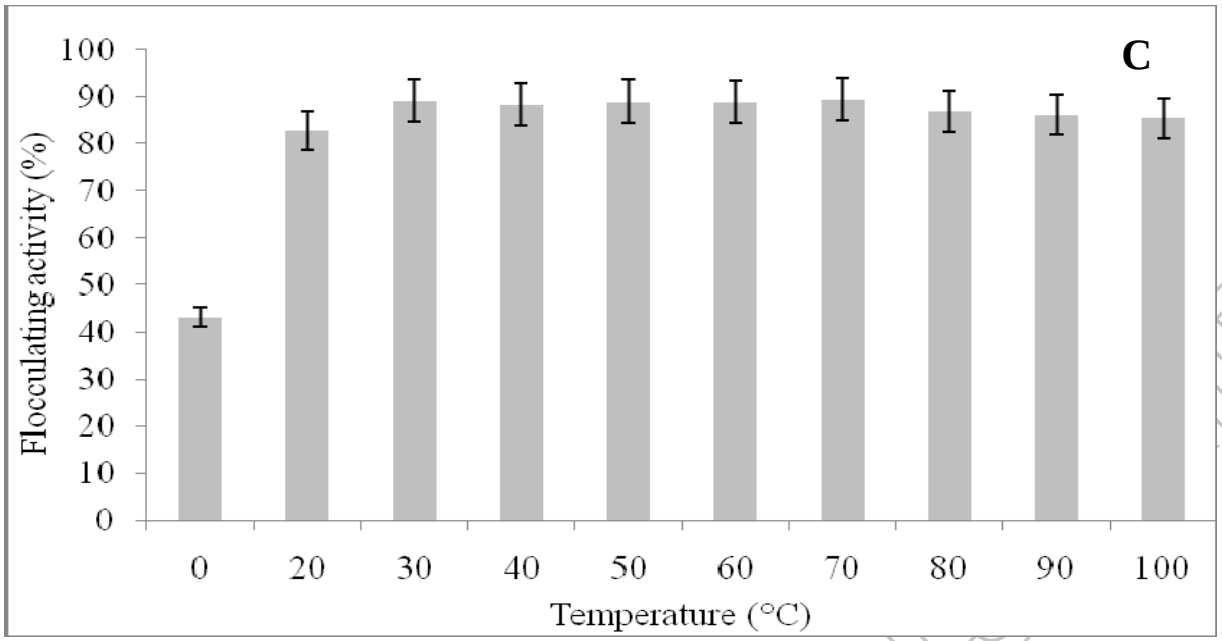

Transmittance [%]
95
90
85
80
75
70
3375.60
3009.38
2954.56
2922.78
2852.73
2377.37
2128.72
2019.68
1741.17
1643.03
1547.15
1493.23
1454.67
1377.10
1153.89
1119.24
4450 4300 4150 4000 3850 3700 3550 3400 3250 3100 2950 2800 2650 2500 2350 2200 2050 1900 1750 1600 1450 1300 1150
Wavenumber cm-1

Figure 6. FTIR spectrum of pKr. Fourier transform infrared (FT-IR) spectroscopy of pKr sample was obtained over a wave number range of 400-4000 cm^{-1} (Bruker Vertex 70 FTIR spectrometer).

Figure 7. Effect of pKr concentration (A), pH (B), temperature (C), and metal ions (D) on the flocculating efficiency of pKr.







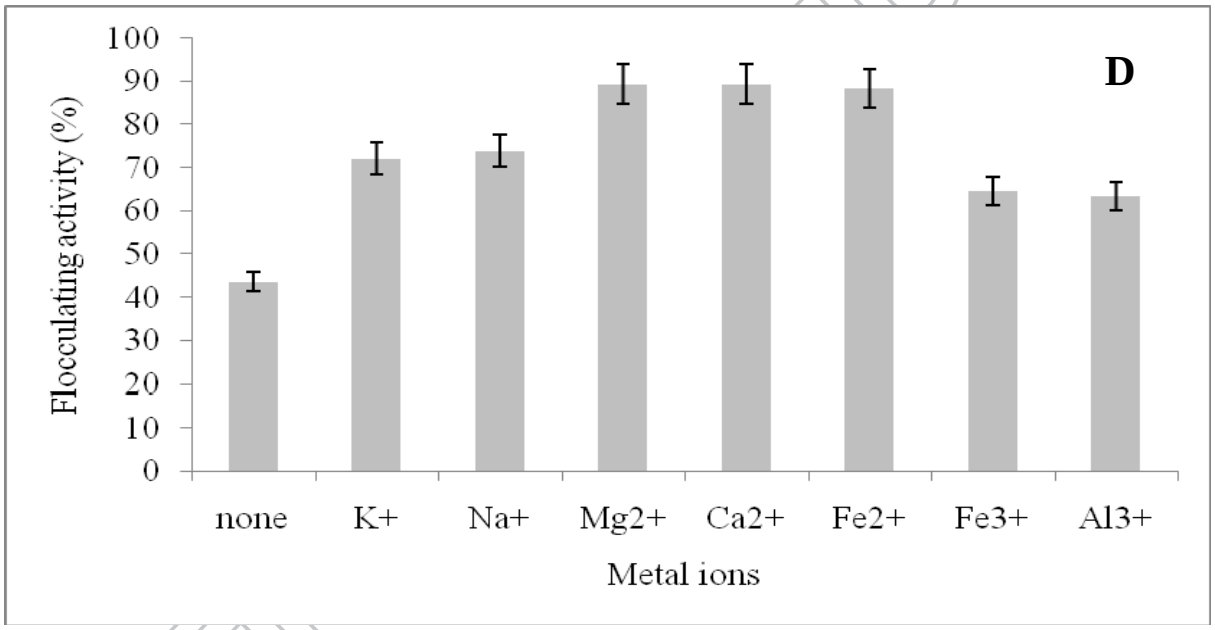


Table 1. Plackett-Burman experimental design for screening significant variables affecting pKr production.

Run	glucose (g/L)	peptone (g/L)	KH_2PO_4 (g/L)	Inoculum size (%)	pH	Temperature (°C)	Incubation time (days)	pKr yield (g/L)
1	1(10)	1(7.5)	1(2.5)	-1(0.5)	1(8)	-1(30)	-1(1)	1.12
2	1(10)	1(7.5)	1(2.5)	-1(0.5)	1(8)	-1(30)	-1(1)	1.02
3	-1(5)	1(7.5)	1(2.5)	1(1.5)	-1(6)	1(37)	-1(1)	0.70
4	-1(5)	1(7.5)	1(2.5)	1(1.5)	-1(6)	1(37)	-1(1)	0.95
5	-1(5)	-1(5)	1(2.5)	1(1.5)	1(8)	-1(30)	1(4)	0.78
6	-1(5)	-1(5)	1(2.5)	1(1.5)	1(8)	-1(30)	1(4)	0.65
7	1(10)	-1(5)	-1(2)	1(1.5)	1(8)	1(37)	-1(1)	0.82
8	1(10)	-1(5)	-1(2)	1(1.5)	1(8)	1(37)	-1(1)	0.89
9	-1(5)	1(7.5)	-1(2)	-1(0.5)	1(8)	1(37)	1(4)	1.00
10	-1(5)	1(7.5)	-1(2)	-1(0.5)	1(8)	1(37)	1(4)	1.03
11	1(10)	-1(5)	1(2.5)	-1(0.5)	-1(6)	1(37)	1(4)	1.20
12	1(10)	-1(5)	1(2.5)	-1(0.5)	-1(6)	1(37)	1(4)	1.41
13	1(10)	1(7.5)	-1(2)	1(1.5)	-1(6)	-1(30)	1(4)	1.26
14	1(10)	1(7.5)	-1(2)	1(1.5)	-1(6)	-1(30)	1(4)	1.42
15	-1(5)	-1(5)	-1(2)	-1(0.5)	-1(6)	-1(30)	-1(1)	0.60
16	-1(5)	-1(5)	-1(2)	-1(0.5)	-1(6)	-1(30)	-1(1)	0.58

Table 2. Experimental domain of the Box-Behnken design

Variable	Factor	Unit	Center	Step of variation
X_1	Glucose	g/L	15.0	10.0
X_2	Peptone	g/L	7.5	2.5
X_3	Incubation time	days	3.0	2.0

Table 3. Experimental conditions of the Box-Benheken design in coded and natural variables and the corresponding experimental and estimated responses.

Run	X_1	X_2	X_3	Glucose (g/L)	Peptone (g/L)	Incubation time(Days)	Experimental pKr (g/L)	Estimated pKr (g/L)
1	-1	-1	0	5.00	5.00	3.00	2.880	2.575
2	1	-1	0	25.00	5.00	3.00	5.110	5.233
3	-1	1	0	5.00	10.00	3.00	3.940	3.813
4	1	1	0	25.00	10.00	3.00	3.250	3.555
5	-1	0	-1	5.00	7.50	1.00	2.710	2.984
6	1	0	-1	25.00	7.50	1.00	3.700	3.546
7	-1	0	1	5.00	7.50	5.00	3.030	3.184
8	1	0	1	25.00	7.50	5.00	5.290	5.016
9	0	-1	-1	15.00	5.00	1.00	3.970	4.001
10	0	1	-1	15.00	10.00	1.00	2.260	2.109
11	0	-1	1	15.00	5.00	5.00	3.010	3.161
12	0	1	1	15.00	10.00	5.00	4.650	4.619
13	0	0	0	15.00	7.50	3.00	5.120	4.810
14	0	0	0	15.00	7.50	3.00	4.610	4.810
15	0	0	0	15.00	7.50	3.00	4.680	4.810
16	0	0	0	15.00	7.50	3.00	4.810	4.810
17	0	0	0	15.00	7.50	3.00	4.830	4.810

Table 4. Growth of bacteria with different hydrocarbon in solid medium (+: growth, - no growth)

Strain	Crude oil	Pr	Ph	Py	Bab	Bph	Na	Car	Oct	Flu	DBT	DBF	Sqa	An
BU22S	+	+	+	+	-	-	+	-	+	+	-	-	+	+

Pr (Pristane),Ph (Phenanthrene), Py (Pyrene), BaP (B(a)Pyrene), Bph (Biphenyl), Na (Naphtalene), Car (Carbazole), Oct (Octadecane), Flu (fluoranthene), DBT (dibenzothiophen) DBF (dibenzofurane), Sqa (squalene) , An (Anthracene).

Table. 5. ANOVA for the response surface quadratic model

Source of variation	Sum of squares	Degrees of freedom	Mean square	Ratio	Significance
Regression	14.6611	9	1.6220	18.5619	***
Residuals	0.6143	7	0.0878		
Validity	0.4609	3	0.1536	4.0063	N.S.
Error	0.1534	4	0.0384		
Total	15.2754	16			

***Significant at the level 99.9% N.S.: non significant