

# Physio-chemical analysis and kinetic modeling of phenol degradation by the endophyte *Kocuria rhizophila* 14asp

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## Research Article

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# Abstract

Phenol is a carcinogenic pollutant found in a range of industrial effluents in concentrations up to several mg L<sup>-1</sup>. Here, we investigated *in vitro* phenol degradation by non-starved endophytic *Kocuria rhizophila* 14asp in batch mode at a wide range of concentrations (1500 mg L<sup>-1</sup> to 6500 mg L<sup>-1</sup>) under a variety of conditions. The optimum temperature and pH for maximum phenol degradation were found to be 35 °C and 7.3, respectively. The maximum extent of phenol degradation was 93% after 144 hrs when the culture was fed 1500 mg phenol L<sup>-1</sup>, and resulted in a maximum cell yield of 0.21. Kinetic modeling with Haldane, Edward and Yano kinetic models showed that the Edward model best described the phenol degradation kinetics by *K. rhizophila* 14asp. Given the ability of *K. rhizophila* 14asp to utilize high concentrations of phenol as sole source of carbon and energy, it may be useful in biodegradation systems.

## Introduction

In Pakistan, phenol level in water resources is increasing due to release by pharmaceutical industries. Phenol polluted environments negatively impacts plants and animals, and inhibits biotransformation process catalyzed by microbial communities when present at high concentrations [1,2]. Phenol has been found to be toxic to humans even at low concentrations (mg L<sup>-1</sup>) if it is ingested, inhaled or makes direct contact with eyes, skin or mucous membranes. In aquatic environments, phenol is detrimental to fish communities. It was reported that phenol levels in various industrial effluents has reached up to 6.8 g L<sup>-1</sup>, while The European-Union has set the upper limits of phenol at 0.5 mg L<sup>-1</sup> for wastewater emissions and 0.0005 mg L<sup>-1</sup> for potable water [3]. The United States Environmental Protection Agency has kept phenol on its priority pollutants list due to its toxicity and environmental effects [4].

Over the past few decades, many techniques have been considered for phenol removal from industrial effluents and contaminated sites. Biodegradation has gained special attention due to its environmentally friendly results and the possibility of achieving complete mineralization without the accumulation of toxic secondary products [5]. Phenol biodegradation in industrial wastewater can be challenging due to variations in temperature, pH, contaminant concentrations and overall water quality. Further phenol bioavailability [6] and its ability to inhibit the biodegradation of other organic compounds [7] pose additional challenges. Despite this, the efficiency of microbes for phenol removal from contaminated wastewater has been demonstrated [8].

To date, research has focused on identifying microorganisms able to degrade high levels of phenol, often under saline conditions. Species of *Pseudomonas*, *Halomonas*, *Bacillus* and *Candida* have been reported as effective degraders [9-12]. The use of genetically engineered microorganism (GEM) for biodegradation is generally not favored by government agencies due to the potential for unexpected ecological influences [1]. Therefore, natural microorganisms with high levels of phenol tolerance are required for effective biodegradation systems.

The current study is focused on the optimization and modeling of batch phenol biodegradation by the endophyte *K. rhizophila* 14asp under a wide range of environmental conditions. To the best of our knowledge, this is the first time that a *Kocuria* spp. has been reported to metabolize phenol. The investigation is aimed at employing kinetic models like Yano, Edward and Haldane for representation of best fit to batch experimental data. The study is designed to evaluate the selected strain which could be used for the treatment of phenol in contaminated effluents.

## Materials And Methods

### Chemicals and microorganism

Phenol and other inorganic salts were purchased from BDH and Merck that were used in preparing growth media. The endophytic strain *Kocuria rhizophila* (14asp) with Gene bank number KF875448 [13] and the whole genome was sequenced with the help of Dr. Jonathan D. Van Hamme (BioProject ID: PRJNA286912). The strain was generously provided by the Plant Microbe Interaction Lab, Quaid-i-Azam University, Islamabad Pakistan based on the biodegradation characteristics. *K. rhizophila* 14asp was grown on solid mineral salt medium (MSM) with different initial substrate concentrations to select it for further phenol degradation study.

### Growth media and culture conditions

The modified and Sterilized growth MSM media [8] with phenol at different initial concentrations was used at the start of experiment. The pH of the medium was adjusted to optimum pH.

### Effect of different physio-chemical parameters on the biomass and phenol biodegradation ability

#### Effect of pH and temperature

The effect of pH on phenol degradation by *K. rhizophila* 14asp was evaluated in range of 6-8 and turbidity of biomass ( $OD_{600}$ ) was observed. The experiment was performed in 250 ml conical flask containing 100 ml mineral salt medium amended with  $15000 \text{ mg L}^{-1}$  of phenol. The flasks were incubated at  $37 \text{ }^\circ\text{C}$  operating at 135 rpm. The effect of temperature on phenol degradation rate and turbidity of biomass ( $OD_{600}$ ), *K. rhizophila* 14asp was obtained from exponential phase culture, cultivated in phenol ( $1500 \text{ mg L}^{-1}$ ). Then it was incubated under 22, 27, 35 and  $40 \text{ }^\circ\text{C}$  temperatures. After every 24 hrs, the reaction mixture was taken and phenol consumption rate was analyzed through HPLC.

### Phenol biodegradation studies

Based on agar plate evaluation (section. 2.1) *K. rhizophila* 14asp was preceded for further degradation study. Selected bacterial strain was grown in nutrient broth by incubation at optimized conditions for 22 hrs. The culture ( $OD_{600} = 1.5$ ) was inoculated (5% v/v) into sterilized MSM growth medium with phenol as only carbon source. Initial degradation studies were carried out in batch mode; with addition of inoculum

in MSM containing  $1500 \text{ mg L}^{-1}$  of phenol and cultivated in 250 ml flask with 130 rpm in incubator shaker. The MSM devoid of bacterial inoculums were used as control to observe any reaction between media and phenol. After every 24 hrs reaction mixture was analyzed for cell and residual phenol concentration.

#### Analysis of cell and phenol residual concentration

One ml sample was taken aseptically from each flask after every 24 hrs. Samples were centrifuged (WiseSpin, CF10) at 3000 rpm for 20 min to remove suspended biomass from supernatant. For measurement of microbial growth, biomass concentration was determined by spectrophotometer (Hitachi, U-1500) measuring the turbidity of diluted sample at  $OD_{600}$ . After that removed suspended mass was measured on weighing balance (SARTORIUS AG, GERMANY) for wet weight of biomass. The weighed biomass was kept in dry oven (SHELDON, USA) at  $100^\circ \text{C}$  for 15 hrs and weighed again for dry biomass.

The residual phenol concentration in supernatant was determined through high performance liquid chromatography HPLC (Shimadzu, Japan), with a column (Shim-Pack CLC-ODS (C-18), 25cm x 4.6mm,  $5\mu\text{m}$ ) with a detector operating at 270 nm UV-VI. Mobile phase was ethanol/water (70/30 v/v). The flow rate was 0.7 ml/minute and column was operated at room temperature. Authentic samples were run for HPLC to determine substrate concentration. The substrate utilization was determined by measuring the peak area of phenol. The whole experiment was setup in three replicates.

#### Phenol degradation kinetics and modeling

Among available theoretical kinetic models, Haldane, Edward and Yano kinetic models were selected for kinetic modeling of experimental data in order to select the best fit model. Experimental degradation rate was analyzed by MATLAB 7.1. Further kinetic parameters of various models were investigated by non-linear regression analysis for selected models (Table 1).

Degradation rate  $h^{-1}$  ( $q$ ) was analyzed from the slope of negative logarithmic graph against  $S_0$ . Further evaluation is done by fitting our experimentally determined values obtained from the batch mode degradation experiments (described in Section 2.4) to different theoretical kinetic models for selection of best suitable model. Whereas  $S_0$  ( $\text{mg L}^{-1}$ ) is initial substrate concentration,  $K_s$  ( $\text{mg L}^{-1}$ ) is the substrate-affinity constant,  $K_i$  ( $\text{mg L}^{-1}$ ) is the substrate-inhibition constant,  $q$  ( $h^{-1}$ ) is degradation rate,  $S$  is final substrate concentration and  $q_{\text{max}}$  ( $h^{-1}$ ) the maximum degradation rate. Final representation of data was done by plotting  $q$  verses  $S_0$  to show best fit of theoretical model.

#### Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) and the mean differences were compared by Tukey's Multiple Comparison Test at  $p < 0.05$ . All the treatments were in three replicates. All the analyses were performed using GraphPad Prism 5 software.

# Results And Discussion

## Selection of phenol degrading microorganism

The endophytic bacterial strain *K. rhizophila* 14asp was selected on the bases of its growth rate and survival at elevated initial phenol concentrations on MSM agar plates. The selected bacteria grew by using phenol as the only carbon source.

During the study it was also observed that after 24 hours of phenolic stress, colonies of *K. rhizophila* 14asp appeared white and lubricious from clear yellow morphology.

## Optimization Analysis

The rate of degradation of a pollutant influenced by various factors, but environmental fluctuations is most affecting ones [6]

The effects of different physio-chemical factors in the degradation efficiency of phenol were studied in order to optimize the performance of bacterium for obtaining the highest biomass and phenol degradation rate.

## Effect of incubation temperature on growth and degradation

The Exponential growth period was observed at 1500 mg L<sup>-1</sup> which was selected for further physio-chemical parameters. The influence of these parameters was evaluated on rate of phenol degradation and growth of *K. rhizophila* 14asp. This growth period was further confirmed by faster consumption rate of phenol.

The experimental data on the cell biomass (OD<sub>600</sub>) of *K. rhizophila* 14asp at four different incubation temperatures (Fig. 1A) showed non-significant differences in biomass (OD<sub>600</sub> nm). As well as Fig. 1(B) also expressed non-significant differences among residual phenol degradation profile in wide range of temperature. However, it was recorded from (Fig. 1B) that degradation profile at 35 °C was higher. The amount of phenol degraded was increased at optimum temperature.

Most field and laboratory studies on phenol biodegradation showed that rate of degradation were more affected by temperature rather than microbial growth [14]. In general, most studies on phenol degradation have been carried out at an optimum temperature of 30 °C [15,8,16]. We found that phenol degradation rate was best at 35 °C and showed non-significant change in degradation rate between 22-40 °C. The reason for this consequence will need further investigations.

## Effect of different pH on growth and biodegradation

The time course profile of *K. rhizophila* 14asp growth in 1500 mg L<sup>-1</sup> phenol containing MSM at pH 6-8 was shown in Fig. 2(A and B). The cell growth was non-significantly affected by change in tested pH

range. However *K. rhizophila* 14asp showed little retarded growth at acidic pH. While 7 and 7.3 pH showed non-significant differences and lead to significantly increase in cell growth. The remaining substrate concentration (%) profile of *K. rhizophila* 14asp was shown in Fig. 2(B). Results of this degradation profile showed that *K. rhizophila* 14asp optimally degraded phenol at 7.3pH.

The optimum pH 7.3 was gradually fluctuated with increase in initial concentration and was in good agreement with the previous reported study [17]. It has also been reported in literature that various strains efficiently degrade phenol at near a neutral pH [18]. A similar observation was made in this study.

## Phenol degradation

### Effect of substrate concentration on growth rate and biodegradation

In order to investigate the effect of substrate concentration on growth rate of bacterium and rate of biodegradation of phenol, experiment was performed at different initial concentrations of phenol. As seen in (Fig. 3A) bacterium showed maximum growth rate  $\text{h}^{-1}$  at  $1500 \text{ mg L}^{-1}$  phenol but with increase in initial concentration growth rate inversely effected. Similar results were reported in earlier studies [19].

Fig. 3(B) showed the results of residual phenol degradation (%) profile of a wide range of initial concentration  $1500 \text{ mg L}^{-1}$  to  $6500 \text{ mg L}^{-1}$  with 24 hrs time intervals. Further evaluation through Tukey's Multiple Comparison Test showed that first initial concentration has comparable a high degradation rate than other initial concentrations. Results illustrated that with the increase in substrate concentration % degradation and degradation rate slows down (Fig. 3B & 6).

### Effect of substrate concentration on cell yield

Cell yield (Y) was expressed in terms of substrate concentration to illustrate the relation between cell increase and substrate loss (Eq.1) [20]. Where  $X_0$  and  $X_m$  are the initial and maximum dry cell concentrations,  $C_0$  and  $C_s$  are initial substrate concentration and substrate concentration at the maximum cell concentration respectively.

$$Y_{x/s} = \frac{(X_m - X_0)}{C_s - C_0} \quad (\text{Eq. 1})$$

Fig. 4 showed effect of substrate concentration on cell yield (Y) which was calculated by linear regression analysis through GraphPad Prism 5 software. Obtained maximum biomass concentration  $\text{mg L}^{-1}$  ( $X_m$ ) for various final substrate concentration was 0.21 ( $R^2 = 0.8087$ ) which was smaller when compared with the values recorded by other researchers [1,21,22]. It might be due to high initial concentration of substrate. However, it ratified the adoptability and resistance of selected bacteria against toxic effects of phenol.

## Kinetics and modeling of phenol degradation

It was previously described that microbial growth relates to consumption or degradation rate of pollutant [23], later on it was proved that substrate degradation was the measure of microbial performance [24]. Most studies were available on specific growth rate but limited data was available on kinetic modeling with respect to degradation rate [25,22]. In concern to existing knowledge, we have calculated influence of substrate concentration on degradation rate with kinetic modeling.

So, phenol degradation study was further established by kinetic modeling by considering growth rate as constant. All the  $q$  values (degradation rate  $h^{-1}$ ) calculated for  $S_0$  values were used in phenol degradation study. Typical plot of various initial concentrations to find the value of  $q$  (section. 2.6) was a straight line which indicated that the degradation rate was first order (Fig. 5).

Fig. 6 displayed  $q$  variations for different  $S_0$  values. The experimental maximum degradation rate ( $q_{max}$ ) was obtained as 0.002 at 1500  $mg L^{-1}$  initial concentration. As the initial concentration increased, degradation rate was decreased.

Kinetic parameters of three theoretical models (already described in Table 1) were estimated by experimentally derived  $q$  values. Table 1 indicated that Edward model was best fit to entire experimental data among three described models.

Evaluation of degradation kinetics by model fitting has shown that all the three models could predict the experimental data (Table 1) [26]. However, in all three models Edward gave the best fit for experimental data ( $R^2 = 0.991$ ,  $SD = 0.018$ ) as the evaluated degradation rates were very similar of experimental values. The Edward model showed high  $R^2$  value which also confirmed its accuracy. The distinctive kinetic characteristic of *K. rhizophila* 14asp was observed as high  $K_s$  (Table 1) better phenol degradation activity. Previous literature has given the fact that high  $K_i$  value revealed the higher resistance to substrate inhibition [27].  $K_i$  value (1766.935) for Edward model showed that *K. rhizophila* 14asp has strong inhibition effect due to increase in initial concentration but proved great tolerance towards phenol biodegradation.

## Conclusion

The endophytic strain *K. rhizophila* 14asp was able to degrade elevated levels of phenol with the increase of biomass. The optimization study resulted in approval of wide range environmental conditions for this strain in biodegradation system. HPLC confirmed degradation analysis for *K. rhizophila* 14asp and maximum rate of biodegradation ( $q_{max}$ ) was determined. The  $q_{max}$  was found at initial concentration of 1500  $mg L^{-1}$ . Phenol degradation by *K. rhizophila* 14asp showed poor degradation efficiency at and above 2500  $mg L^{-1}$  substrate concentration. Overall extent of phenol removal has been observed 32% in 144 hrs. However, this strain displayed great tolerance over high initial concentration of 6500  $mg L^{-1}$  than earlier reported microbes. The modeling of degradation kinetics could well be represented by Edward model. It could be concluded from the present report that *K. rhizophila* has shown slow degradation rate but proved effective for treatment of excessive phenol containing industrial effluents. The study will have

impact as by providing reference literature for biodegradation systems of phenol using *Kocuria rhizophila*.

## Declarations

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### *Conflict of interest statement*

The authors declare that they have no conflict of interest.

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## Figure

Figure 6 is not available with this version

## Table

Table 1 is not available with this version

## Figures

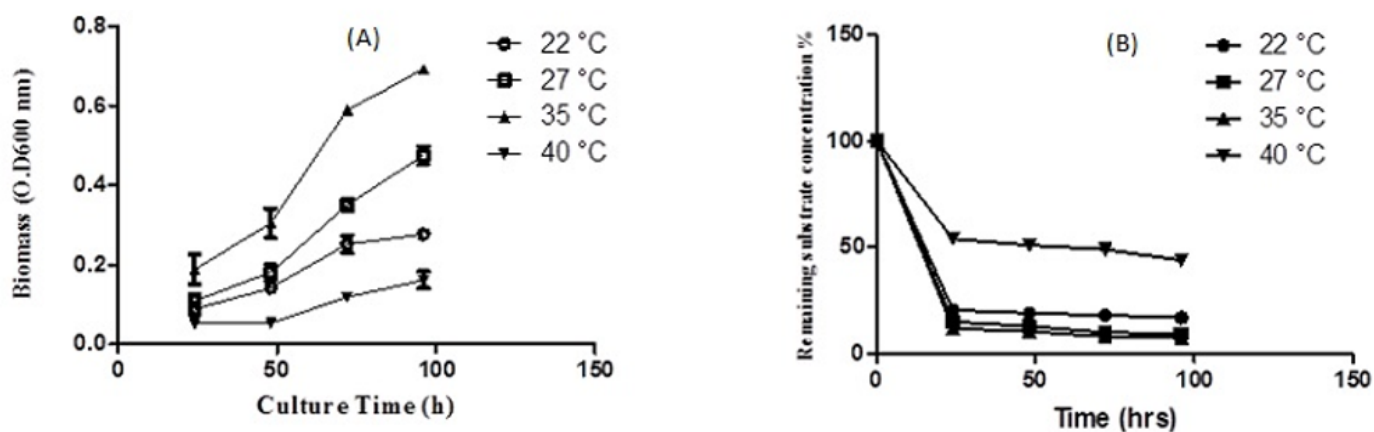


Figure 1

Effect of temperature on biomass (OD) and phenol degradation ability by *K. rhizophila* ( $p < 0.001$ ,  $n = 3$ ) (B) Effect of incubation temperature on remaining phenol degradation (%) profile

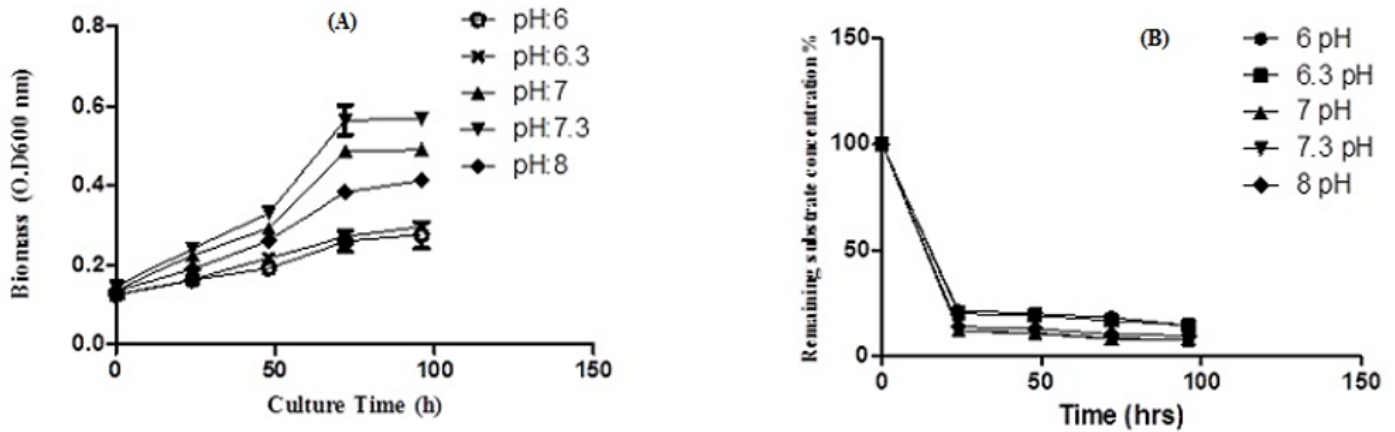


Figure 2

Effect of pH on biomass (OD) and phenol degradation ability by *K. rhizophila* ( $p < 0.05$ ,  $n = 3$ ) (B) Effect of different pH on remaining phenol degradation (%) profile

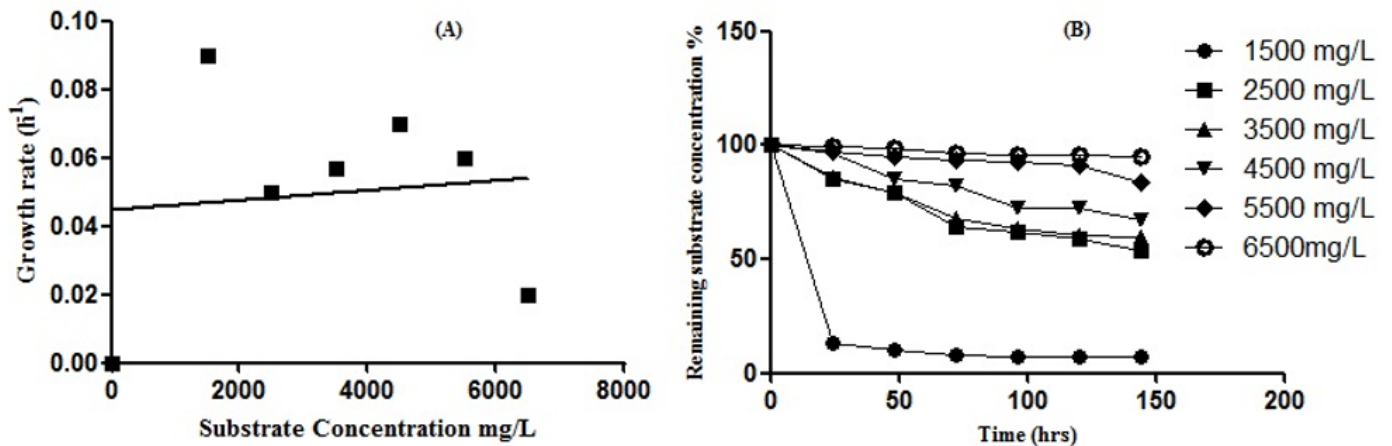


Figure 3

Effect of substrate concentration on growth rate ( $h^{-1}$ ) and biodegradation (A) Growth rate ( $h^{-1}$ ) on phenol by *K. rhizophila*, grown in shake flask culture in MSM at 35 °C (B) Residual phenol degradation (%) profile for its various substrate

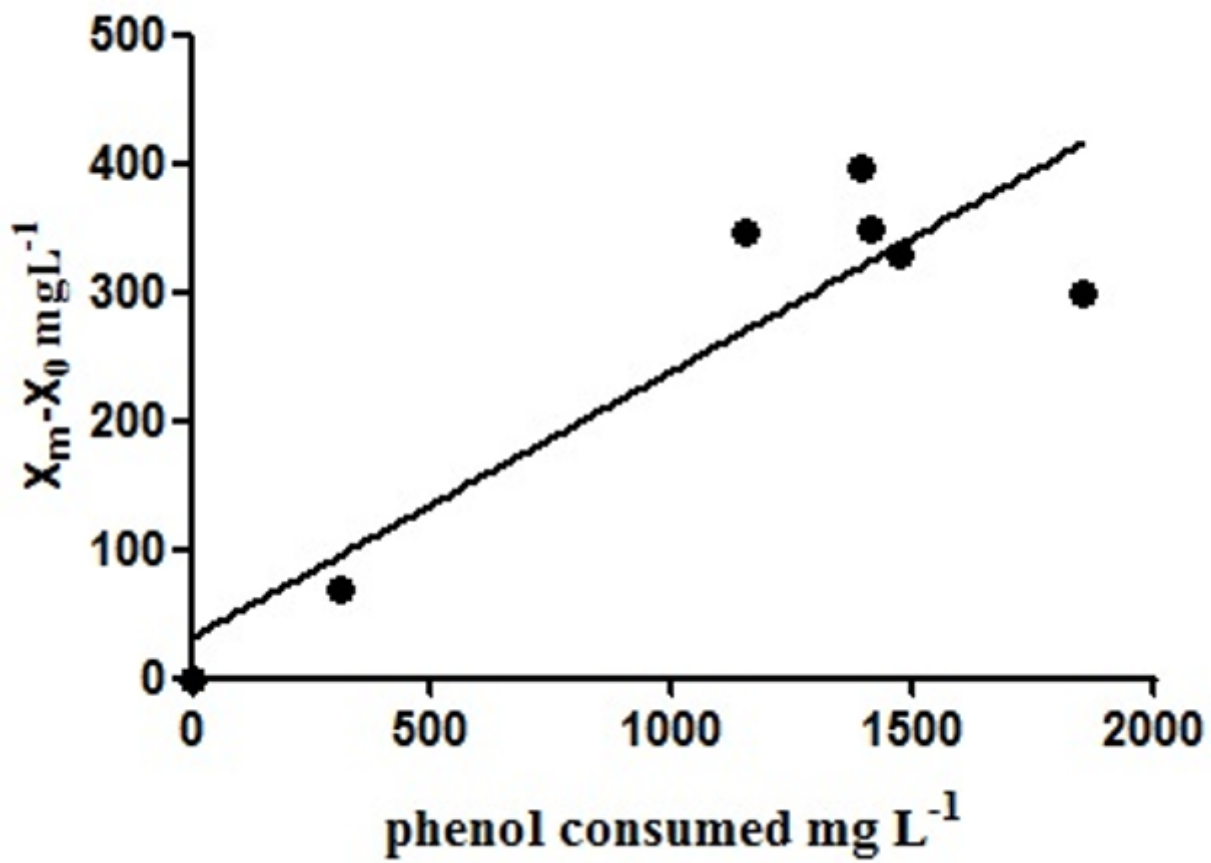


Figure 4

Determination of cell yield of *K. rhizophila*, grown in shake flask culture in MSM containing phenol as a substrate and carbon source

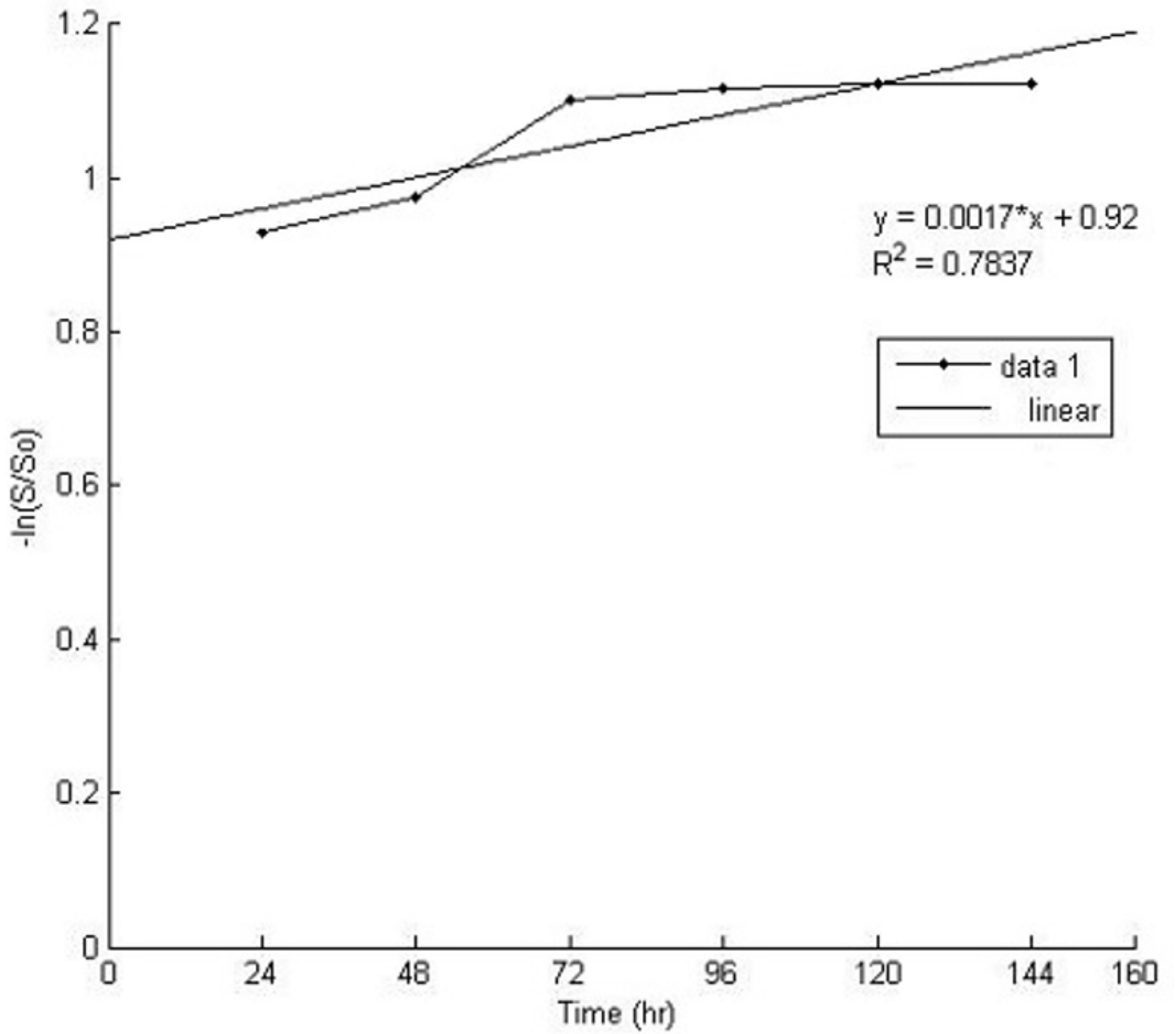


Figure 5

Typical plot of determination of  $q$ , when the initial concentration was  $1500 \text{ mg L}^{-1}$