GROWTH KINETICS OF RHODOCOCCUS ERYTHROPOLIS IGTS8 ON THIOPHENE AND DIMETHYLSULPHOXIDE

BY

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1-Abstract

Sulfur oxides emissions through fossil fuel combustion posse environmental problems because these oxides are major cause of acid rain, which dissolve buildings, kills forests and poisons lakes. That is why world environmental regulations are becoming harder with respect to sulfur content in fossil fuels. The demand for new low-cost desulfurization technologies has led to renewed interest in biodesulfurization.

In this work the growth kinetics of *Rhodococcus Erythopolis* IGTS8 have been studied on two model sulfur compounds that are found among other sulfur compounds in petroleum fractions. These are namely thiophne and dimethyl sulfoxide , DEMSO. Batch reactor and different substrate concentrations ranging between 1 and 8 gl^{-1} were used to study the growth kinetics. Growth on thiophene was shown to follow Monode kinetics with μ_{max} =0.064 h^{-1} and K_s = 2.8gl^{-1}, on the other hand, growth on DEMSO was found to be substrate inhibited.

2- Introduction

The concept of microbial desulfurization is not new, BDS has been reported since 1935 and much work has been done in order to understand the biochemistry of microbial attack on organic sulfur compounds and the mechanisms of BDS reactions (Malik, 1978). The first U.S. patents were issued in the 1950s. This technology has not been commercialized because the biological systems has lacked reaction kinetics and specificity required for commercial application. There have been several reports over the last 20 years of successful lab scale ”microbial desulfurization” that was of limited commercial interest. The problem was that although they desulfurized the oil, they also consumed most of it. (Monticello, 1994).

BDS is a process that is being developed based on naturally occurring bacteria that can remove organically bound sulfur from petroleum without degrading the fuel value of the product. (Monticello, 1993). The use of microorganisms for the BDS of high sulfur coals and oils has attracted attention as a potential pre -combustion technology for the reduction of the organo- sulfur content of fossil fuels. (Finnerty, 1993).

A number of organisms have been shown to be able to metabolize DBT to CO2 and biomass with the destruction of carbon- carbon bonds, but this reduces the fuel value. The mechanism for removal of the sulfur atom from DBT by IGTS8 is the most extensively studied example of microbial mediated thiophene ring cleavage. To a large part, Energy Biosystems Corporation (EBC) also concentrated in their studies on *Rhodococcus* IGTS8 to develop a commercial scale for biodesulfurization process (Monticello, 1994).The Strain IGTS8 (ATCC53968) is able to extract sulfur from a variety of Organo sulfoxides and sulfones (Kayser *et al.*,1993). Further more, the organism is able to remove organic sulfur from petroleum and soluble coal- derived materials without decreasing the caloric value of those substrates (Kilbane and Jackwski, 1992).The metabolism of *Rhodococcus* IGTS8 is under aerobic conditions requiring oxygen with the pH of about 6 to 7, and a temperature of about 28^{o}C to 32˚C. The higher temperature range result in faster metabolism, however, the microorganisms are known to not tolerate temperatures in the order of 37 ^{o}C (Kilbane, 1992).

Wang[a] *et al.,(*1996) Studied the kinetics in batch and fed-batch cultures of *Rh erythropolis* N1-36. In flask cultures, production of the desulfurization product, monohydroxybipheniel (OH-BP), was maximal at pH 6.0. Batch and glucose –fed –batch cultures of N1-36 having either DBT or $DBTO_2$ as the sole sulfur source where used to establish the kinetics of both growth and desulfurization. The specific exponential growth rate (**μ)** of batch cultures with 100 μM DBT was 0.153 hr^{-1}, while the corresponding rate with 100 μM $DBTO_2$ as the sulfur source was 0.180 hr^{-1}. Stationary phase typically occurred after approximately 40 hr of growth.
The standard conditions for kinetic analysis in bioreactors were as follows: temperature, 30 °C, agitation speed 300 rpm, and aeration 1.5 vol./vol./min, the pH was initially 6.0.
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Setti *et al.*, (1999) studied the DBT biodesulfurization activity of *Rhodococcus erythropolis* IGTS8 in batch conditions. Growth of Rh.

IGTS8 on 1% (w/v) glucose and 200 ppm DBT as the sole carbon and sulfur sources respectively was monitored with a turbidity meter and the values reported as turbidity units, the stationary phase was reached after 150 hr and the lag phase was close to 48 hr. The specific growth rate (**μ**) and product formation coefficient of the strain were found close to 0.061 h^{-1} and 2.65 g HBP produced / g dry biomass respectively. Also, the maximum specific rate of HBP production of the strain was close to 0.7 g HBP produced /g of dry biomass $^{-1}$ h^{-1}. The conditions for kinetic analysis were as follows: temperature 30 °C, agitation speed 200 rpm, and pH 6.5.

As shown above, most of BDS kinetic studies are focused on DBT as a model sulfur compound because it is the most calcitrate sulfur compound w.r.t. catalytic hydrodesulfurization. Other compounds like thiophene and dimethylsulfoxide though are among the sulfur compounds found in petroleum fractions, did not receive similar attention.

In this paper growth kinetics of *Rhodococcus erythropolis* IGTS8 are studied on the two sulfur bearing compounds; thiophene and DMSO. The results are compared with those obtained for the same microorganism on DBT and petroleum fractions and published in another communication (El-Temtamy et al. ,2004).

3. Basic Growth Kinetics

Considering growth of microorganisms in a batch culture, it may be assumed that the growth rate is proportional to cell concentration (X).

$$\frac{dx}{dt} = \mu x \qquad \textbf{(1)}$$

where the proportionality constant (μ. is known as the specific growth rate (h^{-1}). Rearranging and integrating equation (1)

$$\int_{xo}^{x} \frac{dx}{x} = \int_{o}^{t} \mu \, dt \qquad \textbf{(2)}$$

at time t=0, X= X_o then

$$\ln \frac{x}{x_\circ} = \mu t \qquad \textbf{(3)}$$

If the growth rate can be described by equation (3) then the microorganism is said to be growing in the exponential phase. In this phase if ln X/X_o is plotted versus time, a straight line would result with a slope equals to μ (Wang et al., 1979). Plotting experimental growth data in the form of natural logarithm of cell concentration versus time will often yield a straight line over a large portion of the curve, as shown in fig.(1). In the range from t_1 to t_2 the curve is linear, and this is the region of exponential growth.

Three other regions can be identified between t_o and t_1, there exists a period of cell adaptation or lag phase and beyond t_2 there is a region where the growth is limited by the lack of a particular substance, the number of new cells is equal to the number of dying cells, thus a stationary phase till t_3. In many cases cells stop multiplying and die after t_3 because of nutrient's depletion, toxic effects or cell aging.

The exponential and deceleration regions can be described by a single relation that sets μ equal to a function of substrate concentrations. It has been observed experimentally that μ is at maximum when the particular limiting substrate concentration , S, is large, and for low concentration μ is proportional to S. Over the whole range from low to high S, μ is described by the following Monod equation:

$$\mu = \mu_{max} \frac{S}{S + Ks} \qquad (4)$$

This is a two parameter equation involving two constants, where μ_{max} h^{-1} is the maximum specific growth rate and *Ks* gl^{-1} is the saturation constant. Monod equation is best considered to be an empirical equation. Taking the reciprocal of Equation (4) then;

$$\frac{1}{\mu} = \frac{1}{\mu \max} + \frac{1}{\mu \max} \frac{Ks}{S} \qquad \textbf{(5)}$$

A plot of $1/\mu$ versus $1/S$ yields a strait line with a slope Ks/μ_{max} and an intercept of $1/\mu$ max if Monod kinetics is valid (Baily et al.,1987).

4.Materials and methods

4.1. Microorganism

Rhodococcus erythropolis IGTS8 (ATCC 53968) was obtained from American Treasury Culture Collection.

4.2. Media

4.2.1.Basal Slat medium (BSM)

A defined BSM consisted of:2.44 g of KH2PO4, 5.57 g of Na2HPO4, 2.0 g of NH4CL, 0.2 g of MgCl2.6H2O, 0.001g of CaCl2.6H2O, 0.001 g of FeCl3.6H2O, 0.004 g of MnCl2.4H2O, 6.4 ml of Glycerol. Completed by distilled deionised water to one litre. Heat was used to completely dissolve the salts and to produce a homogenous solution. pH of medium was adjusted to7.0. by NaOH if necessary. (Piddington *et al.*, 1995) and (Patel *et al.*, 1997).

BSM was used to assay bacteria for their ability to desulfurize the sulfur compound

4.2.2.Luria Broth Medium (LB)

A defined LB medium consisted of 1% Bactotryoptone , 0.5% yeast extract and 1% NaCl. were dissolved in 1 liter of distilled deionized water (Denome, *et al*(1994)):

4.3. Chemicals

Thiophane and Dimethylsulphoxide Analar grade produced by Merk were utilized.

4.4. Equipment

- pH meter model 3071.
- Shaker (with Temperature control)
- Autoclave
- Mixer
- Centrifuge
- Oven
- Heater
- Separation funnel
- Spectrophotometer (UNICAM.5625 UV/VIS, England

4.5. Exprimental Procedure

A set of experiments have been done to follow up the growth of Rhodococcus IGTS8 on sulfur containing compounds. Growth was followed up by monitoring the optical density of the culture media as a function of time using Unicam5625 UV/VIS spectrophotometer.

4.5.1. Preparation of Cell Culture.

The procedure followed here is adopted from (Denome et al., 1994).

- Rh. erythropolis IGTS8 was heavily inoculated in LB and incubated for 24 hours at 130 rpm and 30 ^{o}C.
- Cells were pelted by centrifugation at 5000 rpm for 15 minutes.
- The supernatant was removed and the pellets were washed three times in sterilized BSM and re-suspended in sterilized fresh BSM.
- Washed cells were used to inoculate sterilized fresh BSM containing the test substrate (sulfur compounds) under sterile conditions (the inoculation size was 5% v/v).

4.5.2. Growth experiments

1- BSM containing the sulfur compounds were sterilized in autoclave for 15 minutes and left to cool.
2- Cold BSM containing sulfur compound was inoculated by (5%v/v) cells that were previously prepared.
3- Non- inoculated BSM was used as blank
4- The culture was incubated at 30^{o}C for (4-7) days in a shaking bath incubator at 200 rpm. The above steps were based on studies carried out by (Demone *at al*., 1994).
5- Growth was followed up by withdrawing samples from the cell suspension at the prescribed time then analyzed by a spectrophotometer (UNICM.5625 UV/VIS, England) by measuring the optical density (OD) at 600 μm (Wang et al., 1979).
6- pH of the culture was monitored by pH meter model (3071).

5. Results and Discussions

It is known that it is very difficult to estimate growth kinetic constants from batch culture fermentation experiments because during

most of the batch fermentation the specific growth rate appears to be constant and independent of nutrient concentration. It is possible, however, to employ initial rate method where low initial substrate concentration levels are utilised and when the initial growth rate is measured before substrate concentration changes significantly (Wang et al.,1979).

In the present work very low initial substrate (sulfur compound) concentrations were used, and batch culture was utilized in the investigation of growth kinetics. The specific growth rate was calculated from the plot of ln X/X_0 versus time for each substrate concentration. Monod kinetics was then investigated from the plot of $1/\mu$ versus 1/S. The studied sulfur compounds are namely thiophene and dimethylsulfoxide, DMSO.

5.1.Growth of *Rh. erythropolis* on Thiophene

Growth results of LB activated *Rh.erythropolis* IGTS8 on basal salt media containing (1, 3, 6 and 8 gl^{-1} thiophene are shown in figures (2 to 5). The results are expressed as $\ln X/X_0$ versus time. The usual growth curve is obtained for all the investigated thiophene concentrations.

At low thiophene concentrations (1 to 3 gl^{-1}) growth started immediately after inoculation and stationery phase was reached after 50 h, while higher thiophene concentrations (6 and 8 gl^{-1}) were characterized by an initial time lag period that extended to 45 h after inoculation and stationery phase was reached after 90 h. At higher thiophene concentrations , cells needed more time to synthesis the enzymes responsible for metabolizing the higher substrate concentration. In all cases, however, logarithmic phase growth manifested before the declining growth or stationery phase was reached. The specific growth rate, μ, was calculated from the slope of the straight line (logarithmic phase) in each case. The values of μ are 0.05, 0.059, 0.062 and 0.061 h^{-1} for substrate concentrations of (1, 3, 6 and 8) respectively. Plotting $1/\mu$ versus 1/S Figure (6) shows that the relation can be fitted to a straight line, i.e. Monod equation is valid. The intercept $1/\mu_{max} = 15.6$ i.e. $\mu_{max} = 0.064h^{-1}$ and the slope $Ks/\mu_{max} = 43.8$ i.e. Ks = 2.8 gl

Setti et al.(1999) reported a growth rate of $o.o61h^{-1}$ for the same strain on 200 ppm Dibnsothiophene, also he found that the lag phase was close to 48 h and stationary phase was reached after 150 h. To the authors Knowledge, no growth data on thiophene has been published.

5.2. Growth of *Rh. erythropolis* on DEMSO

Growth of LB activated *Rh. erythropolis* on DEMSO of concentrations 1, 2, 3, 6 and 8 gl^{-1} was investigated. Results are shown in figures (7 to 11). The same specific growth rate of 0.095 h^{-1} was obtained for DEMSO concentrations of 1 and 2 gl^{-1} and very close growth rates for 3 and 6 gl^{-1} namely, 0.081 and 0.087 h^{-1} respectively and a lower growth rate of 0.042 h^{-1} for DEMSO concentration of 8 gl^{-1}.

This indicates that growth rate decreases slightly with increasing DMSO concentration up to 6 gl^{-1} then decrease sharply with further increase in DMSO concentration. This suggests some sort of substrate inhibition. The trend of variation of specific growth rate, μ with substrate concentration did not follow Monod equation as shown in figure(11).

6.Conclusion

Growth of *Rh. erythropolis* on two model sulfur compounds known to be present in crude oil and its distillates has been investigated. The compounds are thiophene and dimethylsulphoxide, DMSO. While growth on thiophene obeyed Monod kinetic model, growth on DEMSO showed some sort of substrate inhibition.

A comparison of the growth rates of the same microorganism on DBT reported elsewhere (El-Temtamy et al.,2004) to the growth rates reported in this work is given in table (1). As can be shown while all growth rates are of the same order of magnitude, growth rates on DMSO are shown to be the highest and those on DBT are the lowest. Another point worth mentioning is that growth rates on model sulfur compounds are of the same magnitude as those on petroleum fractions (El-Temtamy et al.,2004). In other words for evaluation of BDS microorganisms we can use model sulfur compounds and get correct expectations about what happens in reality.

Table (1): Specific growth rates, μ $h,^{-1}$ of *Rh. erythropolis* on different organosulfur compounds

Concentration gl^{-1}	μ(Thiophene) h^{-1}	μ(DMSO) h^{-1}	μ(DBT) h^{-1}
1	0.050	0.096	0.044
3	0.059	0.081	0.026
6	0.062	0.087	0.025
8	0.061	0.042	0.020

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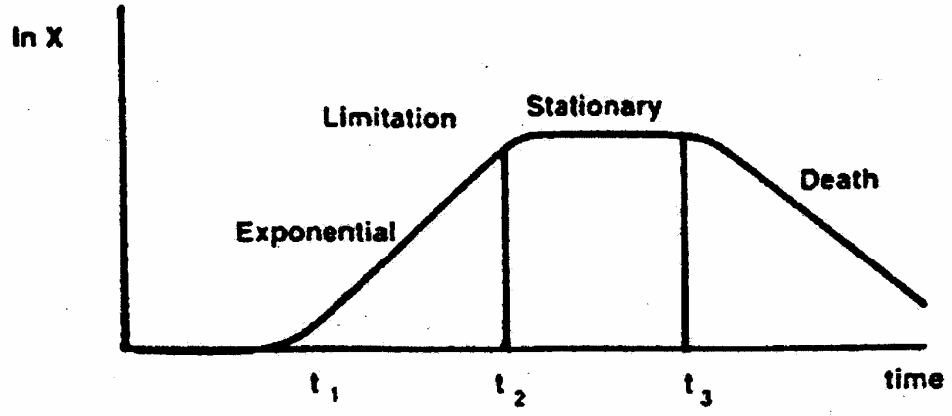


Figure (1): Biomass concentration during batch growth.

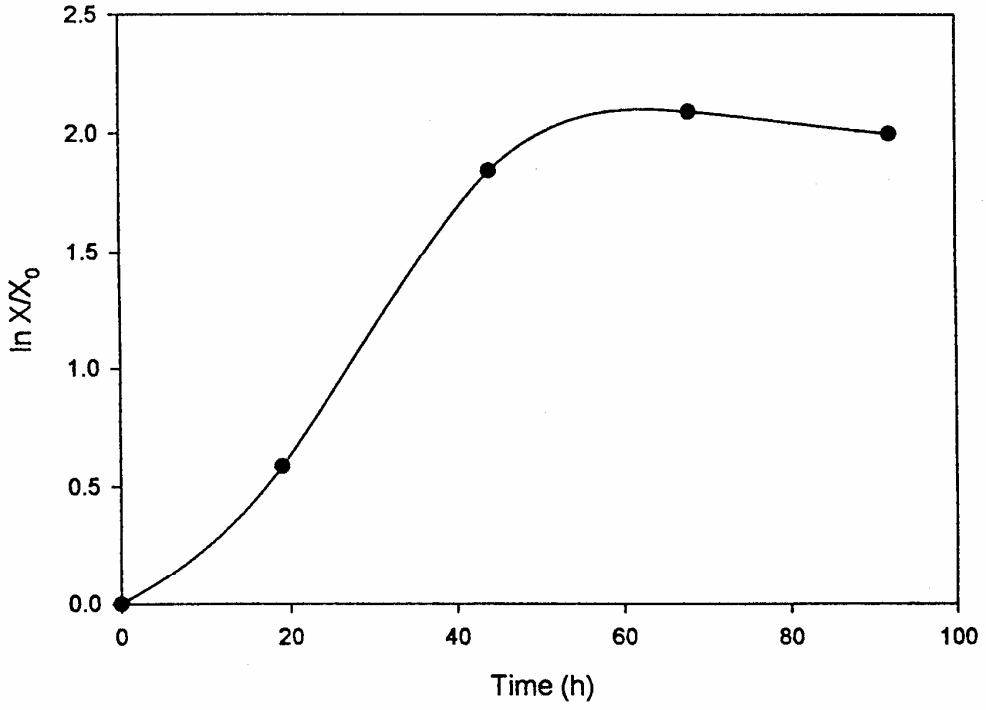


Figure (2): Relative cell concentration versus time for 1 gl^{-1} thiophene.

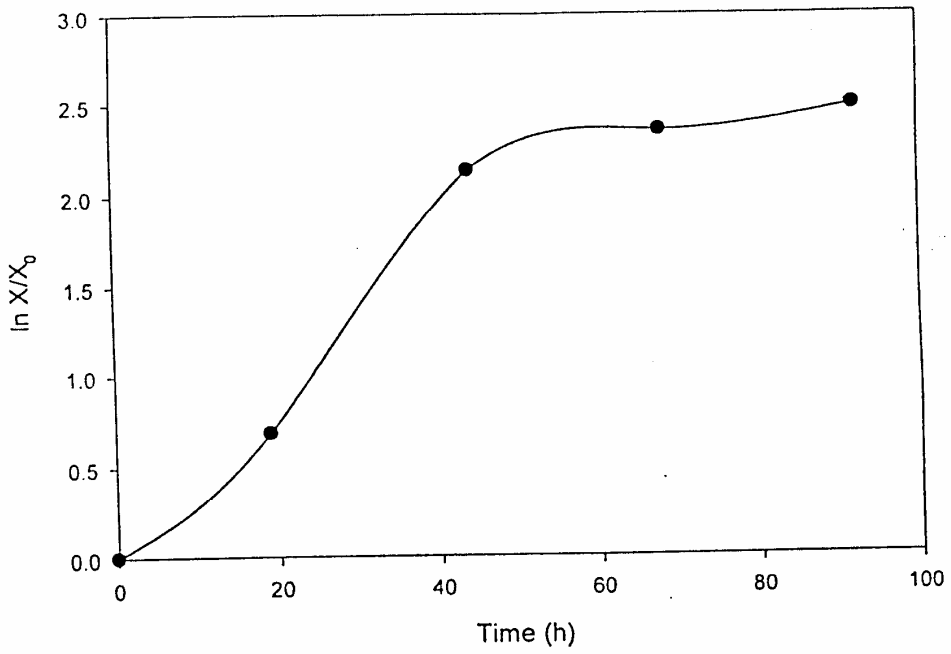


Figure (3): Relative cell concentration versus time for3 gl^{-1} thiophene.

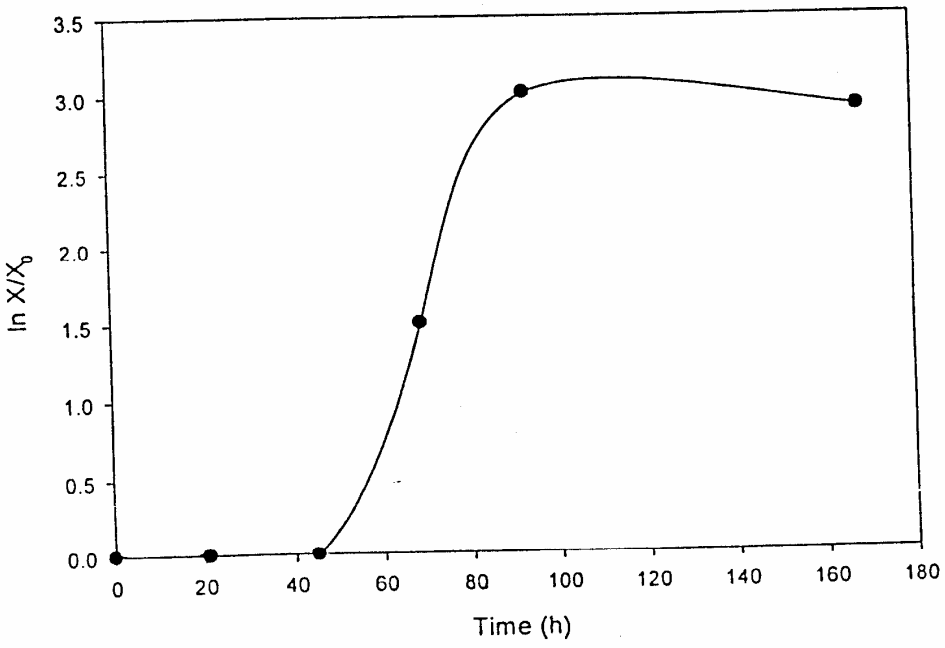


Figure (4): Relative cell concentration versus time for6 gl^{-1} thiophene.

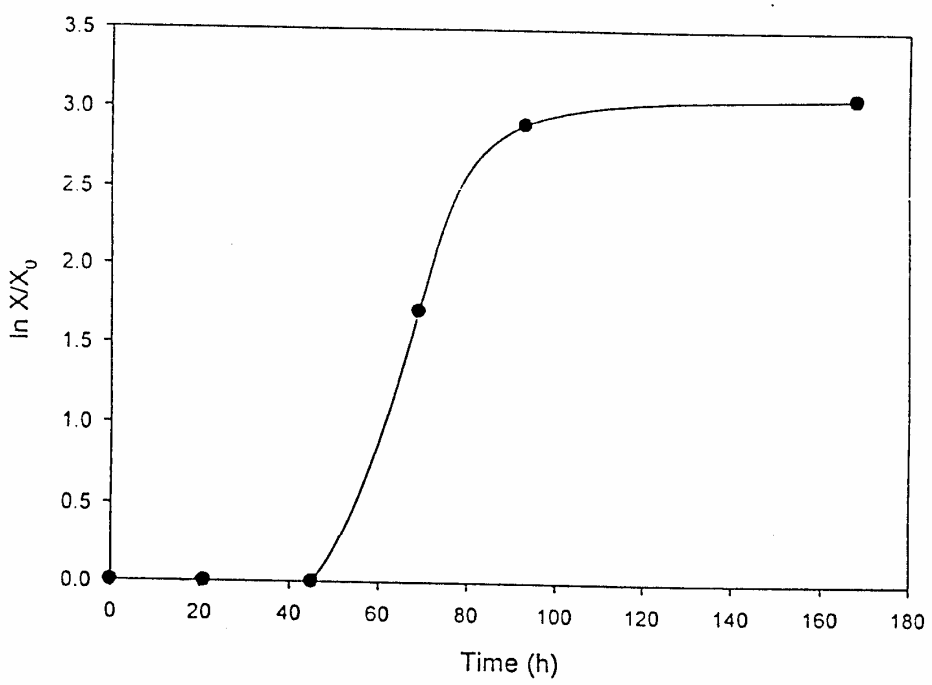


Figure (5): Relative cell concentration versus time for8 gl^{-1} thiophene.

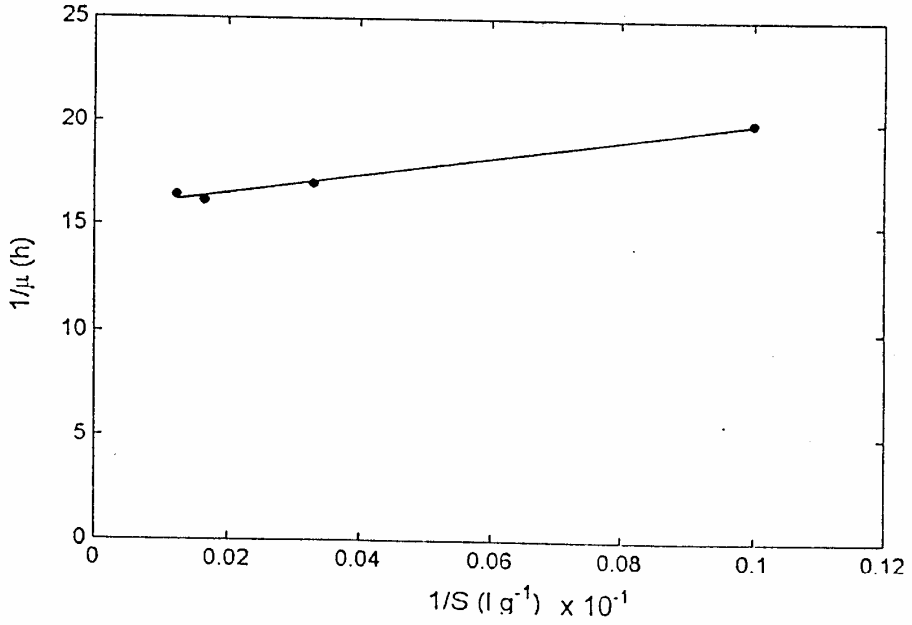


Figure (6): Double reciprocal plot for estimating μmax and *Ks* in Monod Kinetic model for *Rh. erythropolis* growth on thiophene.

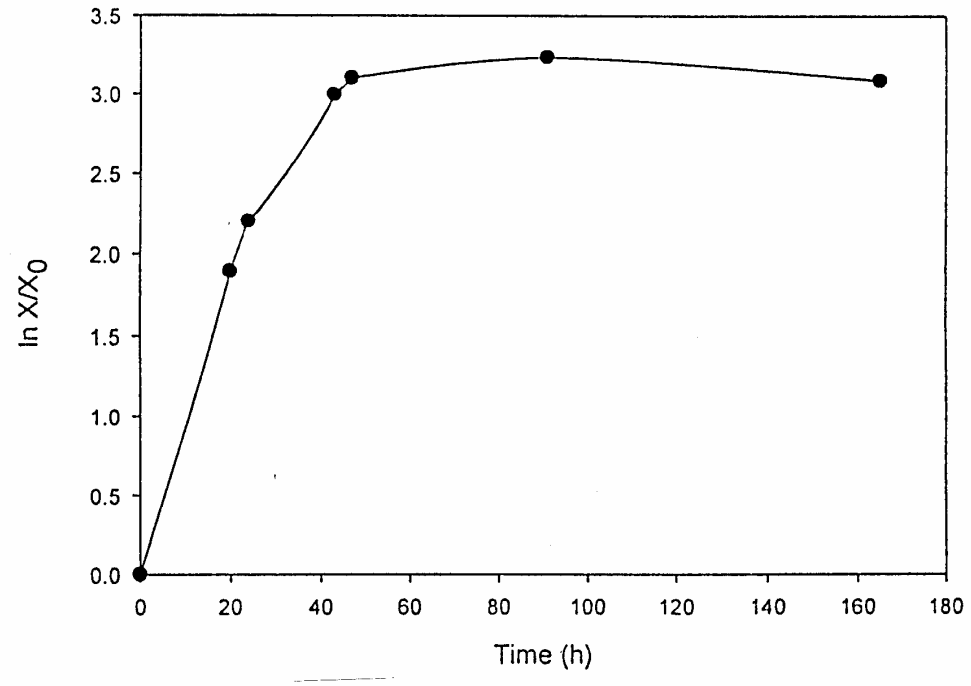


Figure (7): Relative cell concentration vs time for 1 gl^{-1} DEMSO.

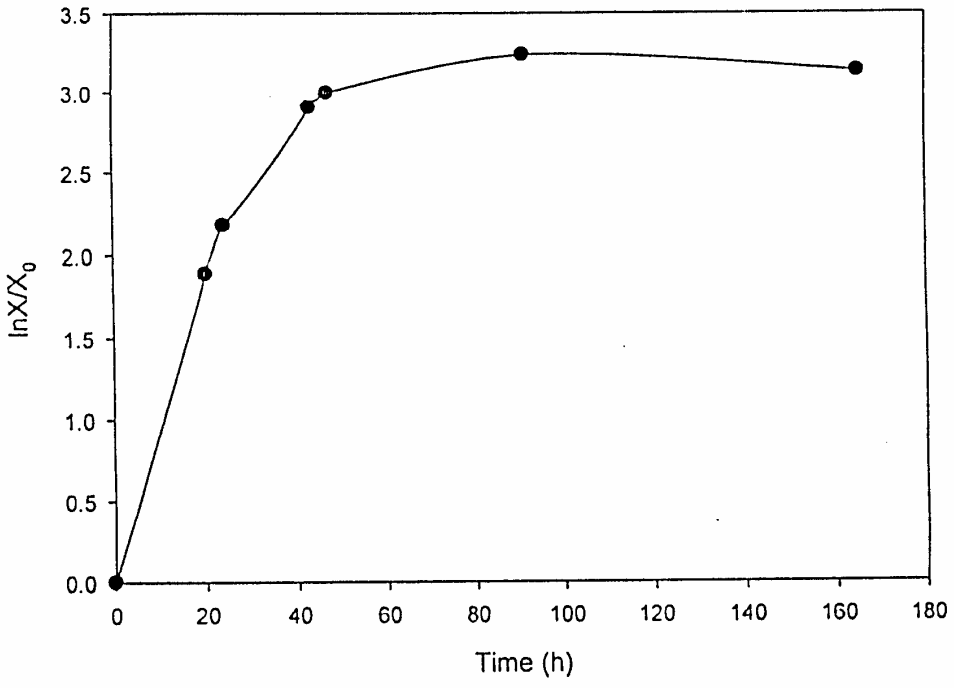


Figure (8): Relative cell concentration versus time for 2 gl^{-1} DEMSO.

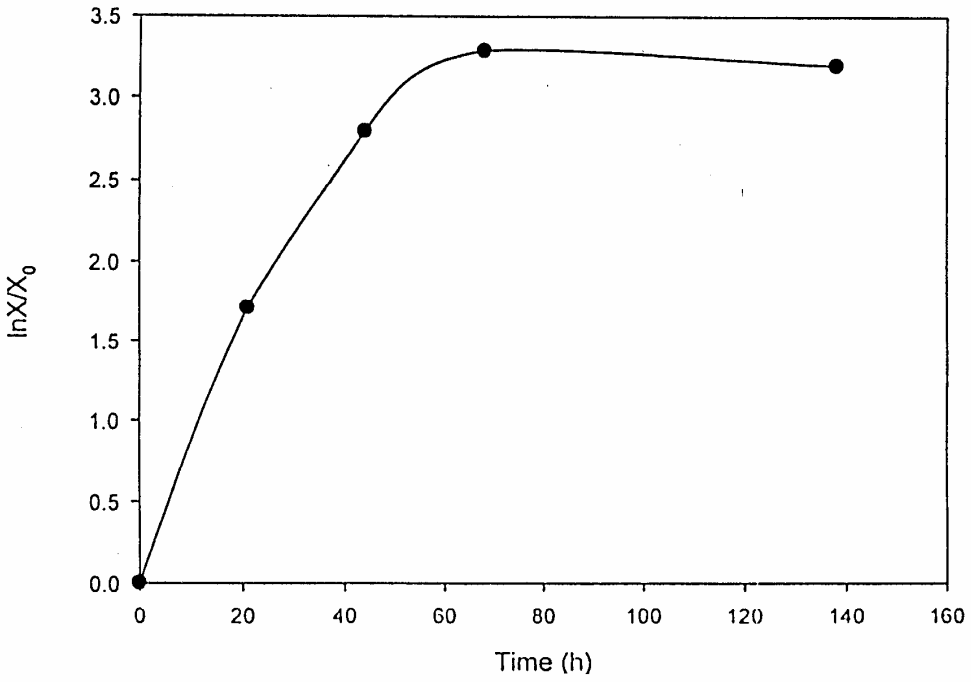
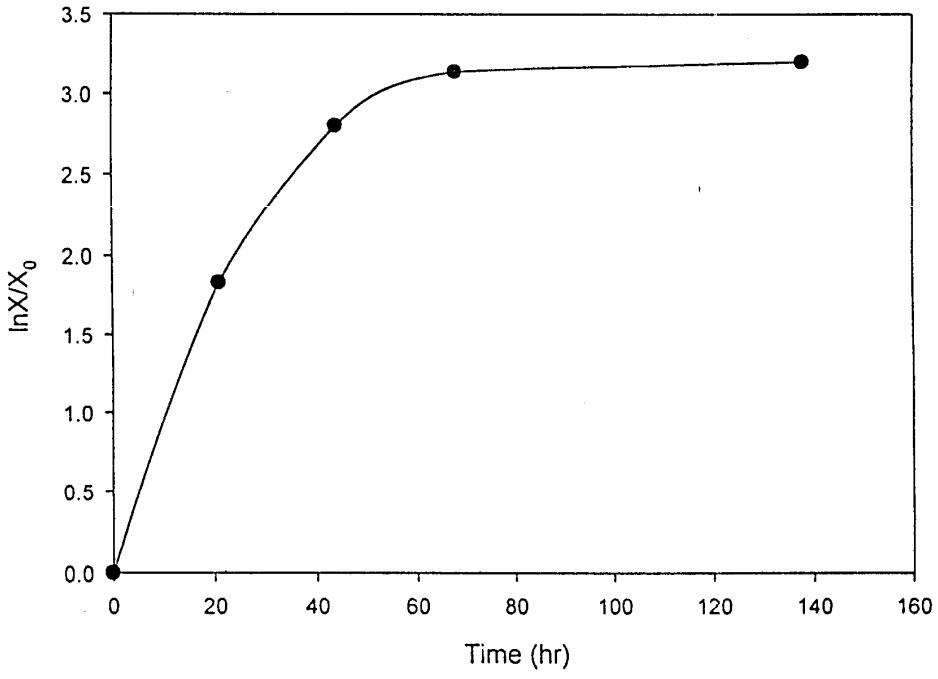
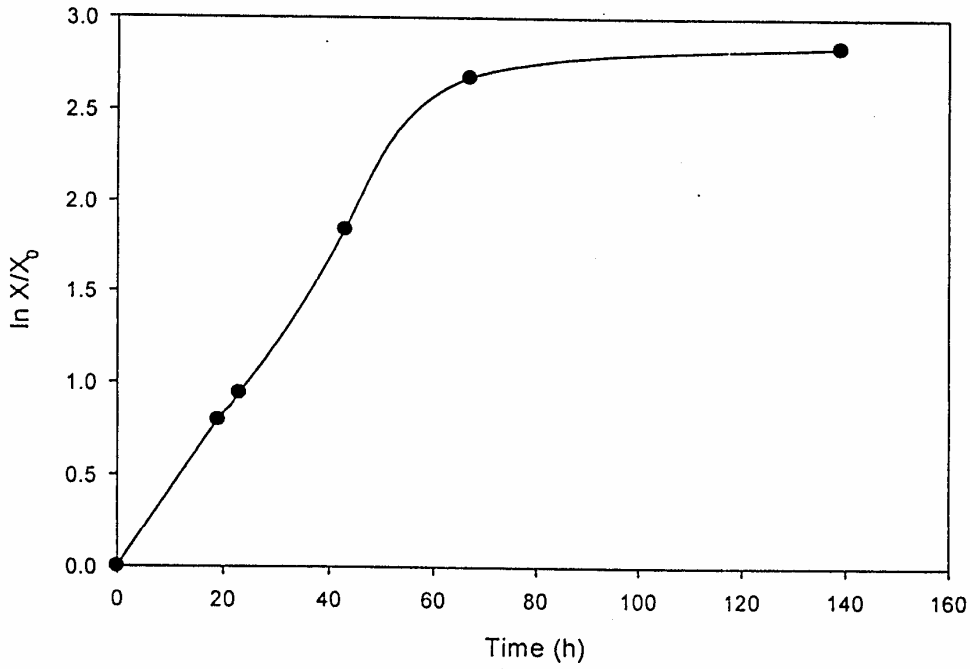


Figure (9): Relative cell concentration versus time for 3 gl^{-1} DEMSO.



Figure(10): Relative cell concentration versus time for 6 gl^{-1} DEMSO.



Figure(11): Relative cell concentration versus time for 8 gl^{-1} DEMSO.

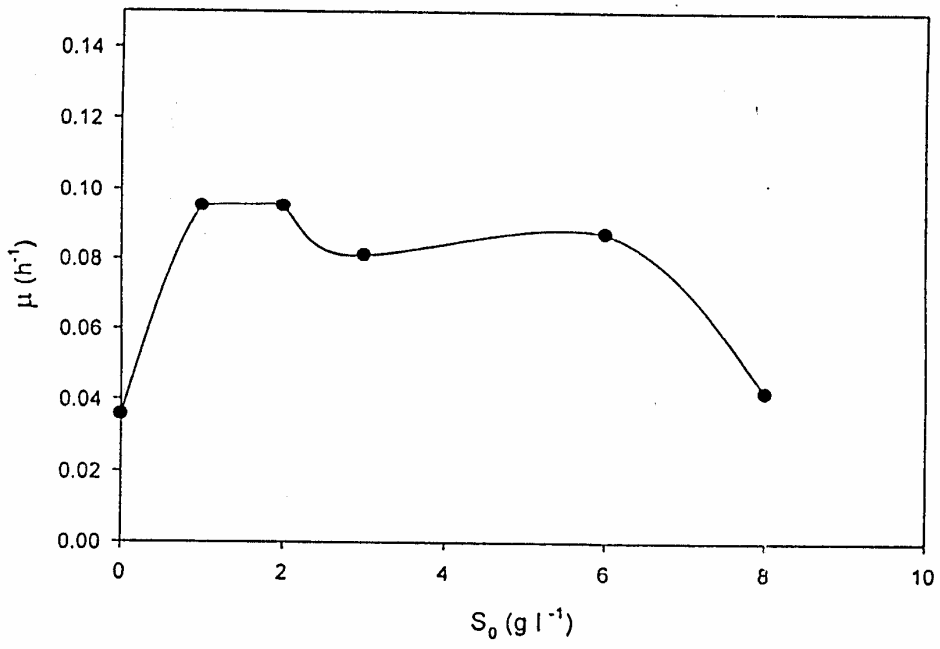


Figure (12): Specific growth rate versus initial substrate concentration for DEMSO.