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Yield coefficient for the growth of *Pseudomonas* sp. AQ5-04 at various concentrations of phenol

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Phenol is widely used by many industries in Malaysia, and is one of the highly toxic environmental pollutants with levels exceeding the regulatory standard that have been reported in Malaysia. A phenol-degrading bacterium; *Pseudomonas* sp. AQ5-04, a metabolically versatile xenobiotic-degrading bacterium, has been isolated from a local contaminated site. The bacterium was able to withstand a high concentration of phenol. This work aims to determine the effect of concentration and to establish the yield factor on phenol as a carbon source. It was established that the isolate can degrade up to 900 mg/L phenol within 72 h; it was discovered that the yield factor ($Y_{X/S}$) varied between 0.3 to 0.6 mg cells per mg phenol for different initial phenol concentrations with increasing phenol concentrations resulting in a lower yield. This shows that growth on phenol is strongly inhibitory and resulted in low yield at an inhibitory concentration of phenol.

Key words: *Pseudomonas* sp. AQ5-04, yield factor, phenol, inhibitory concentration.

INTRODUCTION

Phenols and phenolic compounds are injurious to organisms even at low concentrations with many of them categorized as dangerous pollutants due to their toxicity towards human health for various reasons Some of the phenolic compounds include chlorophenols, nitrophenols, methyl phenols, alkylphenols, aminophenols, butylhydroxytoluene, nonylphenol and Bisphenols A (Maiti and Mannan, 1999; Hirooka et al., 2003). In Malaysia, the 2014 Environmental Quality Report showed that nearly all groundwater monitoring stations had phenol concentrations exceeding the National Guidelines for Drinking Water Quality Standard (0.002 mg/L). Phenol and phenolic compounds continue to be the top scheduled wastes generated in Malaysia as the demand for phenol by the industries are increasing annually (Gami et al., 2014a).

Phenols and phenolic mixes are damaging to living things even at low amount with huge numbers of them

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classified as poisons because of their poisonous quality towards human wellbeing (Das and Maiti, 2007; Zulkharnain et al., 2013; Ghazali and Johari, 2015). Part of the phenolic substances are nitrophenols, chlorophenols, methyl phenols, aminophenols, alkylphenols, nonylphenol, butylhydroxytoluene and Bisphenols A (Anuar, 2013; Ghazali and Johari, 2015). In Malaysia, the National Guidelines for Drinking Water Quality Standard is 0.002 mg/L and the 2014 Environmental Quality Report demonstrated that nearly all of the groundwater observing stations had exceeded this level for phenol. This is due to phenol and phenolic substances are the number one scheduled waste product in Malaysia as their usage in the industrial sector is one of the highest (Ahmad et al., 2011a, 2012, 2017; Arif et al., 2013; Department of Environmental, 2014; Gami et al., 2014b; AbdEl-Mongy et al., 2015; Norazah et al., 2015; Sabullah et al., 2017).

Physicochemical methods for phenol removal are abounded and include photodecomposition (Akbal and Onar, 2003), chemical polymerization (Kulkarni and Kaware, 2013), ion exchange (Alkaram et al., 2009), electrocoagulation (Abdelwahab et al., 2009) and advanced oxidation (Liotta et al., 2009). Having said that, a natural or biological approach continues to be considered as the preferable means of taking care of phenol pollution. Organic methods are powerful at small concentrations of phenol, is value effective and do not create secondary pollutants compared to some physicochemical methods (Hank et al., 2010; Kulkarni and Kaware, 2013; Agarry et al., 2008; Kulkarni and Kaware, 2013).

The ability of several microorganisms to utilise phenol and other phenolic substances are considered as a tool for the removal of noxious waste material (Agarry et al., 2008). In reality, the endeavour of utilizing microorganisms to eliminate phenol and other organic contaminants has been escalating in recent years. There are a considerable variety of microorganisms that coexist in almost all-natural surroundings which includes water and soil. These microorganisms can break down organic substances by generating intracellular or extracellular xenobiotics-degrading enzymes (Agarry et al., 2008; Luo et al., 2009, 2012; Nordin et al., 2013; Zulkharnain et al., 2013).

Phenol biodegradation is affected by various factors such as temperature, pH, nitrogen source and salinity (Ahmad et al., 2011b). In this work, a phenol-degrading bacterium has been isolated from a waste site in the Langat River Basin where intensive studies for many years have shown the severity of the contamination of this area by industrial activities near and surrounding the basin (Yusof et al., 2002; Taweel et al., 2013). In general, there is limited information on the growth yield of bacterial cells on phenol, of which this paper is attempting to address. This value will be very useful in predicting the growth on phenol during bioremediation works.

MATERIALS AND METHODS

Growth and maintenance of phenol-degrading bacteria

The phenol-degrading bacterium AQ5-04 was isolated in 2014 in Selangor and was identified as *Pseudomonas* sp. AQ5-04 with an accession number KT693288.1. A minimal salt medium (MSM) containing (g/L) of NaCl (0.1), $MgSO_4$ (0.1), Fe_2 (SO4).H_2O (0.01), K_2HPO_4 (0.4), KH_2PO_4 (0.2), $MnSO_4.H_2O$ (0.01), $(NH_4)_2SO_4$ (0.4), $NaMoO_4.2H_2O$ (0.01) was utilized in this work. The MSM was supplemented with 500 mg/L of phenol as a carbon source (Ahmad et al., 2012). The best isolate was utilized in this work, its optimization and characterization works (Aisami, 2017). The phenol-degrading activity was determined using the 4-aminoantipyrine colourimetric assay at 510 nm (Ahmad et al., 2012). Bacterial growth was measured using colony forming-units (CFU/ml) and dry cellular weight. Biomass concentration as a function of optical density was estimated from the liquid medium by centrifuging the bacterial cells culture at 10,000 g for 15 min. After centrifugation, residual phenol was measured in the supernatant. The cellular pellet was resuspended in 1 mL of fresh culture medium and the optical density measured at A 600 nm using a UV/visible spectrophotometer (UV 1240 Mini Shimadzu Japan) with the culture medium as the blank. Biomass of the bacterium as dry weight of washed cells was determined by vacuuming filtered culture filtrates onto a pre-weighed Whatman filter disc (0.2 cm, 4.7 cm). The filter was then dried in a vacuum oven at 80°C until a constant weight was achieved and subtraction of this weight to the initial mass of the filter represents cell dry weight. A calibration curve was constructed for the cellular biomass concentration against this wavelength. Samples exceeding an absorbance value of 1.0 were diluted before measured. A linear relationship between OD_{600} nm and cell dry weight of up to 750 mg/L (R^2 of curve was 0.97) was calculated.

Effect of various phenol concentrations on the bacterial growth and phenol removal

Phenol concentration of between 400 and 1200 mg/L was used to determine the effect on the bacterial growth and phenol degradation.

Growth kinetics

In a batch system, the biomass growth rate is typically defined as follows:

$$\left(\frac{dX}{dt}\right) = \mu_g X - k_d X \quad (1)$$

Where the growth rate of biomass (h^{-1}) is represented by μ_g, X is the cellular biomass concentration (mg/l), and the endogenous decay coefficient (h−1) is represented by k_d. The substrate utilisation rate is defined as follows;

$$\left(\frac{dS}{dt}\right) = -\left[\frac{1}{Y_x}\left(\frac{dX}{dt}\right) + mX\right] = -\mu_s X \quad (2)$$

S is defined as the rate-limiting substrate concentration at time t(mg/l), the specific substrate consumption rate (h^{-1}) is μ_s the observed yield coefficient, which is defined as the ratio of biomass formed to the mass of substrate utilized (mg/mg) is represented as

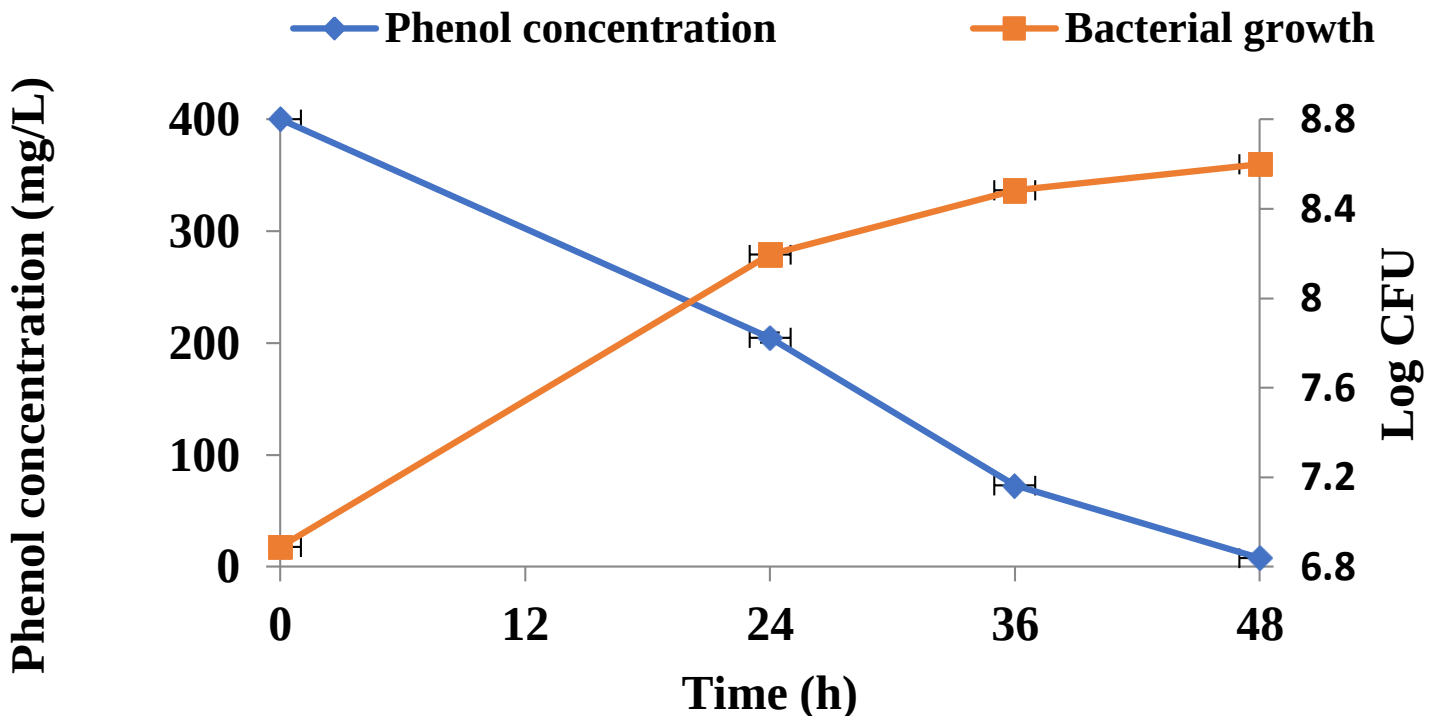

Figure 1. Effect of phenol concentration (400 mg/L) on bacterial growth and phenol degradation.

Y_X, and m is maintenance energy coefficient (h^{-1}) or the percentage of the energy sources utilized by the bacterial cells for needs other than growth. The endogenous decay coefficient at the exponential growth phase can in many cases be neglected.

Hence, Equation 2, upon integrating with the boundary condition ($X = X_0$ at $t = 0$), is reduced to;

$$\ln \frac{X}{X_0} = -\mu_g t \quad (3)$$

Yield coefficient on phenol

The plot of (X–X_0) versus (S_0–S) at the exponential growth region of the bacterial growth curve at any particular value of an initial concentration of substrate gives a straight line. The value of the observed yield coefficient or Y_{xs} can be obtained from the slope of this line. The formula for the observed yield coefficient is as follows

$$(Y_{xs})_{obs} = \frac{(X - X_0)}{(S_{l0} - S_l)} \quad (4)$$

RESULTS AND DISCUSSION

As shown in Figure 1, *Pseudomonas* sp. AQ5-02 can tolerate 400 mg/L phenol and up to 50% (200 mg/L) was removed within the first 24 h and there is an increase in the bacterial growth and almost 100% was removed within 48 h. But at 800 mg/L, it took the isolate 60 h for complete degradation (Figure 2) while for 1000 mg/L, almost 90% was removed after 60 h and no increase in the percentage removal after 72 h (Figure 3). At the concentration of 1200 mg/L, the bacterial growth increases after 24 h but no further significant increase in the growth at 48, 60 and 72 h. This indicates that at concentration, the phenol became very toxic to the bacterial leading to the inhibition of the growth and also phenol degradation as shown in Figure 4. This result is in agreement with some previous studies that reported that there is decrease in phenol degradation at higher phenol concentrations (Mohanty and Jena, 2017). Also, as the concentration of phenol increases there is increased in degradation time and decreased in bacterial growth as reported by other authors (Bakhshi et al., 2011).

Yield coefficient ($Y_{X/S}$)

The apparent bacterial yield on phenol was determined through linear regression of the corresponding slope of the plot of cell dry weight concentration (mg/mL) vs. initial phenol concentration (mg/mL) during the exponential portion of the growth phase (published elsewhere).

As shown in Figure 5, the highest yield coefficient was 0.6 mg cells per mg phenol at 200 mg/L of initial phenol concentration. The yield was down to 0.3 mg cells per mg

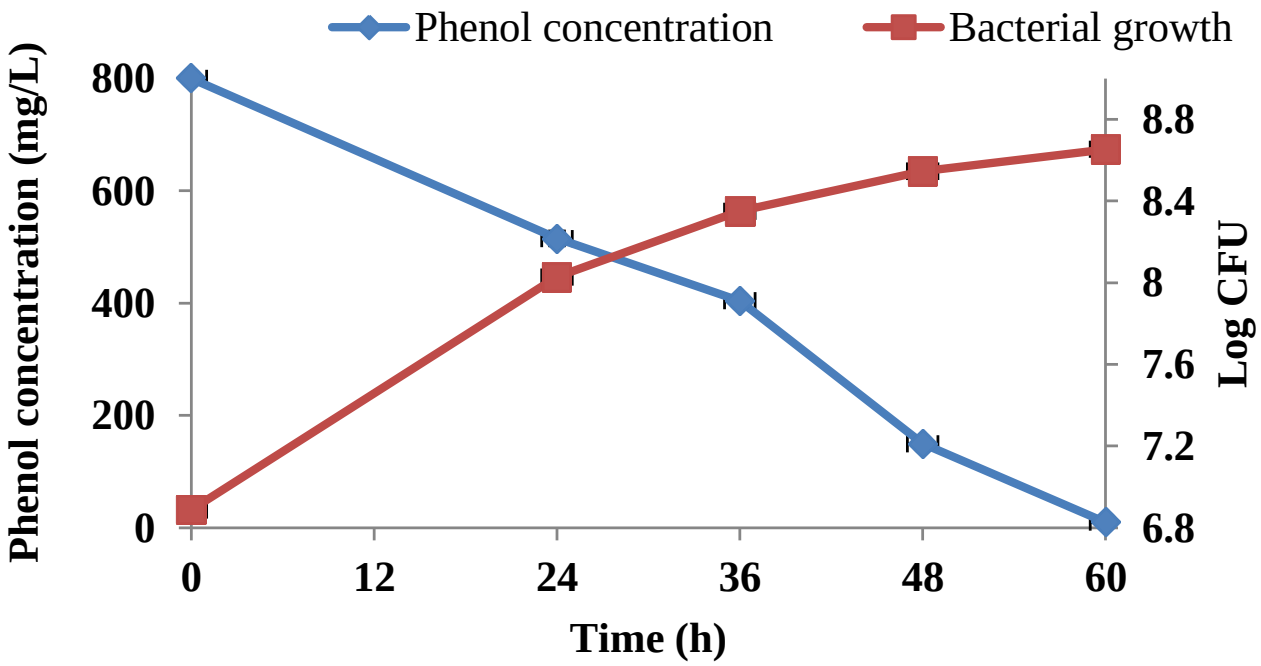

Figure 2. Effect of phenol concentration (800 mg/L) on bacterial growth and phenol degradation.

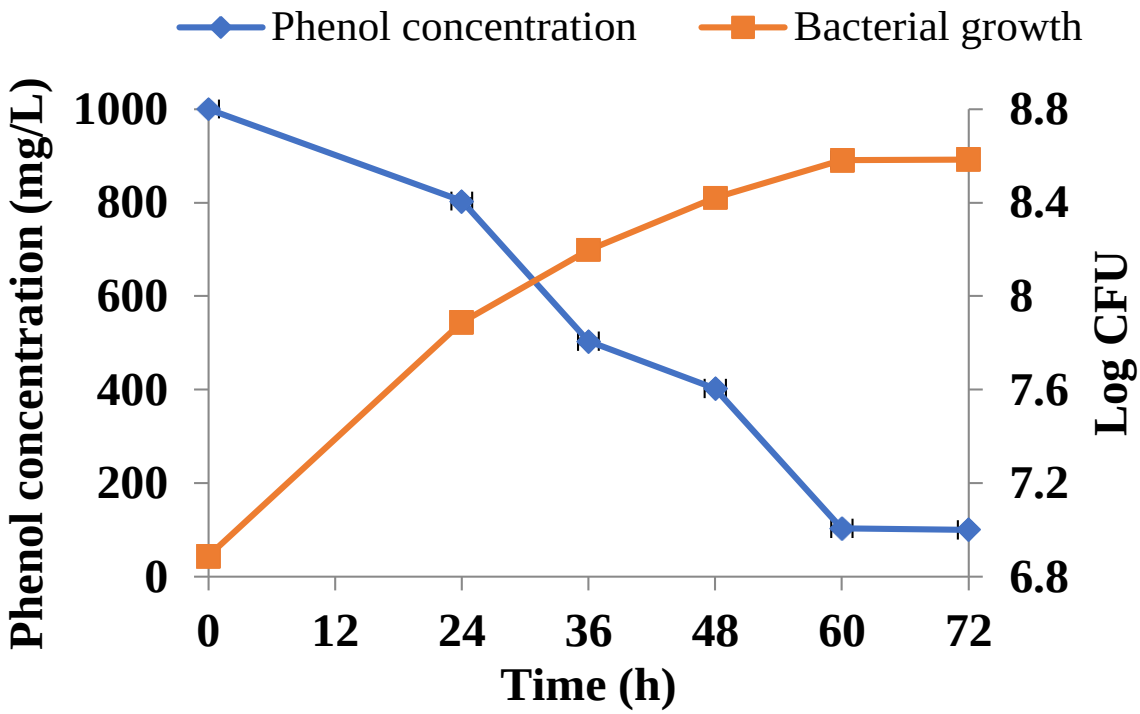

Figure 3. Effect of phenol concentration (1000 mg/L) on bacterial growth and phenol degradation.

phenol at 2000 mg/L of initial phenol concentration, indicating a trend of lower growth yield at inhibiting phenol concentrations. The value obtained in this work is quite far from the highest theoretical value of $Y_{X/S}$ for growth on phenol, which is 0.94 g/g (Reardon et al., 2000). This is not surprising since the conditions of

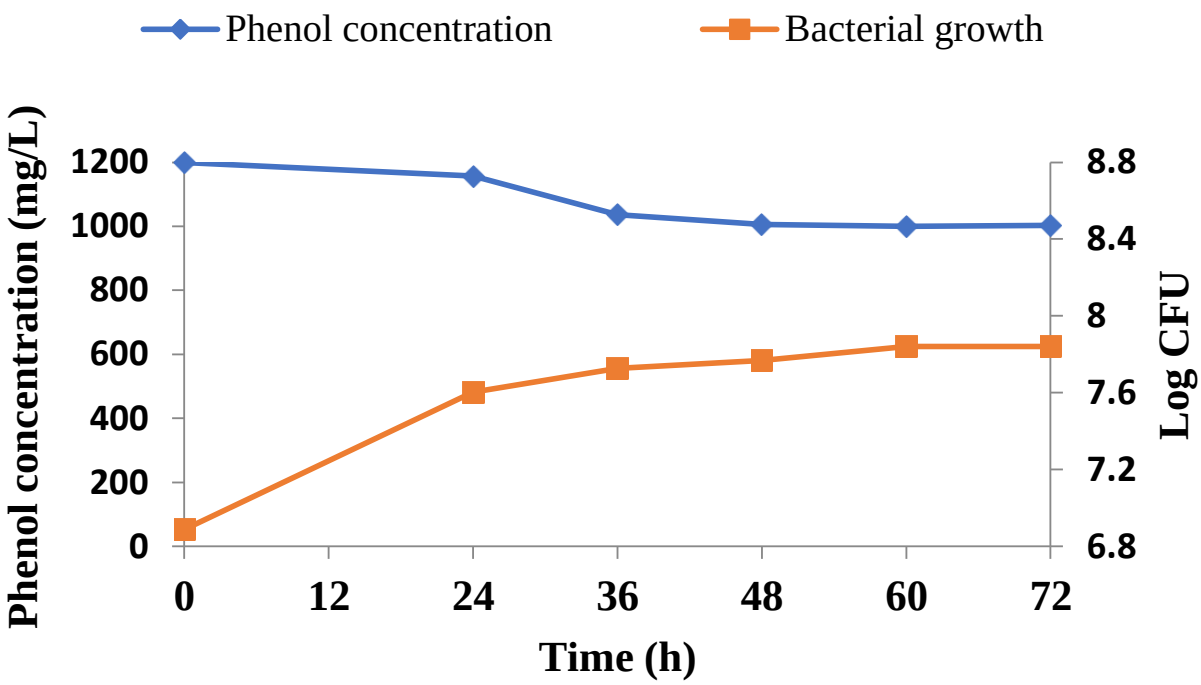

Figure 4. Effect of phenol concentration (1200 mg/L) on bacterial growth and phenol degradation.

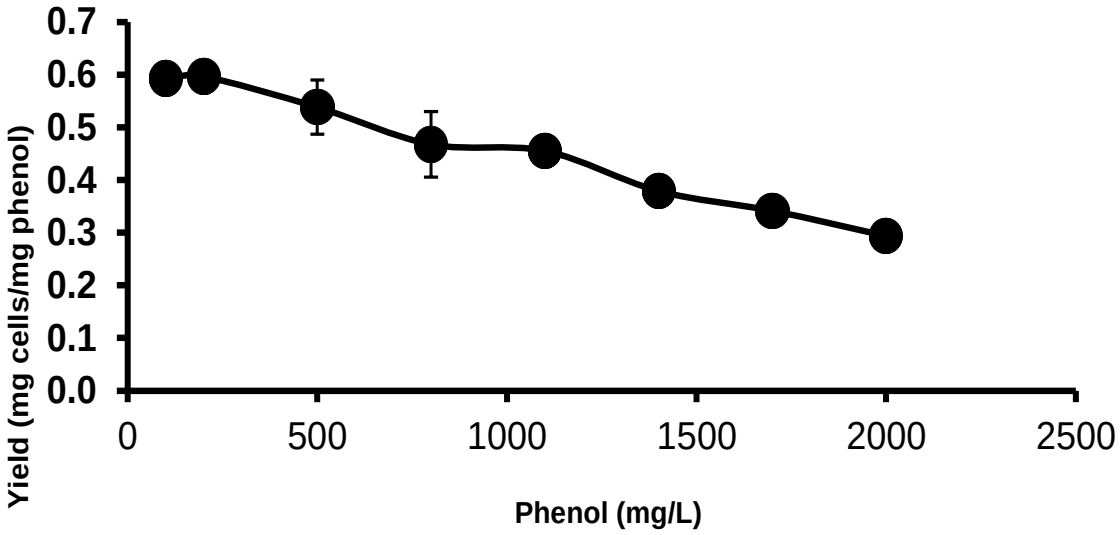

Figure 5. Variation in yield coefficient with initial phenol concentration.

growth are not as optimal as in chemostat conditions. Comparison of various growth yield date on phenol for several phenol-degrading bacteria available from the literature indicates that the yield reported on phenol varies dramatically with the lowest yield exhibited by *Pseudomonas putida* DSM 548 at 0.0017 mg cells/mg phenol while the highest yield is exhibited by *Bacillus cereus* at 0.88 mg cells/mg phenol. However, the yield is reflected by the tolerance of the phenol-degrading bacteria wherein *P. putida* DSM 548, the inhibition constant or K_i value is just 54.1 mg/L, a low value compared to the better phenol-tolerant bacterium *B. cereus* where the K_i value is 238.1 mg/L.

In general, the higher the concentration of phenol, the lower the yield as phenol is toxic and much of the energy from the substrate is needed to maintain cellular function to combat the toxic phenol (Onysko et al., 2000). Phenol can easily cross bacterial cell wall and membrane and

Table 1. Yield factors observed by some previous studies.

Bacterial strain	Phenol concentration range (mg/L)	$Y_{x/s}$ observed (mg cells/mg phenol)	Reference
Bacillus cereus	50-600	0.102-0.880	Zhang et al. (2013)
Pseudomonas putida MTCC 1194	10-1000	0.65	Kumar et al. (2005)
Peudomonas putida DSM 548	1-100	0.0017	Monteiro et al. (2000)
Alcaligenes faecalis	0-1800	-	Jiang et al. (2007)
Pseudomonas putida LY1	20-800	0.765 (for concentration 50 mg/L)	Wang and Loh (1999)
Pseudomanas putida (Tan1) *Staphylococcus aureus* (Tan2)	100-800	0.4258	Li et al. (2010)
Pseudomonas putida F1 strain ATCC 700007	10-200	0.44	Abuhamed et al. (2004)
Mixed cultures	25-1450	0.6	Senthilvelan et al. (2014)
Stenotrophomonas maltophilia strain KB2	25-500	0.65	Szczyrba et al. (2016)
P. putida (PTCC 1694)	300-1000	0.012-0.177,	Bakhshi et al. (2011)
Pseudomonas putida Q5	20-600	0.42-0.89 (estimated)	Onysko et al. (2000)
P. putida ATCC 17484	5-200	0.70	Hutchinson and Robinson (1988)
C. tropicalis PHB5	20-2400	0.185 -0.96	Basak et al. (2014)
Mixed culture	1000-1500	0.16 - 0.27	

can transform the presence of oxygen by various oxygenases to form toxic phenoxy radicals that attack various cellular structures and cause lipid peroxidation. Furthermore, the production of phenol-degrading metabolites including quinine methides and semiquinones can damage protein and DNA (Gami et al. 2014a). The yield coefficient is dependent on initial phenol concentration, with higher concentrations reducing yield significantly as was observed in many studies on phenol-degrading bacteria (Chi and Howell, 1976; Hutchinson and Robinson, 1988; Wang and Loh, 1999; Onysko et al*.,* 2000; Abuhamed et al*.,* 2004; Jiang et al*.,* 2007; Li et al*.,* 2010; Bakhshi et al*.,* 2011; Senthilvelan et al*.,* 2014). Hence, the average value of the yield coefficient is usually not reported. The trend in the yield of cellular biomass on phenol for many phenol-degrading microorganisms is a diminishing yield as the phenol concentrations were increased. Yields in many cases started to dramatically decline at concentrations of phenol of above 100 mg/L (Onysko et al*.,* 2000; Bakhshi et al*.* 2011; Wolski et al*.,* 2012; Zhang et al*.,* 2013; Basak et al*.* 2014) indicating the toxicity of phenol is causing cells to direct their resources for survival. Under toxic concentrations of phenol, it is anticipated that the maintenance energy will be quite high. Table 1 shows some established yield factors, the concentration range and also the name and strain of the bacteria.

Conclusion

Pseudomonas sp. strain AQ5-04, a phenol-degrading bacterium showed tolerace of 1000 mg/L phenol and growth yield inhibition in the presence of phenol. The yield obtained in this work is within the range observed on the growth on phenol by several other bacteria, although the yield obtained in this work is well below the theoretical value of yield on phenol, which is 0.94 g cells per g phenol. The inhibition of the growth yield at high concentration of phenol is anticipated but this bacterium showed some resilience in terms of growth yield data at very high concentration of phenol.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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