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Biotransformation using resting cells of *Rhodococcus* UKMP-5M for phenol degradation

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ABSTRACT

Phenol is a toxic compound that may be transformed into non-toxic compounds by the activity of microbial cells. The possibility of using biotransformation method for the degradation of phenol was studied using the whole cells of *Rhodococcus* UKMP-5M suspended in 250 mL shake flask with buffered liquid containing phenol. The cells of *Rhodococcus* UKMP-5M were produced by cultivation in Minimal Salt Medium (MSM) with the addition of phenol and/or glucose as carbon source. The biotransformation conditions to obtain the highest percentage of phenol degradation were as follows; pH 7.4, 0.5 g/L phenol in MSM as biotransformation medium, cells were produced by cultivation in MSM supplemented with 0.5 g/L phenol and the optimal cell concentration was 10%. The phenol degradation rate obtained in biotransformation using *Rhodococcus* UKMP-5M cells correlated well with phenol hydroxylase activity. The highest percentage of phenol degradation in biotransformation using suspended cells of *Rhodococcus* UKMP-5M was only up to 89%, which was slightly lower than those obtained in growing cell system (98%).

1. Introduction

Contamination of soil, water and underground water by aromatic organic pollutants in many areas has caused great concern around the world. Among these organic pollutants, phenolic compound is well known for its high toxicity to humans and animals (Shourian et al., 2009; Wang et al., 2010., Khleifat, 2006, Liu et al., 2016, Nor Suhaila et al., 2013a,b, Mohanty and Jena, 2017). The types of polyphenol oxidase-catalysed processes and reactors for the treatment of industrial wastewaters were discussed by Mukherjee et al. (2013). Beside Polyphenols, phenol is widely found in many industrial waste waters and effluents. Phenol can be removed through bioremediation, in which, it is metabolized as carbon and energy sources by phenol degrading microorganisms (Varma and Gaikwad, 2009; Mohanty and Jena, 2017; Nor Suhaila et al., 2016). The possible savings using bioremediation as compared to other methods have been estimated, ranging from 65 to 85% (Karigar et al., 2006). Rotating biological contactor (RBC) is among the well known method used for the pass many years back. Three-stages cross flow laboratory-scale RBC was applied in this system and microbial was isolated from a mixture of municipal and slaughter house activated sludge. Effectiveness of evaporation is play an important role in phenol removal efficiency in RCB, However, increasing the hydraulic load in this system cause a decrease in treatment efficiency (Alemzadeh at al., 2002). Dey and Somnath Mukherjee, 2013 reported that trickling filter performs better than the aeration tank in phenol removal. However, a sudden drop of removal percentage by a mix microbial sludge collected from an effluent treatment plant was observed at influent phenolics concentrations of 300 mg/L.

Biotransformation using whole cells or enzymes is an area of immense importance (Devi et al., 2006). The use of growing cells for phenol bioremediation faces some problems such as limited growth, low cell concentration during the cultivation, low reaction rate and low percentage of degradation. The presence of high phenol concentration may also inhibit growth of phenol degrading microorganism (Rocha et al., 2007). The use of resting cells as catalyst in the biotransformation for the degradation of toxic compounds has been reported (Karim and Gupta., 2003; Dekant, 2009). In biotransformation, the substrate added to the system did not support cell growth but it is transformed to other

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compounds in the presence of a primary substrate (Chee Loh and Jing Wang, 1998). Co-metabolism is a transformation of secondary substrate (substrate for non-growth) in the presence of primary substrate (substrate for growth) (Hao et al., 2002). Several microorganisms such as *Pseudomonas putida* (Jing Wang and Chee Loh, 2001), *Arthrobacter citreus* (Karigar et al., 2006), *Candida tropicalis* (Varma and Gaikwad, 2009) and *Acinetobacter* (Hao et al., 2002) have been used in the biotransformation of phenol.

Rhodococcus has been known as a robust microorganism for the degradation of harmful pollutants such as cyanide (Maniyam et al., 2019), azo dyes (Maniyam et al., 2018a,b), nitriles (Sjahrir et al., 2018), and chlorinated compounds (Maniyam et al., 2016). In addition, phenol is first converted by phenol hydroxylase to catechol (1,2-dihydroxybenzene) which is further degraded through ortho- or meta-cleavage by *Rhodococcus* (Martinkova et al., 2009). Hydroxylation to catechol, ring cleavage via catechol-2,3-dioxygenase to 2-hydroxymuconic semialdehyde (HMSA) for meta-pathway (extra diol, between one of the hydroxyl groups and a non-hydroxylated carbon), and via catechol-1, 2-dioxygenase to cis, cis-muconate for ortho-pathway (intradiol, carbon bond between two hydroxyl groups) is the sequence involved in the degradation of phenol (Banerjee and Ghoshal, 2010). *Rhodococcus* sp was used because the ability to produce phenol hydroxylase that can transform the phenol into 2-hydroxymuconic semialdehyde or cis, cis-muconate.

However, detailed descriptions of biotransformation of phenol by the resting whole cells are lacking. In addition, information on the use of the whole cells of *Rhodococcus* spp in biotransformation of phenol into non-toxic compounds is not available in the literature.

The objective of this study was to investigate the possibility of using resting whole cells of *Rhodococcus* UKMP-5M in the biotransformation of phenol. The effect of different types of carbon source, as non-growth substrate, on the performance of phenol biotransformation was studied. The biotransformation was carried out using resuspended whole cells of *Rhodococcus* UKMP-5M in shake flask. The activity of enzyme relevant to phenol degradation during the biotransformation process was also evaluated. For comparison, degradation of phenol using growing cell of *Rhodococcus* UKMP-5M was also conducted in shake flask culture.

2. Materials and methods

2.1. Microorganism

Rhodococcus UKMP-5M isolated from a petroleum contaminated soil at an oil refinery in Malacca, Malaysia was used throughout this study. This bacterium was maintained at the Universiti Selangor Culture Collection Centre, Selangor, Malaysia. The bacterium from 50% glycerol stock culture was grown in 30 °C at pH 7 in nutrient broth (NB) for 24 h and was used as an inoculum for all cultivation experiments. Culture was stored in −80 °C for subsequent used.

2.2. Production of Rhodococcus UKMP-5M cells

Liquid minimal salt medium (MSM) consisted of (g/L): K_2HPO_4, 0.4; KH_2PO4,0.2; NaCl, 1; $MgSO_4$ 0.1; $MnSO_4$ 0.01; $FeSO_4H_2O$, 0.01 and $Na_2MoO_4{\cdot}2H_2O$, 0.01 (Bai et al., 2007) was used as the basal medium for the cultivation of *Rhodococcus* UKMP-5M. The cultivation of *Rhodococcus* UKMP-5M was carried out in 250 mL shake flask containing 100 mL medium. Minimal salt medium (MSM) containing different carbon sources; i) MSM with 0.5 g/L phenol, ii) MSM with 2 g/L glucose, and iii) MSM with a mixture of 0.5 g/L phenol and 2 g/L glucose, were tested in this study. The flask was inoculated with 10% (v/v) inoculum and incubated at 30 °C on a rotary shaker (Jeiotech; SI-600R, Korea), agitated at 160 rpm for 24 h. At the end of cultivation, the cells from the culture broth were harvested by centrifugation at 29,568 g (TOMY; MX-305, Japan) for 15 min. Supernatant was discarded and the cells were washed using sterilise phosphate buffer (pH 7.0) for three times. The cell suspension in phosphate buffer with a concentration of 1 g/L was stored at 4 °C, for subsequent used in phenol biotransformation.

2.3. Phenol biotransformation using resuspended cells of Rhodococcus UKMP-5M

The *Rhodococcus* UKMP-5M cell suspension stored in phosphate buffer was resuspended in various biotransformation media (phosphate buffer containing phenol and MSM containing phenol) to initiate phenol biotransformation. The medium was sterilized by autoclaving at 121 °C for 20 min. Phenol was sterilized separately by filtration using 0.2 μm regenerated cellulose membrane filter and added to the sterilized medium after cooled down to room temperature.

All biotransformation experiments were performed in triplicate and the results were presented as average value. During the cultivation, 10 mL of culture samples were withdrawn at time intervals (between 0 and 24 h) for analysis.

2.4. Analytical methods

The optical density of the culture was measured at 680 nm using a spectrophotometer (BioMate 3 UV–Vis Spectrophotometer, Thermoscientific, USA). Cell concentration, in term of dry cell weight, was determined by filtration and oven dried method (Ariff et al., 1996). The known volume of culture sample was filtered through a known weight of dried membrane filter with the pore size of 0.2 μm using a vacuum pump. The membrane filter with the bacterial cells was dried at 80 °C until a constant weight was achieved.

Phenol concentration in the culture was determined by a colorimetric method based on rapid condensation with 4-aminoantipyrene (4-AAP), followed by oxidation with potassium ferricyanide under alkaline conditions to give a red-colored product (APHA, 1998). To perform the analysis, 1 mL of supernatant (adjusted to pH 10 using 1 N NaOH) was mixed with 100 μL of potassium ferric cynide $K_3Fe(CN)_6$. The mixture was mixed with 100 μL of 4-aminoantipyrine. The absorbance of the resulting solution after incubation for 15 min (red in colour) was measured at 500 nm.

Phenol hydroxylase (EC. 1.14.13.7) activity was determined by the oxidation of NADH in the presence of phenol, where the absorbance was monitored at 340 nm. The cell extract (100 μL) containing the enzyme was added into a mixture (3 mL) of 50 mm KH_2PO_4:K_2HPO_4 buffer pH 7.2, 100 μmol of NADH and 100 μmol of phenol. One unit of phenol hydroxylase activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADH min^{-1} (Ali et al., 1998). Protein content in cells was extracted using glass beads disruption method (Ramanan et al., 2010) and protein concentration was determined according to the method as described by Bradford (1976), using bovine serum albumin as a standard.

3. Results and discussion

3.1. Effect of two media on phenol degradation by growing cell of Rhodococcus UKMP-5M

The time course of phenol degradation by growing cell in MSM containing only phenol as carbon source is given in Fig. 1. 0.5 g/L phenol was completely degraded after 21 h and the phenol degradation rate was 0.02 g/L.h. as compared to only 99.92% out of 0.230 g/L phenol was degraded when the degradation process take place in Tricking Filter (Dey at al., 2013). The final cell concentration obtained at the end of the cultivation was 0.209 g/L. Substantially high phenol hydroxylase activity, 38.71 U/mL ($P > 0.05$) was detected in growing cell system, indicating that this enzyme was responsible in the degradation of phenol in comparison to the system containing MSM with the addition of glucose. Fig. 2 shows the time course of phenol degradation using

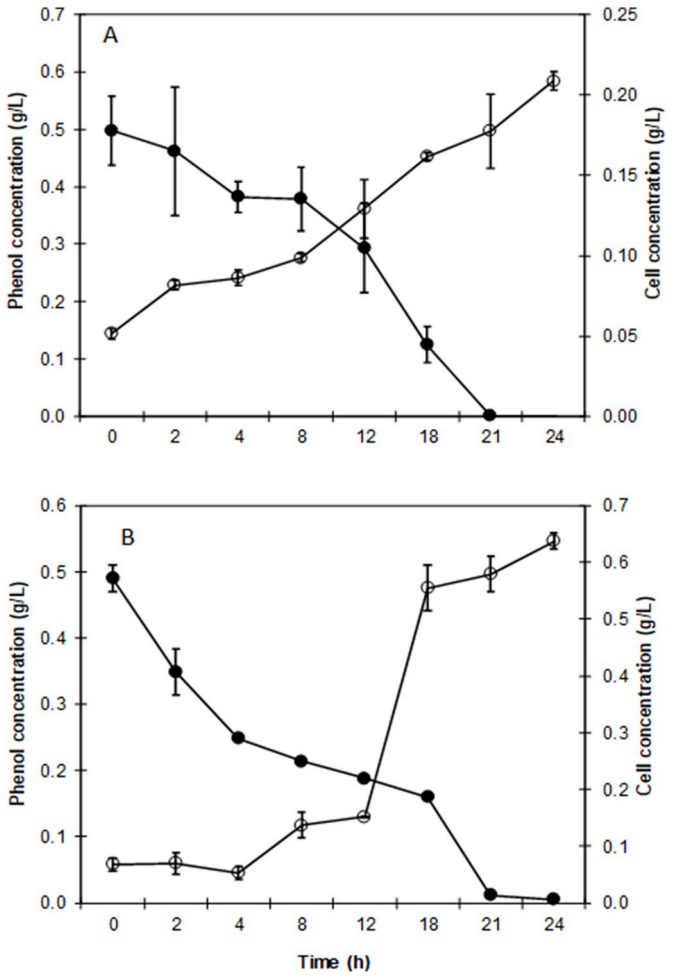

Fig. 1. Time course of phenol degradation by growing *Rhodococcus* UKMP-5 cell in two different cultivation media, (A) MSM with the addition of 0.5 g/L phenol, and (B) MSM with the addition of 0.5 g/L phenol and 2 g/L glucose. Symbols represent: (●) phenol degradation; (○) cell concentration. Statistically Significant Differences ($P < 0.05$) were observed.

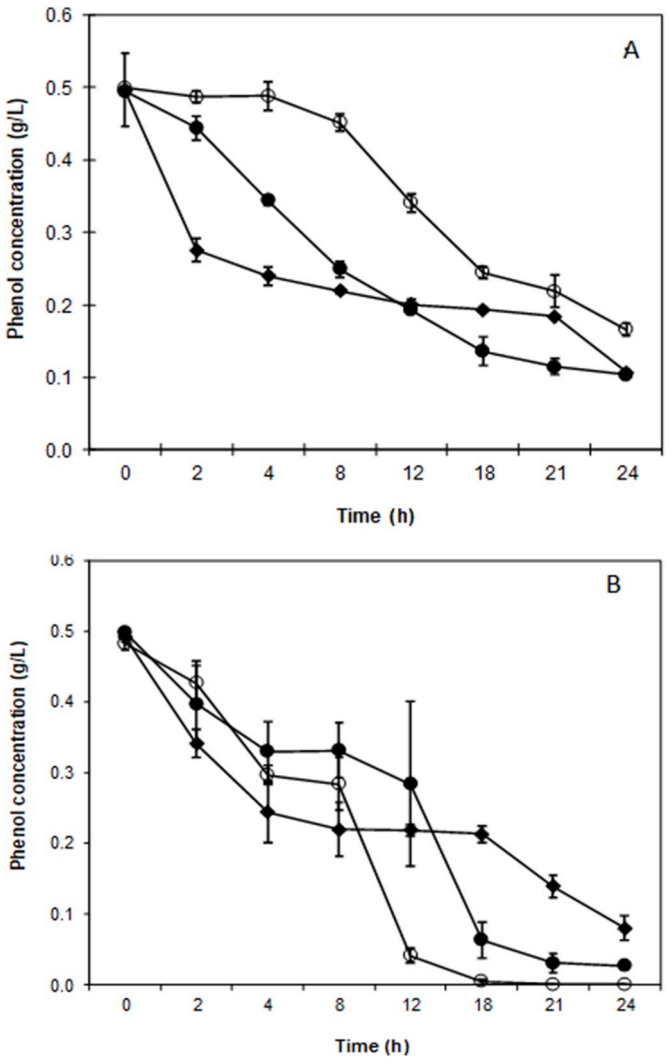

Fig. 2. Profile of phenol degradation during biotransformation by *Rhodococcus* UKMP-5M resting cell in two different suspension media, (A) phosphate buffer with 0.5 g/L phenol at pH 7.4, and (B) MSM with 0.5 g/L at pH 7.4. The cells of *Rhodococcus* UKMP-5M were produced in cultivation using three different media, (◆) MSM with 0.5 g/L phenol; (○) MSM with 2 g/L glucose; (●) MSM with 0.5 g/L phenol and 2 g/L glucose. Statistically Significant Differences ($P < 0.05$) were observed.

growing cell in MSM containing phenol with the addition of glucose as carbon source. In this growing cell system, phenol was degraded completely only after 24 h of cultivation. With the addition of glucose, higher cell concentration (0.638 g/L) with similar phenol degradation rate 0.020 g/L.h were observed. Reduced phenol hydroxylase activity (25.81 U/mL) in this growing cell system was related to a slight reduction in phenol degradation rate. From the results of this study, it can be concluded that the supplementation of extra carbon source (glucose) to the growing cell system of *Rhodococcus* UKMP-5M enhanced cell growth but phenol hydroxylase activity was inhibited. Phenol degrading aerobic bacteria are able to convert phenol into non-toxic intermediates of the tricarboxylic acid cycle (Powlowski and Shingler, 1994). Zaki (2006) claimed that the highest phenol mineralization rate was related to higher activity of phenol hydroxylase.

3.2. *Effect of pH on biotransformation of phenol by Rhodococcus UKMP-5M cell produced by cultivation using different carbon sources*

The effect of pH on the performance of phenol biotransformation by *Rhodococcus* UKMP-5M cell produced by cultivation using different carbon sources is summarized in Table 1. In all cases, the percentage of phenol degradation was significantly decreased with decreasing pH, where decreased in phenol hydroxylase activity was also observed. In biotransformation using cells produced by cultivation in MSM with phenol, the highest phenol hydoxylase activity (16.13 U/mL) was measured at pH 7.4. The activity was reduced to 9.68 U/mL and 3.23 U/mL at pH 7 and pH 6, respectively. The percentage of phenol degradation at pH from 7 to 8 was not significantly different ($P > 0.05$) and ranged from 73.39 to 78.68. Shourian et al. (2009) reported that phenol degradation by *Pseudomonas* sp. SA01 was optimal at pH 6.5. The consortium was able to grow on a wide range of pH from 5.5 to 8.5 achieving the phenol degradation efficiency in a range of 5%–99% for only up to 100 mg/L phenol (Veenagayathri and Vasudevan, 2010).

Biotransformation carried out using cells produced by cultivation in MSM containing only glucose as a carbon source shows almost similar result where the highest percentage of phenol degradation was also observed at pH 7.4, which also showed the highest phenol hydroxylase activity (12.90 U/mL). Significant reduction in the percentage of phenol degradation was noted at low pH (pH 6 to 6.4), which also corresponded well with decrease in phenol hydroxylase activity (6.45–9.68 U/mL).

In biotransformation using cells of *Rhodococcus* UKMP-5M produced by cultivation in MSM containing phenol and glucose as carbon source, the highest percentage of phenol degradation (79.23%) was also

Table 1
Effect of pH on biotransformation of phenol by *Rhodococcus* UKMP-5M cell produced by cultivation using different carbon sources.

Biodegradation technique	Cultivation medium for cell production	pH	[a]Final phenol at the end of process (g/L)	Phenol degradation rate (g/L.h)	Percentage of Phenol degradation (%)	Phenol hydroxylase activity (U/mL)
Biotransformation with resting cells						
	MSM with 0.5 g/L phenol					
		6	0.191	0.013	61.79 ± 0.11^{c}	3.23 ± 0.16^{c}
		6.4	0.185	0.013	62.98 ± 0.18^{c}	3.23 ± 0.11^{c}
		7	0.133	0.015	73.39 ± 0.21^{b}	9.68 ± 0.21^{b}
		7.4	0.107	0.016	78.68 ± 0.28^{a}	16.13 ± 0.38^{a}
		8	0.121	0.015	75.80 ± 0.26^{b}	12.90 ± 0.31^{a}
	MSM with 2 g/L glucose					
		6	0.311	0.079	37.82 ± 0.23^{c}	6.45 ± 0.24^{c}
		6.4	0.285	0.089	42.91 ± 0.36^{c}	9.68 ± 0.32^{b}
		7	0.209	0.012	58.24 ± 0.38^{b}	6.45 ± 0.18^{c}
		7.4	0.166	0.014	66.78 ± 0.41^{a}	12.90 ± 0.42^{a}
		8	0.210	0.012	58.04 ± 0.37^{b}	9.68 ± 0.37^{b}
	MSM with 0.5 g/L phenol and 2 g/L glucose					
		6	0.124	0.016	75.20 ± 0.35^{b}	6.45 ± 0.22^{c}
		6.4	0.126	0.016	74.71 ± 0.33^{b}	9.68 ± 0.37^{b}
		7	0.169	0.014	66.24 ± 0.18^{c}	12.90 ± 0.48^{a}
		7.4	0.104	0.017	79.23 ± 0.43^{a}	12.90 ± 0.42^{a}
		8	0.124	0.016	75.20 ± 0.37^{b}	6.45 ± 0.35^{b}
Cultivation with growing cells						
	MSM with phenol	7.5	0.006	0.029	98.90 ± 0.45^{a}	38.71 ± 0.51^{c}
	MSM with phenol and glucose	7.5	0.006	0.020	98.90 ± 0.45^{a}	25.81 ± 0.48^{c}

Data were obtained from the time course of each fermentation run.
Values shown are mean of three replicates with +SD.
[a-c] Mean value in same row with different superscripts are significant different ($P < 0.05$).
[a] Biotransformation was performed in phosphate buffer containing 0.5 g/L phenol.

observed at pH 7.4. The highest activity of phenol hydroxylase (12.90 U/mL) was observed at pH 7.0 and 7.4, and the activity was decreased significantly ($P < 0.05$) at low pH (pH 6.0 and 6.4). However, the percentage of phenol degradation at low pH was not significantly different with that obtained at pH 7.4. It is interesting to note that the percentage of phenol degradation (58.04%) and phenol hydroxlase activity (9.68 U/mL) was significantly reduced at high pH (pH 8) for biotransformation using cells produced by cultivation using MSM with only glucose. Similar observation did not occur in biotransformation using cells produced by cultivation using MSM with phenol or a mixture of phenol and glucose. In addition, the percentage of phenol degradation in biotransformation using cells produced by cultivation in MSM without phenol (i.e., MSM with glucose alone) was comparatively lower than those obtained by biotransformation using cells cultivated in MSM with the addition of phenol. From the results of this study, it can be concluded that the presence of phenol in cultivation media is the critical factor in producing cells of *Rhodococcus* UKMP-5M with high ability in phenol degradation during biotransformation. The present of phenol in the cultivation media enhanced the secretion of phenol hydroxylase. This phenomena has been describe by Paca et al., 2007. They claimed that the NADPH-dependent phenol hydroxylase activity in *C. tropicalis* cells was significantly increased for about 20 fold higher when cells were grown on media containing phenol. On the other hand, low levels of NADPH-dependent phenol hydroxylase activity were detected in the cytosolic fraction of *C. tropicalis* grown on glucose as the carbon source.

3.3. Effect of cell suspension media on phenol degradation during biotransformation using Rhodococcus UKMP-5M cells

The profiles of phenol degradation during biotransformation using cells of *Rhodococcus* UKMP-5M suspended in two different suspension media, phosphate buffer with phenol at pH 7.4 and MSM with phenol at pH 7.4, are shown in Fig. 2. Similar to results presented earlier, the phenol degradation rate in biotransformation using resting cells in phosphate buffer with phenol was influenced by the medium used in cell production. However, the effect of different media used in cell cultivation for biotransformation in MSM with phenol was not clear. The cells produced by cultivation in MSM with phenol gave higher phenol degradation rate in the initial stages of biotransformation (0–6 h), but the rate was greatly reduced in the later stages (6–24 h). On the other hand, cells produced by cultivation in MSM with glucose had low phenol degradation rate during the initial stages, but the rate was increased in the later stages of biotransformation.

In general, biotransformation performed using suspended resting cells in MSM with 0.5 g/L phenol buffered at pH 7.4 showed higher percentage of phenol degradation as compared to cells suspended in 0.5 g/L in phosphate buffer. Results from this study indicate that the percentage of phenol degradation (only up to 78%) was not enhanced ($P > 0.05$) when phosphate buffer was used as a transformation medium, suggesting that the secretion of phenol hydroxylase and phenol degradation were not efficiently occurred during non-growth condition of *Rhodococcus* UKMP-5M. The degradation of phenol during biotransformation in MSM with addition of phenol as carbon source was found to be significantly higher (84.0–99.8%) than those obtained in biotransformation medium in phosphate buffer with additional of phenol as carbon source. The higher percentage of phenol degradation in biotransformation with the presence of nutrients, supplied by MSM, confirms the important role of nutrient on phenol biodegradation.

3.4. Effect of various concentrations of Rhodococcus UKMP-5M cells on biotransformation of phenol

The effect of *Rhodococcus* UKMP-5M cells concentration of biotransformation of phenol is shown in Fig. 3. In this experiment, the cells were produced by cultivation in MSM with 0.5 g/L phenol and were suspended in MSM containing 0.5 g/L phenol buffered at pH 7.4. A significant increase ($P < 0.05$) in protein concentration was observed with increasing cell concentration used in biotransformation. The protein concentration detected in biotransformation using 5%, 19%, 15% and 20% cell concentration was 0.11, 0.43, 0.51 and 0.65 mg/mL, respectively. The increment in protein concentration was also paralleled with increasing phenol hydroxylase activity. Phenol hydroxylase activity measured in biotransformation using 2, 5 and 10% cell concentration was 12.90, 16.13 and 22.58 U/mL, respectively. Although protein

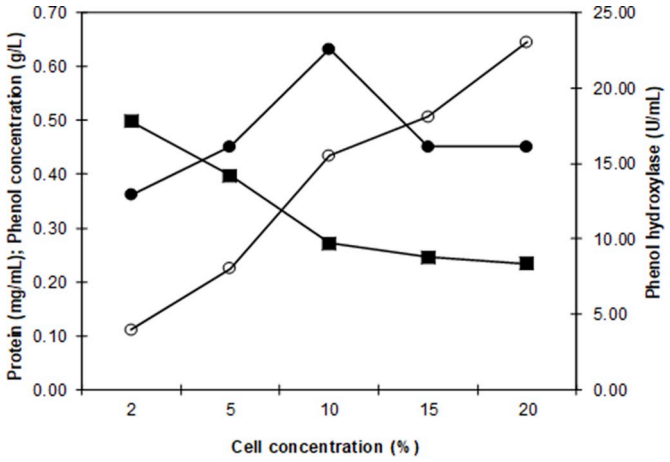

Fig. 3. Effect of different concentrations of *Rhodococcus* UKMP-5M resting cells on the performance of phenol biotransformation. The cells were produced by cultivation in MSM with phenol and biotransformation was performed in MSM with phenol buffered at pH 7.4. Symbols represent: (○) total protein; (●) phenol hydroxylase activity; (■) phenol concentration.

concentration was higher when high cell concentration was used in biotransformation, reduction of phenol hydroxylase activity to 16.13 U/mL was observed in biotransformation using high cell concentrations (15 and 20%). Thus, it can be concluded that cell concentration of 10% was optimal for the synthesis of high activity of phenol hydroxylase (22.58 U/mL) with the percentage of phenol degradation of about 78%. The transformation reaction is limited by substrate transport process at high cell concentrations (Wang et al., 2006).

The removal of phenol from liquid up to about 99% could be achieved in cultivation with growing cells of *Rhodococcus* UKMP-5M (see Table 1). The removal of phenol from liquid using biotransformation technique as reported in this study was only up to about 89%. Biotransformation of phenol as a toxic compound to non-toxic compound by *Rhodococcus* UKMP-5M was more efficient in growing cell culture supplied with nutrients for enhancement of microbial activity, especially enhancement of phenol hydroxylase secretion. The toxicity of non-growth substrate or its transformation products may result in the injuries of some compounds and therefore inactivate the cells (Si-Jing Wang et al., 2003). The benefit of biotransformation could be exploited by using this technique in subsequent stage after the biodegradation using the cultivation with growing cells. In the first stage, the phenol in liquid waste will be degraded by the growing cells of *Rhodococcus* UKMP-5M in batch cultivation. The cells harvested may be used subsequently in the second stage using biotransformation technique and the cells may be recycled for several stages of biotransformation.

4. Conclusions

Results from this study have demonstrated that the biotransformation using suspended cells of *Rhodococcus* UKMP-5M in buffered MSM containing phenol can be used as the efficient method for the removal of phenol from the liquid waste. The phenol degradation rate in biotransformation was greatly affected by the pH, cultivation medium for cell production, addition of nutrients in medium for biotransformation and cell concentration used in biotransformation. The optimal biotransformation conditions for phenol degradation were as follows; pH 7.4, 0.5 g/L phenol in MSM as biotransformation medium, cells were produced by cultivation in MSM supplemented with phenol and 10% cell concentration was used in biotransformation. The phenol degradation rate obtained in biotransformation correlated well with phenol hydroxylase activity, suggesting that this enzyme play important role in phenol biodegradation by *Rhodococcus* UKMP-5M. Unlike in growing cell system, complete degradation of phenol in biotransformation with initial concentration of phenol at 0.5 g/L cannot be achieved. The highest percentage of phenol degradation in biotransformation using suspended cells of *Rhodococcus* UKMP-5M was only up to 89%.

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