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Research Article

OPTIMIZATION OF OPERATING VARIABLES FOR PROTEIN DISRUPTION BY *RHODOCOCCUS* UKMP-5M AND PHENOL DEGRADATION PATHWAY

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ABSTRACT
The feasibility of using *Rhodococcus* UKMP-5M in phenol biodegradation is the main focus of this study. High biodegradation of phenol was corresponded well with high concentration of protein, suggesting that this compound was responsible in phenol biodegradation. The cells of *Rhodococcus* UKMP-5M were successfully disrupted using glass bead technique for the extraction of protein. The optimal cell disruption was obtained at this condition: 50 mL falcon bottle, glass bead with the diameter of 425-600 µm, cell concentration of 10%, and disruption time of 30 min. *Rhodococcus* UKMP-5M was metabolized phenol via the meta-pathway and it was confirmed by the detection of catechol 2, 3- dioxygenase at 375 nm wavelength which converted catechol into 2-hydroxymyconic semialdehyde which is a non-toxic compound.

Keywords: *Biodegradation, Phenol, Protein, Rhodococcus UKMP-5M, Meta-Pathway*

INTRODUCTION
Phenols are distributed either as natural or artificial mono-aromatic compounds in various environmental sites as major pollutants. Their existence in wastes from industrial processes such as oil refineries, coking plants, wastewater treatment plants, petroleum-based processing, textile manufacture, pharmaceuticals and phenol resin industry manufacturing and plants, has been well established (Kumar *et al.*, 2005; Scragg, 2006).
The World Health Organization (WHO) recommends that the maximum permissible concentration of phenol in drinking water is 1 µg/L (Kumaran and Paruchuri, 1997) as the concentration above this level may cause systemic poisoning (Basha *et al*., 2010) and has resulted in environmental contamination and contributed to many deleterious effects on living systems (Mailin and Firdaus, 2006).
The versatility and adaptability of the microbial degradation of hazardous wastes have demonstrated that it is a potential and the preferred technology for cleaning the contaminated soils and waters (Santos *et al.*, 2009) due to cost effective and environmental friendly (Ariana *et al.*, 2004). A vast research on phenol degradation have been conducted on a diverse group of microorganisms including *Candida tropicalis, Chlorella vulgaris, Pseudomonas putida, Aureobasidium pullulans, Bacillus cereus* (Wang *et al.*, 2011; Feng Liu *et al.*, 2010; Scragg *et al.*, 2006; Min Chen *et al.*, 2007; Santos *et al.*, 2009 and Benerjee and Ghoshal, 2010).
However, reports on the application of *Rhodococcus* spp. through the action of phenol hydroxylase enzyme for phenol degradation are very limited. Phenol hydroxylase is an intracellular degrading enzyme, in which, cell disruption is considered as an important step in downstream processing for the recovery of intracellular products (Ramanan *et al.*, 2008; Shynkaryk *et al.*, 2009). Established protocols of cell wall disruption involve breaking the cell wall either mechanically (high-speed agitation in a bead mill with glass beads) or enzymatically (Ying Lim *et al.*, 2008).
The objective of this study was to optimize the disruption procedure for protein release and to determine to metabolic pathway of phenol degradation by *Rhodococcus* UKMP- 5M.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

The bacterium, *Rhodococcus* UKMP-5M, was used throughout this study. This bacterium was isolated from a petroleum contaminated soil at an oil refinery in Malacca, Malaysia. The bacterium from stock culture maintained in glycerol was transferred into shake flask containing nutrient broth (Merck, Germany). The flask was incubated at 30^{o}C in an incubator shaker (Jeio Tech SI-600R, Korea) agitated at 160 rpm for 24 hours. This culture was used as a standard inoculum throughout this study.

Cultivation and Phenol Degradation Experiments

Minimal Salt Medium (MSM) was used in this study. The optimized $(NH_4)_2SO_4$; 0.3 g/L was used to obtain the highest concentration of cell. The medium (100 ml) in 250 ml shake flask was inoculated with 10% (v/v) inoculum to initiate the cultivation and degradation of phenol. The flask was incubated at 36^{o}C on a rotary shaker, agitated at 160 rpm. During the cultivation, 10 ml of culture samples were withdrawn at time intervals for analysis.

Cell Disruption

Disruption of *Rhodococcus* UKMP-5M cells for protein extraction was performed using bead mill. The effect of different milling times (30, 60, 90 and 120 sec) on the secretion of protein from the disrupted cells of *Rhodococcus* UKMP-5M was studied. Subsequently, the effect of bead diameter (<106, 150-212, 212-300,425-600, 710-1180 µm) was studied at the milling time of 30 sec, which was the preferred time for the protein release. The effects of shape and container size (50 ml Falcon, 15 ml Falcon, 30 ml universal bottle, 50 ml volumetric flask, 50 ml scotch bottle and 10 ml white cap bottle) and different cell concentrations (2, 5, 10 and 15%) on the efficiency of cell disruption and protein extraction were also studied.

Analytical Procedures

During the cultivation, samples were taken for analysis. The samples were centrifuged at 40 000 rpm for 20 minutes. Total protein content was measured using Bradford method (Bradford, 1976).

Phenol hydroxylase enzyme assay was based on method carried out by Ali *et al.*, (1998) with some modification. The oxidation of NADH in the presence of phenol by physically-treated cell extracts was monitored at 340 nm for every 1.5 min. Three milliliters of reaction mixtures containing 50 mm KH_2PO_4: K_2HPO_4 buffer pH 7.2, 100 µmol NADH, and 100 µmol phenol was used before the addition of the 100 µl cell extract.

One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NADH per minute. The phenol hydroxylase molar extinction coefficient at 340 nm is 6200 $M^{-1}cm^{-1}$ (Dawson, 1985) and the enzyme activity was calculated based on Beer's Law

Catechol-2, 3-dioxygenase enzyme assay was based on method as described by Ali *et al.*, (1998). Crude protein extract (100 µl) was added into the reaction mixture (3 ml) containing 50 mm KH_2PO_4:K_2HPO_4 buffer pH 7.2 and 1 µmol catechol, which was equilibrated at 55°C. The increase in absorbance at 375 nm caused by the formations of the reaction product 2-hydroxymuconic semialdehyde ($\varepsilon M = 4.4 \times 10^4$ $M^{-1}cm^{-1}$) was monitored for the assay of catechol-2, 3-dioxygenase.

For determination of catechol-1, 2-dioxygenase activity, similar reaction conditions as for catechol-2, 3 dioxygenase assay was used. The formation of cis, cis-muconic acid was monitored by reading the absorbance of the reaction mixture at 260 nm ($\varepsilon M = 1.75 \times 10^3$ $M^{-1}cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 µmol 2-hydroxymuconic semialdehyde min^{-1} at 55°C (Ali *et al.*, 1998).

RESULTS AND DISCUSSION

Optimization of Operating Variables for Protein Disruption

The effect of glass bead operating variables on the disruption of *Rhodococcus* UKMP-5M cells and the amount of protein release is summarized in Table 1. Among the milling time (30, 60, 90 and 120 sec) tested in this study, the highest protein release (0.782 mg/ml) was obtained at 30 sec. Decreased in the amount of protein release with increasing milling time was observed. This may be due to protein

denaturation after prolonged milling time. The ratio of glass bead to cell of 1:1.5 was used in this study (Ramanan *et al.*, 2008).

The glass beads diameter of less than 106 μm was not suitable to be used for the disruption of *Rhodococcus* UKMP-5M, as shown by very low protein release (0.09 mg/ml) (Table 1). The efficiency of cell disruption with higher release of protein was improved with increasing glass bead diameter. The highest protein release (1.108 mg/mL) was obtained when glass bead with the diameter of 425-600 μm was used.

However, protein release (0.352 mg/mL) was greatly reduced when bead with large diameter (710-1180 μm) were used. Bead milling is a simple, gentle, efficient and inexpensive method for cell disruption without the need for specialized equipment. The technique can be used as a first step in both small and large scale protein purification (Song and Jacques, 1997). Beads are preferable as the simplest cell disruption technique when high protein concentration with minimal loss in activity is needed (Benov and Al-Ibraheem, 2002).

Size and shape of container also significantly ($P<0.05$) affects the release of protein from the disrupted cells of *Rhodococcus* UKMP-5M. The highest amount of protein release (1.197 mg/mL) was obtained when 50 mL falcon tube was used. The lowest protein release (0.162 mg/mL) was obtained when 10 mL white cap bottle was used. The influence of shape and the size of the container on cell disruption and protein recovery has also been reported by several researchers (Ramanan *et al.*, 2008; Benov and Al-Ibraheem, 2002).

Cell concentration in the feed also affects the efficiency of the disruption of *Rhodococcus* UKMP-5M cells by bead milling. The lowest protein release (0.440 mg/mL) was obtained at the lowest cell concentration (2%) tested in this study. Protein release was significantly increased ($P<0.05$) with increasing cell concentration.

The highest protein released was obtained when 20% cell concentration was used in the milling. However, cell concentration at 10% was selected for further used due to comparable results and limited amount of cell produced by *Rhodococcus* UKMP-5M.

***Meta and Ortho-Cleavage Pathway Determination for Phenol Degradation by Rhodococcus* UKMP-5M**

A typical time course of phenol degradation by *Rhodococcus* UKMP-5M is shown in Figure 1, in which, meta-pathway of phenol degradation was determined. Results from this study showed that *Rhodococcus* UKMP-5M metabolized phenol via the meta-pathway and it was confirmed by the detection of catechol 2,3- dioxygenase at 375 nm wavelength which converted catechol into 2-hydroxymyconic semialdehyde. The profile of the enzyme activity was parallelled with phenol concentration, where the enzyme activity was increased with decreasing phenol concentration.

Limited activity of catechol 1, 2-dioxygenase was detected in the culture supernatant, indicating that phenol degradation by *Rhodococcus* UKMP-5M followed the meta-pathway. Phenol degrading bacteria are able to convert non-toxic intermediate of the tricarboxylic acid cycle via an ortho and meta pathway (Powlowski and Shingler, 1994).

Phenol degradation using similar pathway was also shared with *Bacillus* strain Cro 3.2 and *Bacillus cereus* (Ali *et al.*, 1998; Benerjee and Ghoshal, 2010).

On the other hand, *Fusarium* sp. exhibited both catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase suggesting that the intermediate catechol can be oxidized in the catabolic pathway of ortho and meta fission (Cai *et al.*, 2007).

Some bacterial strains have the capability to degrade substances with multiple pathways. In addition, Tuah (2006) assumed that *Candida tropicalis* RETL-Cr1 probably metabolized phenol via the ortho-pathway. As a confirmation, meta-cleavage dioxgenase assays was performed to prove that *Rhodococus* UKMP-5M was followed the meta-pathway.

Result for spray plate method show the yellow colour formation as a result of meta cleavage of catechol by meta-cleavage dioxygenase when *Rhodococcus* UKMP-5M was sprayed with an ether solution of catechol (Figure 1).

Table 1: Effect of Operating Variables on Protein Release by *Rhodococcus* UKMP-5M Using Glass Beads Technique

Operating Variables	Protein Concentration (mg/ml)
A. Optimization of Disruption Time	
* Bead Diameter 425-600 µm	
* *Falcon 50 mL*	
Time (sec)	
30	0.782
60	0.761
90	0.693
120	0.686
Blank	0
B. Optimization of Bead Diameter	
**Beads Diameter (µm)*	
< 106	0.009
150-212	0.153
212-300	0.884
425-600	1.108
710-1180	0.352
C. Optimization of Shape and Size of Container	
**Bead Diameter 425- 600 µm*	
Container Shape and Size	
50 mL Falcon	1.197
15 mL Falcon	0.918
30 mL Universal Bottle	0.547
50 mL Volumetric Flask	0.916
50 mL Scotch Bottle	0.753
10 mL White Cap Bottle	0.162
D. Optimization of Cell Concentration	
**Bead Diameter 425-600 µm*	
**Falcon 50 mL*	
Cell Concentration (%)	
2	0.440
5	0.931
10	1.129
15	1.205
20	1.218

*variables; disruption was performed for 30 min in B, C and D

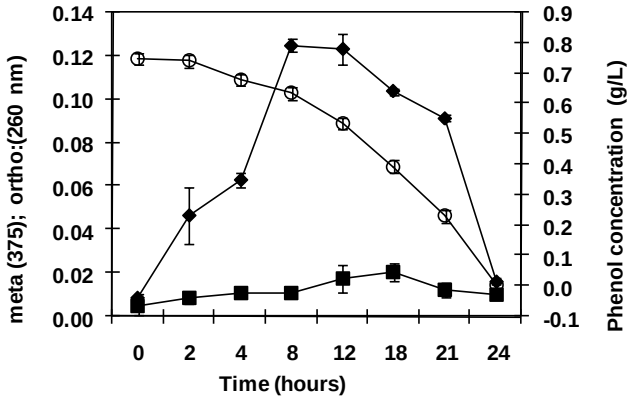

Figure 1: Time Course of Phenol Degradation by *Rhodococcus* UKMP-5M via Ortho and Meta Pathway
Symbol Represent: (■) Ortho-Pathway (♦) Meta-Pathway; (○) Phenol Degradation. Error Bars represent Standard Error between Three Determinations. Statistically Significant Differences (P<0.05) were Observed

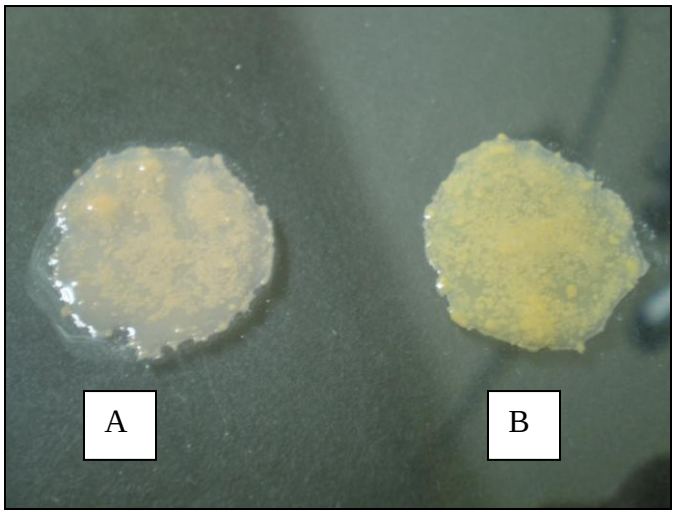

Figure 2. Photograph of *Rhodococcus* UKMP-5M Cells Using Meta-Cleavage Dioxygenase Assays A) Orange Color B) Yellow Color

Conclusion

The cells of *Rhodococcus* UKMP-5M were successfully disrupted using glass bead technique for the extraction of phenol hydroxylase. The optimal cell disruption was obtained at this condition: 50 ml falcon bottle, glass bead with the diameter of 425-600 µm, cell concentration of 10%, and disruption time of 30 min. Therefore, sufficient amount of phenol hydroxylase enzyme can be obtained for phenol degradation by using this technique.

Rhodococcus UKMP-5M was successfully confirmed to follow meta-pathway by detection of catechol 2,3- dioxygenase at 375 nm wavelength which converted catechol into 2-hydroxymyconic semialdehyde. Results of this study have demonstrated that *Rhodococcus* UKMP-5M is a versatile bacterium which has a great potential to be used industrially in the removal of xenobiotic compounds especially phenol by producing useful non-toxic metabolite.

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