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Research Article

BIOTRANSFORMATION OF PHENOL BY THE RESTING CELLS OF *RHODOCOCCUS* SP. NAM 81

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ABSTRACT

Continuous accumulation of phenol contaminant in wastewater liberated from different resources affect the natural biodiversity of the environment. A locally isolated *Rhodococcus* sp. was successfully shown it ability to remove phenol. This study demonstrated that the employment of resting cells of *Rhodococcus* sp. NAM 81 from late exponential phase, suspended in phosphate buffer can be used as an alternative in comparison to free and immobilized cells for phenol biodegradation process in liquid waste. Before the resting cells are being tested in different phenol concentrations as a source of biocatalyst, the suitability of the type of inoculums was investigated. The results showed that acclimatised cells towards phenol with 3g/L as the initial cell concentration were the most competent in degrading phenol.

Keywords: *Rhodococcus, Phenol, Biodegradation, Resting Cells, Phenol Hydroxylase*

INTRODUCTION

The widespread discharge of aromatic, halogenated aliphatic and polycyclic aromatic hydrocarbons, synthethic polymers and azo dye compounds through leakage and spillages had caused extensive pollution of surface soils, sea and groundwater environments. Demands for clean water has drawn significant attentions towards the monitoring of water supplies as wells as the reprocessing and recycling of wastewater as there is not less than two thousand pollutants that existed in wastewater (Annadurai *et al.,* 2000). Phenol is among the pollutant that is classified as a priority pollutants worldwide in account of its toxicity (Agarry and Aremu, 2012). It can be found naturally in crops, microbes, foods and cosmetics, the broad existence of man-made phenol in the environment due to many activities in different major industrial sectors which have also affected the quality of water. Phenol contaminant in wastewater and environment originates from industrial effluents such as from the petrochemicals, polymeric resins, pharmaceutical, steel, dye, food, oil refineries and coal conversion industries (Yemendzhiev *et al.,* 2008; Passos *et al.,* 2010). It is being widely applied as fuels, as solvents and starting materials for the creation of plastics, synthetic fibres and pesticides (González *et al.,* 2001; Nunes *et al.,* 2008; Ali *et al.,* 2009). Phenol signifies a long-term cause of contamination and poses acute risks to the environmental health as it is being manufactured in huge amounts annually. Due to its harmful side effects, various treatment processes were developed for removing of phenol from wastewater including biological-based technology or bioremediation. It is environmentally friendly method for pollution remediation and offers a safe and cost effective approach compared to that of physico-chemical strategies (Wang *et al.,* 2007). The continuous increase in advances in science and technology has escalated the interest towards the use of natural resources to a great extent. Aerobic, anaerobic bacteria, yeast, and fungi are involved in bioremediation of phenol with these microorganisms metabolising the compound as an energy and carbon source (Varma and Gaikwad, 2009).

Rhodococcus is a remarkable bacterial species that shows feasibility in decomposing numerous pollutants and is a valuable candidate in the field of bioremediation. This genus has attracted attention because of its resistance towards the toxicity of xenobiotics and the existence of extensive catabolic activities. In this study, a bacterial strain that can degrade elevated concentration of phenol, *Rhodococcus* sp. NAM 81, was

utilized in phenol bioremediation. This strain is capable to degrade phenol by using it as a carbon source for growing bacterial cells and immobilized cells. This study was aimed to determine the prospect of employing the resting whole cells of *Rhodococcus* sp. NAM 81 in degradation of phenol. Various factors that could affect the efficiency of phenol removal by resting cells of *Rhodococcus* sp. NAM 81 was investigated in order to determine the most desired condition for maximum phenol biotransformation.

MATERIALS AND METHODS

Microorganism and Culture Conditions

Rhodococcus sp. NAM 81 that is capable to degrade phenol was obtained from UNISEL Culture Collection Unit, Institute of Bio-IT Selangor. Nutrient broth and minimal salt medium (MSM) was applied for the experiments in this study. MSM consisting of (g/L); K_2HPO_4, 0.4; KH_2PO_4, 0.2; NaCl, 1; $MgSO_4$, 0.1; $MnSO_4$, 0.01; $FeSO_4H_2O$, 0.01 and $Na_2MoO_4.2H_2O$, 0.01 was prepared according to Ahmad *et al.*, (2011). For phenol degradation experiments, sterilised MSM broth was prepared in 250mL conical flask, while phenol was sterilised separately. It was filtered using 0.2µm regenerated cellulose membrane filter (Sartorius Stedim, Germany) and was added subsequently to an autoclaved medium. The initial pH of the medium was adjusted to 7.5 using 30% NaOH prior to autoclaving.

Determination of Growth Curve

An amount of 100ml of nutrient broth in 250ml Erlenmeyer flask was inoculated under sterile conditions with 1% of seed culture with an optical density of 1.00 at 600nm in triplicate. The flasks were incubated at 30°C on a rotary incubator shaker, agitated at 160rpm for seven days. The growth of bacteria cells was monitored at 6hrs intervals for the first and second day followed by every 12hrs monitoring for a week.

Preparation of Inoculum

Two types of inoculums have been tested; growing cells in nutrient broth and acclimatized cells in minimal salt medium (MSM) containing 0.5g/L phenol. Preparation of primary culture was started by inoculation of 1% of actively growing culture into the feed medium consisting of 30ml of MSM and carbon source and was incubated at 30°C on a rotary shaker, agitated at 160rpm for 24hrs. The fully adapted cell was used as the free cells. Resting cells were prepared by collecting the grown cells by centrifugation at 14000rpm for 15mins at 4°C. The supernatants were decanted and the cells were washed twice using phosphate buffer (pH 7) and resuspended in the same buffer with a concentration of 1g/L and refrigerated until further use.

Effect of Cells Suspension Media and Cells Concentration

The capability of resting *Rhodococcus* sp. NAM 81 cells (1g/L) suspended in different suspension solution (phosphate buffer (pH 7), normal saline, basal medium, Tris-HCl (pH 7) was tested towards 0.5g/L of phenol concentration in MSM. Effect of various initial cells biomass (1, 2, 3, 4 and 5g/L) as the inoculum was also tested. The biodegradation of phenol was done in 100ml of MSM containing phenol in triplicate.

Analytical Procedures

MSM containing phenol were withdrawn at interval time to monitor residual phenol concentration from the spent medium which was determined quantitatively by the spectrophotometric method at 500nm using 4-aminoantipyrine as the color reagent (APHA, 2005). Optical density at 600nm (OD_{600}) as an indication of bacterial growth rate was also read by the spectrophotometer (Thermoscientific, USA).

RESULTS AND DISCUSSION

Growth Curve of Rhodococcus sp. NAM 81

The growth curve of *Rhodococcus* sp. NAM 81 was monitored in order to determine the reproduction profile of the strain. The increase of cell concentration and optical density over time for *Rhodococcus* sp. NAM 81 is exhibited in Figure 1 that shows a typical shape of growth pattern that comprised of lag, log and stationary phases. The strain started to multiply after 3hrs of incubation and the lag phase was observed within 12hrs of inoculation. The strain rapidly increases in the number of cells as a fresh grown seed culture has been used. Known optical density culture was used in order to know the starting cells

number. It was found that the optical density gradually increases over time and the strain demonstrated an exponential pattern after 18hrs of incubation with 0.87g/Lof cell density achieved at this point. The mid log phase was achieved at 30hrs with 0.3 absorbance value of optical density which corresponded to 1.42g/L of dry cell weight. The fast growth rate of this bacterium in nutrient broth is expected as the medium is rich and provided sufficient nutritional conditions that facilitated its growth and maintenance of the cells.

As shown of Figure 1, *Rhodococcus* sp. NAM 81 entered the stationary phase after 42hrs of incubation. Since there is no significant difference in the optical density of the cells harvested between 36hrs and 42hrs of incubation which accumulated 2.88g/L cells at 1.2 optical density and 3.02g/L cells at 1.35 optical density respectively, 36hrs of incubation was chosen to be the best time to harvest the cells. The cells obtained after 36hrs of incubation period produced high cell concentrations in the late exponential phase and was found to be suitably used as an inoculum. This locally isolated *Rhodococcus* strain exhibited rapid growth compared to Northern hemisphere species as reported by Ganguly (2005) who obtained the exponential phase at 36 to 65hrs of incubation and attained stationary phase after 70hrs. The difference might also be probably due to the component of the media used as *Rhodococcus rhodochrous* was grown in yeast and malt extract medium (Ganguly, 2005; Keusgen *et al.*, 2001). It was reported that log-phase resting cells for *Rhodococcus* sp. MB-P1 (Khan *et al.*, 2013) and *Trichosporon cutaneum* R57 have been utilized for phenol degradation (Lazarova *et al.*, 2015). In the present study, in order to verify the suitability of the cells to be kept as resting cells, the ability of cells from each phase was tested for phenol biotransformation. It was found that cells harvested from the log phase exhibited 90% degradation of phenol in MSM which was the most competent in comparison to lag phase and stationary phase cells, respectively.

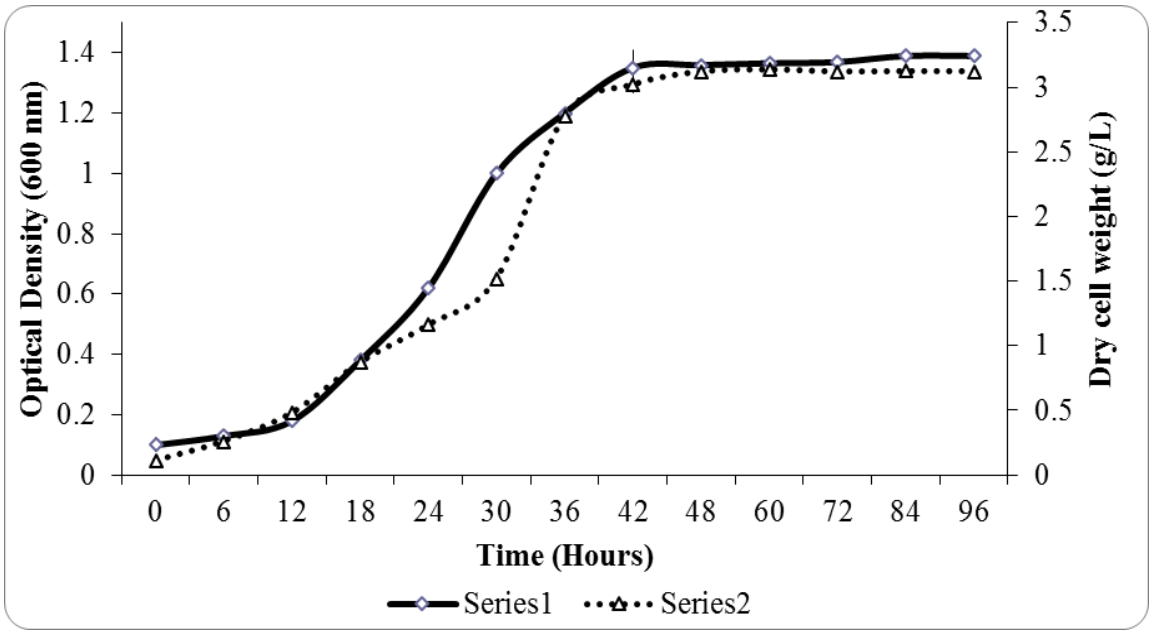

Figure 1: Growth Curve of *Rhodococcus* sp. NAM 81 in Nutrient Broth. The Strain was Grown at 30°C, 160rpm Agitation. Error Bars Represent Standard Error between Three Determinations

Types of Inoculums

Biodegradation of phenol using cells produced by cultivation in nutrient broth achieved 50% of phenol degradation and 0.321g/L cells while cells in MSM containing 0.5g/L phenol as a carbon source exhibited moderate cell concentration with only 0.456g/L as the highest within 24hrs of incubation compared to MSM containing glucose and phenol that was able to produced 0.771g/L cells. However, cells produced

in both media were able to degrade almost 75% of phenol within 24hrs of incubation in MSM containing 0.5g/L phenol. Resting cells from MSM with the addition of glucose demonstrated low degradation rate as it only achieved 50% of phenol degradation at 16hrs while cells from MSM with the addition of glucose and phenol achieved 50% of phenol degradation in less than 10hrs of incubation. Low concentration of glucose influenced the growth of *Rhodococcus* sp. NAM 81 but inhibited the activity of phenol hydroxylase as the degrading enzyme (Suhaila *et al.,* 2012). The addition of glucose supported higher metabolic activity and production of energy due to increase of biomass and linearly enhanced phenol-degrading activity of *Rhodococcus* NAM 81.

The utilisation of acclimatised cells increases the degradation capability of *Rhodococcus* cells because it shortens the lag phase of the cells as the existing phenol in the media enhanced the secretion of phenol-degrading enzymes (Paca Jr *et al.,* 2007) as high phenol hydroxylase activity is responsible for the degradation of phenol.

Hence, acclimatization is crucial in ensuring the activeness of organisms and adaptation to the carbon and energy source before being employed for phenol degradation process. The preparation of resting cell will be cultivated in MSM containing phenol before being suspended in buffer. A similar approach is applied to produce the resting cells of *Pseudomonas putida* EKII for degradation of phenol and phenolic compounds (Hinteregger *et al.,* 1992).

Effect of Cells Suspension Solution and Cells Concentration on Phenol Degradation

The effort to produce high performance resting cells for phenol degradation was continued with identification of the best solution to resuspend the cells. Buffers are aqueous system that is able to resist changes of its pH on addition of acid or base in small quantity. Biological buffer should not exhibit toxicity towards cells, should not infuse cells membranes, is able to resist enzymatic degradation and do not interfere with biological processes.

Hence, determination of suitable buffer in the preparation of *Rhodococcus* resting cells is essential especially for biodegradation and biotransformation purposes. The profiles of phenol biodegradation using resting cells in various type of solution are shown in Figure 2. Highest phenol degradation was demonstrated by resting cells in phosphate buffer (pH 7) resulting in 75.25±6% of degradation followed by 60.96±4% in Tris-HCl (pH 7), 45.06±1.9% in normal saline and 21.19±1% in 0.85% basal medium. Phosphate buffer was used for the suspension of *Rhodococcus rhodochrous* PA 34 in the production of nitrile hydratase (Prasad *et al.,* 2007).

It was also utilized in the preparation of resting cells of *Rhodococcus* UKMP-5M for cyanide degradation (Maegala *et al.,* 2011), for the cells of *Bacillus subtilis* ZJB-063 for nitrile-converting activity (Zheng *et al.,* 2007) and for *Nocardia globerula* for nitrile amide degrading acivity (Kumar *et al.,* 2005). Normal saline was utilized by Ainon *et al.,* (2011) for toluene biodegradation experiment. Basal medium exhibits low phenol degradation ability as it contains $CaCl_2$ that has been reported as one of the factors that inhibits degradation (Suhaila *et al.,* 2012). High concentration of $CaCl_2$ reduce the growth of microorganism and repress the excretion of phenol hydroxylase as suspension buffer and basal medium contained $CaCl_2$ (Antony *et al.,* 2013; Boumaaza *et al.,* 2015).

The suitable amount of resting cells for phenol degradation in is one of the key factors that determines the degradation performance of phenol. The effect of resting cells quantity on phenol degradation is as observed in Figure 3. The resting cells were incubated in MSM-containing phenol before it was resuspended in phosphate buffer. The cells were then employed in the experiment to determinant the efficiency of phenol removal in MSM containing phenol.

A significant increase in protein concentration and phenol hydroxylase activity was observed concurrent with the reduction of phenol concentration and an increase in the concentration of cells. The increasing trend of phenol hydroxylase activity was paralleled with protein concentration only from 1 to 4g/L of cell concentration but dropped when 5g/L of cells were used. It was observed that 3g/L of resting cells were the optimal concentration for high phenol hydroxylase activity with 75% phenol degradation. Substrate mass transfer is reported as the limitation in transformation reaction at high cell concentrations (Wang *et al.,* 2006).

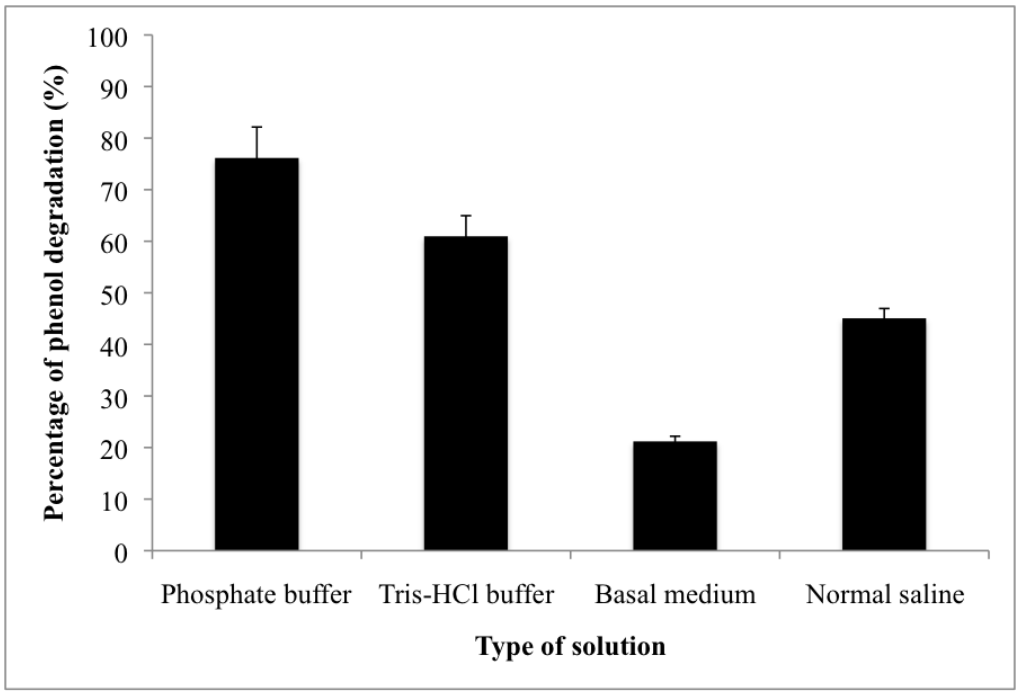

Figure 2: Phenol Degradation by Resting Cells of *Rhodococcus* sp. NAM 81 Resuspended in Different Solution. Error Bar Represents Standard Error between Three Determinations

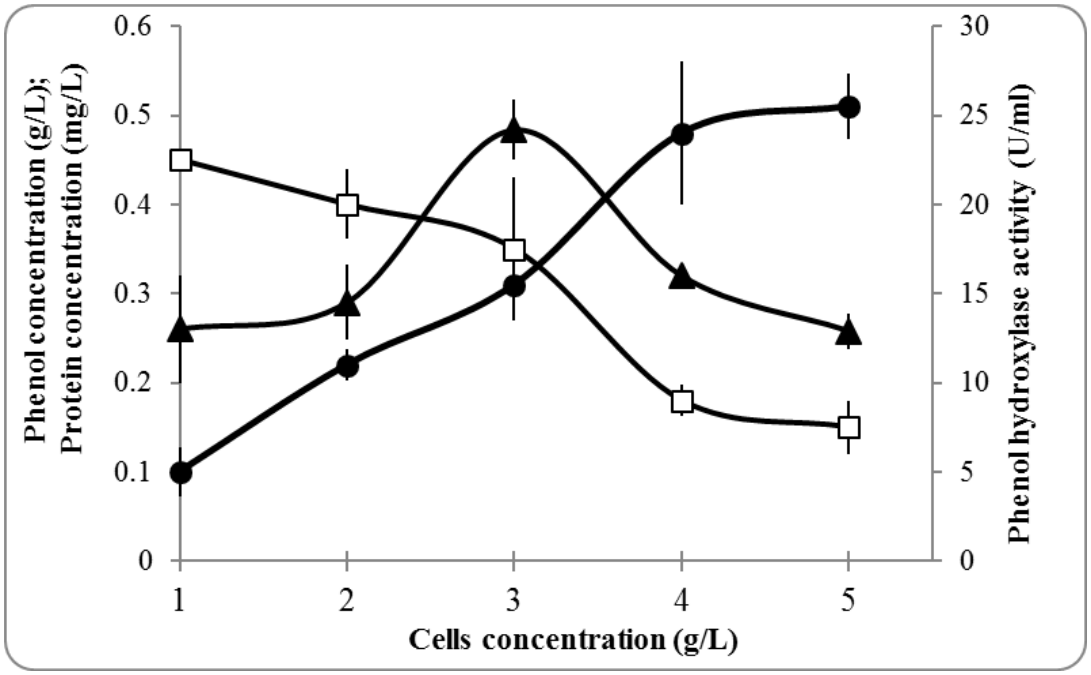

Figure 3: Phenol Degradation in MSM Containing 0.5 g/L Phenol for 24 hours at 30 °C by Various Cell Concentration of Resting Cells of *Rhodococcus* sp. NAM 81
Symbol Represent: □ Phenol Concentration; ▲ Phenol Hydroxylase Activity; ○ Protein Concentration. Error Bar Represents Standard Error between Three Determinations

Conclusion

This study investigated the removal of phenol by resting cells of *Rhodococcus* sp. NAM 81 and this method demonstrates promising potential as an alternative for growing and immobilized cells system for the biotransformation of phenol. Normally, biodegradation involves microbial communities using the enzymes or whole cells either as growing or resting cells. The employment of growing cells for phenol bioremediation sometimes have limitation such as minimum or inadequate growth, minimal concentration of cells, inhibition of growth and minimum percentage and rate of degradation especially at high phenol concentrations. High concentration of resting cells could be collected prior to its application as the cells are stable for long term storage. The following conclusions were drawn from this study: (1) The best time to harvest the cells is at the late exponential phase prior to phenol acclimatization (2) Phosphate buffer is the most suitable solution to preserve the resting *Rhodococcus* sp. NAM 81 cells (3) A concentration of 3 g/L is the most appropriate initial cell concentration for maximum phenol degradation by resting cells of *Rhodococcus* sp. NAM 81.

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