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Research Article

BIOTRANSFORMATION OF ACRYLONITRILE USING IMMOBILIZED CELLS OF *RHODOCOCCUS* UKMP-5M AS BIOCATALYST

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ABSTRACT
The ability of *Rhodococcus* sp either as free resting cells or immobilized cells to transform nitrile-based substances has been proven. These substances exist in industrial wastewater. In this study, the potential use of immobilized cells of a Malaysia strain, *Rhodococcus* UKMP-5M in the biotransformation of acrylonitrile was evaluated. Biotransformation using resting cells of *Rhodococcus* UKMP-5M entrapped in calcium alginate, agar-agar and polyurethane foam was compared with free resting cells. Highest nitrile-hydrolyzing enzymes activity was observed with immobilized resting cells in polyurethane foam. Hence, this method was further optimized by varying types of cells (growing or resting cells), size of the polyurethane foam and its quantity using one- variable-at-a-time method and one-way analysis of variance. The utilization of immobilized of growing cells of *Rhodococcus* UKMP-5M in polyurethane foam cubes at a size of 1cm × 1cm × 1cm with a quantity of 7, respectively yielded the most optimum of nitrile-hydrolyzing enzymes activity. In addition, the immobilized cells were found can tolerate higher concentrations of acrylonitrile as compared to free resting cells since immobilized cells were protected against the toxic effects of the substrate which in turn made the immobilized cells possess higher stability. On top of that, the immobilized cells can be reused up to 46 times with 56% of initial activity which proved to be beneficial economically. Based on these findings, it can be presumed that the immobilized cells of *Rhodococcus* UKMP-5M have a good potential as biocatalysts in the biotransformation of acrylonitrile.

Keywords: *Rhodococcus, Biotransformation, Biocatalyst, Immobilization, Nitrile-Hydrolyzing Enzymes, Polyurethane Foam*

INTRODUCTION
Nitrile substances are found in abundance as wastes from industries and has creates some of the environmental and cost production issues. Therefore, to overcome this issue, the green chemistry methods such as biodegradation, biotransformation and biocatalyst with a utilization of cells and enzymes, usage of bioreactors etc, has been introduced. The application of those methods is actively developed and enhanced in order to achieve the effective reaction which includes higher degradation rate, detoxification of pollutants and wastes as well as the formation of valuable products. The application of free resting cells in a bioreactor has encountered problems in maintaining the enzyme activity in repeated use as well as preservation (Chen *et al.*, 2008a). Therefore, immobilization of cells has become the solution of the problem. The benefits of the utilisation of immobilized cells in the bioremediation are higher stability of immobilized cells or enzymes as biocatalyst enable a continuous process of bioremediation, the ability to catalyse a broad range of pollutants as compared to the resting cells (Velankar *et al*., 2010) and protected against the toxic effects of the substrate which in turn makes the immobilized cells have higher stability (Gong *et al.*, 2012). In addition to that, it also can tolerate higher temperature and concentration of substrate, can be exploited to reach the maximum potential of catalytic of a process (Raj *et al.*, 2008) and helps in separating and protecting the cells from the unfavourable environment (Guo *et al*., 2006). One of

the interesting features of using immobilized cells is it can be reused a few times which in turn lowered the cost involved. Therefore, most of the industries which applied the green chemistry methods use immobilized cells in the production of chemicals.

One of the weaknesses of the immobilized cell is the physical loss of the biocatalyst from the system and its biocatalytic activity (Nigam *et al.*, 2009). It can be solved by selecting a good support matrix (such as agar, alginate, sodium alginate, k-carrageenan, hydrogel, polyurethane sponge, sea weed sponge, loofa etc) and immobilization procedure (such as adsorption onto, entrapment into, membrane immobilization and encapsulation (Martínková and Křen, 2010; Salter and Dell, 1991).

Nitrile can be degraded or transformed by the nitrile-hydrolyzing enzymes (namely nitrilase, nitrile hydratase and amidase) in two different pathways with ammonia and carboxylic acids as the end products in both of the pathways. In addition to that, most of the nitrile biotransformation in commercial scale, in industrial process and in the field application, was performed with the employment of immobilized cells as compared to free cell (Gong *et al.*, 2012). The advantages of the employment of immobilized cells in the bioremediation include enable to perform continuous process, the higher stability of biocatalyst, more economical biocatalytic process, ability to catalyze a broad range of nitrile substrate as compared to the resting cells and can be exploited to reach the maximum potential of catalytic of a process (Gong *et al.*, 2012; Velankar *et al.*, 2010; Raj *et al.*, 2008). However, the weaknesses of the immobilized cell are also need to be considered as the biocatalytic activity might loss or the physical loss of the biocatalyst from the system (Nigam *et al.*, 2009). It can be solved by selecting a good support matrix and immobilization procedure. Therefore, in this present study, the potential of immobilized cells of Malaysia strain, *Rhodococcus* UKMP-5M, in biotransformation of nitrile was investigated.

MATERIALS AND METHODS

All chemicals used in this study were of analytical grade and commercially available. For the preparation of all solution and media in this experiment, the deionized water was a 18.3 MΩ.cm unless stated otherwise. All the experiments involving the preparation of biotransformation mixture and sampling out the biotransformation mixture prior to centrifugation was conducted under sterile environment using aseptic technique. All the glassware used in the biotransformation reaction as well as in the quantification of ammonia was acid washed with warm 0.5 M hydrochloric acid, washed thoroughly with deionized water and air dried prior to autoclaving.

Microorganisms and Culture Conditions

A starter culture of *Rhodococcus* UKMP-5M was formulated by inoculating a loop full of *Rhodococcus* UKMP-5M from an agar plate into a 50mL of sterile minimal salts medium and incubated at 160rpm, 30ºC for 24hrs or until the OD reading at 600nm (OD_{600}) of the starter culture was 0.9 – 1.0. Approximately 1.0mL of a grown starter culture was inoculated into the sterilized minimal salts medium and supplemented with a fresh filter-sterilized propionitrile (0.1mL of 0.02M) followed by incubation at 30°C, 160rpm for 24hrs. The minimal salts medium was prepared by dissolving 3.0g yeast extract, 0.5g KH_2PO_4, 0.5g K_2HPO_4, 0.5g $MgSO_4$ and 0.01g of $CaCl_2$ in 1.0L of 18.3MΩ.cm Milli-Q deionized water (Mauger *et al.*., 1998).

After the period, the cells were harvested by centrifugation at 4ºC, 17800×*g* for 15min. The supernatant was discarded and the cells pellet was washed twice with 0.1M phosphate buffer solution (pH 7). The washed cell pellet was then resuspended in the same buffer and the OD_{600} of cell suspension was adjusted to 0.8 to 0.85 which corresponded to 0.8mg/mL dry cell weight (dcw) and kept refrigerated at 4ºC until further use.

Preparation of Different Types of Matrices for Immobilization

In the immobilization study, a few materials of support matrices as carrier has been evaluated, i.e. calcium alginate, agar and polyurethane foam. The immobilization technique used was the entrapment of the cell into the matrices.

Calcium Alginate: Approximately 1.6g of sodium alginate was dissolved in 40mL of deionized water. The solution was mixed well and divided into 20mL for each conical flask prior autoclaving at 121ºC for

20min for sterilization. After sterilization, the solution was cooled down until approximately 40ºC and added with 5mL of resting cells of *Rhodococcus* UKMP-5M. As for control, only 5mL of sterilized phosphate buffer (pH 7, 0.1 M) was added. The mixture was slowly extruded as drops using 1mL pipettor tip into a cold 0.2M calcium chloride, $CaCl_2$ (Chen *et al*.., 2008a; Graham, *et al*.., 2000; Almatawah & Cowan, 1999). The calcium alginate and resting cells of *Rhodococcus* UKMP-5M were gelled together in the aqueous solution of 0.2M $CaCl_2$, to produce spherical entrapment beads (Guo *et al*., 2006). The beads were then allowed to harden in $CaCl_2$ solution, at room temperature for two hours. After two hours, the beads were filtered through a sterilized siever and washed twice with sterilized deionized water and once with sterilized phosphate buffer to eliminate remaining Ca^{2+}. The beads were then stored in phosphate buffer solution at 4ºC for further use (Chen *et al*., 2008a; Chen *et al*., 2008b; Graham *et al*., 2000; Almatawah and Cowan, 1999).

Agar: The agar was prepared by dissolving 1.6g of agar powder into 40mL of deionized water and mixed well. The mixture was then divided into two which contained 20mL of mixture in each beaker prior to autoclaving.

After sterilization, the solution was cooled down until approximately 40ºC and added with 5mL of resting cells of *Rhodococcus* UKMP-5M. The beaker was swirled to ensure the solution was homogenous and poured into a sterilized petri dish. As for control agar, only sterilized phosphate buffer was added. The agar was allowed to harden at room temperature for 15min. The agar was cut into a 0.5cm × 0.5cm × 0.5cm cube using a clean and sterilized cutter. The agar cubes were then washed and kept as described above (Nigam *et al*., 2009).

Polyurethane Foam: The polyurethane foam (PUF) with a density of 38kgm^{-1} was used in this study was purchased from Fischer Scientific Sdn Bhd (Malaysia) A PUF was cut with a clean cutter to a size of 1cm × 1cm × 1cm. The PUF cubes were rinsed twice with distilled water and dried overnight at 30ºC. The dried PUF cubes were then weighed and autoclaved for sterilization (Chung *et al*., 2005; Quek *et al*., 2006 and Gad *et al*., 2010). Two types of cells, i.e. resting cells and growing cells were used in the entrapment of cells into PUF cubes.

For the entrapment of growing cells into PUF, 23 cubes of PUF, which were weighed earlier, were added into a 250mL conical flask containing 50mL of medium and followed by sterilization. After sterilization, a fresh filter sterilized of propionitrile, (0.4%v/v, 0.02M) was added into the solution. Approximately, 1.0mL of starter culture was inoculated into the solution and incubated at 30ºC, 160rpm for three days. As for control, no inoculum was added (Chung *et al*., 2005, Quek *et al*., 2006 and Gad *et al*., 2010). After three days, the cubes were then washed and kept as stated above.

For the entrapment of resting cells, an amount of 5.0mL of resting cells was dispensed into 20mL of phosphate buffer solution in a 100 mL Erlenmeyer flask followed by the addition of 14 autoclaved PUF cubes. The flask containing the solution of PUF cubes, resting cells and phosphate buffer solution was shaken for two hours at 160rpm. The solution was then discarded and the cubes were washed and kept as mentioned previously.

Optimization of Immobilized Cells of Rhodococcus UKMP-5M in Biotransformation

A few parameters were varied in order to obtain the optimum immobilized cells of *Rhodococcus* UKMP-5M, i.e. different types of matrices as support of immobilization, types of the cells of *Rhodococcus* UKMP-5M, as well as the size and quantity of the PUF cubes. Biotransformation was conducted by placing the reaction flasks in a water bath shaker and left for reaction at 30ºC and 80rpm for two hours. The reactions flasks and controls were performed in triplicates.

Different Types of Matrices: Biotransformation of acrylonitrile was conducted using resting cells immobilized in PUF, calcium alginate and agar and compared against free resting cells. In the resting cells, 10 mL of phosphate buffer solution (pH 6, 0.1M) containing acrylonitrile (5 mM) as substrate and 5%v/v resting cell in 50mL conical flask. For the immobilized cell, the same solution contained 9 cubes of PUF and approximately 40 agar cubes and 130 calcium alginate beads.

Types of Cells of Rhodococcus UKMP-5M: Biotransformation mixture consists of 10mL of phosphate buffer (0.1M, pH 6) and 5mM of acrylonitrile and added with PUF cubes. There were two types of PUF

cubes used in this study, i.e the PUF cubes with immobilized of growing cells of *Rhodococcus* UKMP-5M and PUF cubes with immobilized of resting cells of *Rhodococcus* UKMP-5M.

Sizes of PUF Cubes : PUF was cut into different sizes; 2cm × 2cm × 2cm, 1cm × 1cm × 1cm and 0.5 cm × 0.5cm × 0.5cm using a clean cutter. The quantities of PUF cubes added in 50 mL medium in a 250 mL Erlenmeyer flask were 23 cubes of 1cm × 1cm × 1cm, 40 cubes of 0.5cm × 0.5cm × 0.5cm and 3 cubes of 2cm × 2cm × 2cm. The procedure on the immobilization of growing cells of *Rhodococcus* UKMP-5M was performed as stated above. The biotransformation reaction mixture was conducted as described previously. The PUF cubes which have been immobilized with growing cells of *Rhodococcus* UKMP-5M was added as follows: 1 cubes of 2cm × 2cm × 2cm size, 9 cubes of 1cm × 1cm × 1cm size and 16 cubes of 0.5cm × 0.5cm × 0.5cm size.

Quantity of PUF Cubes: The growing cells immobilized in 1cm × 1cm × 1cm size of PUF cubes were used in the study based on the previous result. The quantity of PUF cubes added in the biotransformation of acrylonitrile was 3, 5, 7, 9 and 11, respectively.

Reusability of PUF Immobilized Cell

The reusability of PUF immobilized cells was evaluated by formulation of 10mL reaction mixture which contained 5mM acrylonitrile and 7 PUF cubes at a size of 1cm × 1cm × 1cm in a 50mL Erlenmeyer flask by two hours reaction at the temperature of 30°C and agitation of 80rpm. After each of the reaction, both of the sample and control PUF cubes were separated from the biotransformation mixture by filtration, washing as standard procedures. The washed PUF cubes were then added in the subsequent biotransformation mixture and continued with a similar reaction conditions as previous reaction. After each cycle, the reaction mixture (0.5mL) were dispensed and followed by the Indophenol Blue Methods for the quantification of ammonia the remaining reaction was transferred to 50mL falcon tube and spun down to determine the cell leakage.

Analytical Methods

Measurement of Growth using Dry Cell Weight and Measurement of Cell Leakage: Measurement of dry cell weight (dcw) of resting cells or growing cells was conducted by dispensing 1mL of the solution into sterilized 1.5mL Eppendorf tube and centrifuged (17,800×g, 15minutes, 4°C). The supernatant was discarded and the cell palate was then rinsed twice with phosphate buffer. Meanwhile, measurement of cell leakage was performed by transferring the remaining reaction solution into a sterilized 50mL falcon tube, spun down and washed. The washed cell pellet was then dried at 80°C for overnight or until the constant weight was achieved.

*Concentration of Ammonia Released***:** A sufficient amount of biotransformation mixture was dispensed in 1.5mL Eppendorf tubes, added with equal amount of 0.5M of hydrochloric acid to terminate the reaction followed by centrifugation. The samples were then transferred to test tubes and added with reagents according to the Indophenol Blue Method and left to stand in a dark room for one hour. After the period, the samples were measured the colorimetrically for ammonia quantification with reagent as blank (Biospectrophotometer Biomate 3, Thermo Scientific, USA). The concentration of ammonia was measured in mm and the nitrile-hydrolyzing enzymes activity (U) was then calculated from this measurement. 1U is defined as the amount of ammonia released in nmoles, per mL per min. The value of nitrile-hydrolyzing enzymes activity recorded was the average values with its standard error within the triplicates.

Statistical Analysis

All the experiments were conducted in triplicates and the values obtained were the means ± standard error of the triplicates. The experiments in optimization study were conducted as one-variable-at-a -time (OVAT) method. The comparison between groups was performed using one way analysis of variance (ANOVA) IBM SPSS version 20 with post hoc analysis by Duncan test. $p<0.05$ was considered statistically significant. The parameter results were ranked from *a* (most preferable optimum parameter) to *d* (least preferable optimum parameter) and the optimum parameter obtained from the current experiment was applied in the determination of the following optimum parameter.

RESULTS AND DISCUSSION

Optimization of Methods of Immobilization Method for Biotransformation

The optimization of immobilization methods for biotransformation was tabulated in the Table 1.

Table 1: Nitrile-Hydrolyzing Enzymes Activity of *Rhodococcus* UKMP-5M in the Optimization of Immobilization Methods

Parameter	Nitrile-Hydrolyzing Enzymes Activity (u/mg dcw)
Matrices	
Resting Cell	$0.3323^{a} \pm 0.0182$
Agar-Agar	$0.0516^{c} \pm 0.0114$
Calcium Alginate	$0.1438^{b} \pm 0.0141$
Polyurethane Foam	$0.1450^{b} \pm 0.0039$
Types of cell	
Immobilized Resting Cells + Acrylonitrile	0.0356 ± 0.0038
Immobilised Growing Cells + Acrylonitrile	0.0558 ± 0.0034
Resting Cells + Acrylonitrile	0.0350 ± 0.0023
PUF + Acrylonitrile	-
Sizes of PUF cubes (cm × cm × cm)	
0.5 × 0.5 × 0.5	$0.0394^{b} \pm 0.0112$
1.0 × 1.0 × 1.0	$0.0812^{a} \pm 0.0112$
2.0 × 2.0 × 2.0	$0.0662^{b} \pm 0.0288$
Quantity of PUF cubes	
5	$0.0651^{b} \pm 0.0282$
7	$0.1726^{a} \pm 0.0437$
9	$0.0649^{b} \pm 0.0245$
11	$0.0524^{b} \pm 0.0094$

Nitrile-hydrolyzing enzymes activity of immobilized cells of *Rhodococcus* UKMP-5M was measured in the system containing the immobilized cells and 5 mm acrylonitrile and run at 30 °C, 80 rpm, pH 6 for 120 minutes. Statistically significant difference ($p<0.05$) was observed among the tested matrices and ranked from the most favoured to the least favoured following alphabetical orders in subscript format (most favoured = ^{a}, least favoured = ^{d}). Statistical analysis was not conducted for types of cells as it only involved two parameters, i.e. immobilized of growing and resting cells.

Types of Support Matrices: The nitrile-hydrolyzing enzymes activity in biotransformation by immobilized cells of *Rhodococcus* UKMP-5M was observed and compared with free resting cells. Similar comparative of matrices study has been conducted by many researchers, such as Nigam *et al.*., (2009), Mirdamadi *et al*., (2008) and Graham *et al.*, (2000). Based on Table 1, it can be clearly seen that the nitrile-hydrolyzing enzymes activity of immobilized cells of *Rhodococcus* UKMP-5M in the PUF is the highest (0.1450 ± 0.0039 U/mg dry cell weight) among other support matrices. The properties of PUF give an advantage in the entrapment of cells as well as the diffusion of substrate. In addition to that, other physical properties of PUF such as elasticity of rubber, toughness and durability of metal as well as resistant to oils, solvent and fats has in turn made PUF applicable in the field of biochemistry and biotechnology, such as in enzyme immobilization and a membrane in analytical biosensors (Romaškevič *et al.*, 2006).

The highest value in nitrile-hydrolyzing enzymes activity from free resting cells of *Rhodococcus* UKMP-5M was expected due to the limitation of mass transfer of substrate to the immobilized cells (Nigam *et al.*, 2009). Park *et al*., (2010) also reported higher activity of amidase was observed in free soluble amidase as compared to amidase immobilized in Co-Cross-Linked Enzyme Aggregate (Co-CLEA).

Lower nitrile-hydrolyzing enzymes activity detected in immobilized cells in agar and calcium alginate as compared to resting cells and immobilized cells in PUF was believed caused by slow diffusion of acrylonitrile across the agar and calcium alginate matrices (Raj *et al.*, 2008). Chen *et al.*, (2008a) studied the effects of varying concentration of sodium alginate (1–5%w/v) and $CaCl_2$ (1.0–6.0%w/v) for the preparation of calcium alginate as support matrices for immobilization in the biosynthesis of p-methoxyphenylacetic acid from p-methoxyphenylacetonitrile. In the study, the best concentration of sodium alginate and $CaCl_2$ was found to be 2.5%w/v and 4.0%w/v, respectively whereas in the present study the concentration were at 4%w/v and 1.6%w/v respectively. Others were 2%w/v for sodium alginate and 2%w/v of $BaCl_2$ (Mirdamadi *et al.*, 2008) and 3% and 1.6%, respectively was reported by Naidu *et al.*, (2011). It can be concluded, the composition of calcium alginate used was not the ideal recipe as the structure of the gel beads of calcium alginate hindered the diffusion of substrate.

Interestingly, in all types of matrices, none of the system showed a measurable cell leakage with the minimum detection limit for cell leakage in this study was 0.1mg/ml. This was due to the lower speed of agitation and time reaction, 80rpm and two hours respectively, applied in the reaction. Cell leakage from calcium alginate matrices was found detected in the reaction of 180rpm for 7 hours (Chen *et al.*, 2008a). In addition to that, Kabainova *et al.*, (2005) reported no cell leakage occurred when the cell immobilized via entrapment method.

Types of Cells of Rhodococcus UKMP-5M: Most of the researchers applied the immobilization of growing cells in the polyurethane foam and immobilization of resting cells in agar and calcium alginate, which due to the conditions of the materials and methods used. Therefore, to proceed with the different types of immobilization matrices, the enzyme activity in two types of cells immobilized in PUF, i.e. resting cells and growing cells, was examined.

The enzyme activity of different types of *Rhodococcus* cells immobilized in PUF with two types of control sets. The first control set was PUF and resting cells of *Rhodococcus* UKMP-5M, and 5mm acrylonitrile as substrate was added after the biotransformation process stopped with the addition of hydrochloric acid (0.5M). The second set was only the PUF cubes and the substrate. Immobilization of growing cells showed 43% higher in enzyme activity as compared to the immobilization of resting cells. In addition, the immediate conversion of acrylonitrile to ammonia by resting cells of *Rhodococcus* UKMP-5M (0.4700 ± 0.1483mm NH_3) was found 17% higher as compared to the chemical hydrolysis of acrylonitrile (0.4000 ± 0.0434 mm NH_3). The finding shows the immobilized of growing cells of *Rhodococcus* UKMP-5M in PUF exhibited greater nitrile-hydrolyzing enzymes activity. Although the addition of PUF in the growth medium caused longer time of incubation, it did support the growth of cell (Romaškevič *et al.*, 2006). In addition, immobilization of growing cells offers an advantage in terms of the enzymes activity due to its superior stability which caused by the self-generating nature of catalytic system of the cells (Fukui and Tanaka, 1984).

Sizes of PUF Cubes: The size of the PUF cubes affects the amount of cell entrapped in the cubes and subsequently the amount of cells involved in the reaction. Theoretically, smaller cubes will yield higher nitrile-hydrolyzing enzymes activity as the high ratio in surface area which in contact with reaction mixture. However, the trend did not occur in the present study. The lower concentration of ammonia detected in the sample set of 0.5cm × 0.5cm × 0.5cm PUF cubes (8.9333 ± 0.4807mg dcw) cubes as compared to the control sets may due to the high quantity of the PUF cubes which caused a small space for the cell to roam and little amount of substrate to diffuse. As for the PUF cube at a size of 2cm × 2cm × 2cm (0.7333 ± 0.1453mg dcw), it can be observed that the placement of the biggest size of PUF cubes showed not all surfaces of the cubes was in contact with the reaction mixture which caused the lower nitrile-hydrolyzing enzymes activity. The PUF cubes at a size of 1cm × 1cm × 1cm (2.7000 ± 0.1523mg dcw) was proven statistically as the optimum support matrices for immobilization of growing cells of *Rhodococcus* UKMP-5M.

Quantity of PUF Cubes: One of the factors that affects the enzymes activity is the amount of cells used. Generally, higher amount of cells entrapped in per unit volume of cells matrices will result in higher activity (Shen *et al.*, 2009a). The experiment was normally conducted by varying the concentration of

resting cells immobilized in matrices. However, since the optimum cells used was growing cells, a modification was made by varying the amount of PUF cubes used in biotransformation. The quantity of PUF cubes added will represent the cell densities in the form of cubes and the effect of the initial cell loading was evaluated.

The finding was a significant difference in the concentration of ammonia from the sample and control sets, when 7 PUF cubes were applied in the system with highest nitrile-hydrolyzing enzymes activity recorded, i.e. 0.1726 ± 0.0437U/mg dcw or near to 163% increment from the utilization of 5 or 9 PUF cubes. The lowest nitrile-hydrolyzing enzymes activity detected in 11 PUF cubes of biotransformation mixture.

This may due to the limited diffusion of substrate across the matrices. Another factor that may contribute to this observation is the competition towards the substrate is higher, with high ratio of cell-to-substrate, which in turn lowered the activity. The lowest nitrile-hydrolyzing enzymes activity was recorded when 7 PUF cubes were employed in the system. This similar finding was reported by Nigam *et al.,* (2009), whom stated the employment of one bead of immobilized cells of *Streptomyces* sp. demonstrated lower conversion of acrylonitrile as compared to two beads. Hence, in order to maintain high nitrile-hydrolyzing enzymes activity throughout the study, a number of PUF cubes of 7 were chosen to be applied in the subsequent experiment.

Substrate Tolerance Study

The immobilized cells were proven to have stability towards higher concentrations of substrates and are protected to other toxicants, which give a special feature as good potential of biocatalyst. Therefore, comparative of substrate tolerance study between the optimized resting and immobilized cells of *Rhodococcus* UKMP-5M was evaluated.

Two concentrations of acrylonitrile i.e. 150 and 200mm were chosen based on the preliminary result of tolerance study of resting cells of *Rhodococcus* UKMP-5M.

Table 2: Enzyme Activity by Different Types of Cells at 150 and 200 mm of Acrylonitrile

Concentration of Acrylonitrile (mm) and Types of Cells of *Rhodococcus* UKMP-5M	**Nitrile-Hydrolyzing Enzymes Activity (U/mg dcw)**
150 mm Immobilized Cells	0.1099 ± 0.0153
150 mm Resting Cells	0.0977 ± 0.0196
200 mm Immobilized Cells	0.1007 ± 0.0196
200 mm Resting Cells	0.0790 ± 0.0266

The nitrile-hydrolyzing enzymes activity of immobilized cells of *Rhodococcus* UKMP-5M in both concentrations of acrylonitrile was almost equal (Table 2). Both of the types of cells demonstrated a decrease in the nitrile-hydrolyzing enzymes activity in 150mm of acrylonitrile to 200mm, with a significant drop (20%) in nitrile-hydrolyzing enzymes activity of resting cells. At 200mm of acrylonitrile, immobilized cells exhibited 28% higher in nitrile-hydrolyzing enzymes activity than resting cells, i.e. 0.1007 ± 0.0196U/mg dcw and 0.0790 ± 0.0266U/mg dcw, respectively. Therefore, it can be deduced that the immobilized cells can tolerate higher concentrations of substrate with comparison of resting cells. Chen *et al*., (2010) reported the removal rate of propionitrile by immobilized cells of *Klebsiella oxytoca* was higher when the concentration of the substance is more than 100mm. Raj *et al.,* (2008) reported the maximum nitrile hydratase activity of immobilized cells of *Rhodococcus rhodochrous* PA-34 in the biotransformation of acrylonitrile was achieved at 1500mM of acrylonitrile.

Reusability

One of the significant features of immobilized cells is the cell can be recycled or reused for several times. Therefore, this work was conducted to evaluate the potential of optimum immobilized cells of *Rhodococcus* UKMP-5M to be recycled. The findings are as presented in Figure 1.

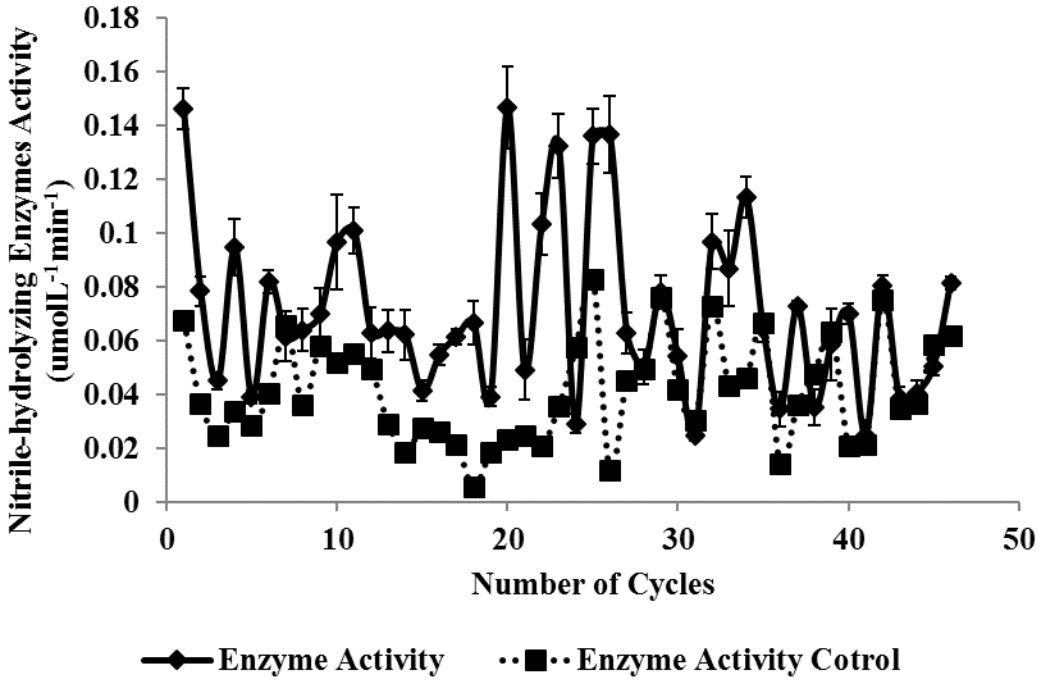

Figure 1: Reusability of the Optimum Immobilized Cells of *Rhodococcus* UKMP-5M. Nitrile-Hydrolyzing Enzymes Activity was Measured in System Containing the Immobilized Cells Exposed with 5 mm Acrylonitrile. The Control Sets Contained the Immobilized Cells and the Substrate was only Added After the Reaction (30 °C, 80 rpm, pH 6, 120 minutes) was Halted

A fluctuate trend of enzymes activity through the whole course of reusability study was also found by Nigam *et al*., (2009). Raj *et al.,* (2008) reported the activity was decreased to 15% at the sixth cycle of agar entrapped cells of *Rhodococcus rhodochrous* PA-34 while Nigam *et al.,* (2009) reported an approximate of 30% of activity of *Streptomyces* sp. was lost after 25 cycles. However, the present study showed better outcome, i.e. the immobilized cells can be recycled up to 46 times with nitrile-hydrolyzing enzymes activity retained roughly at 56% from its initial activity. The nitrile-hydrolyzing enzymes activity was observed almost constant after 35 cycles. Therefore, with a comparison with other findings in reusability study, the immobilized cells in PUF cubes showed better performance.

The control sets in the immobilization study was established using a common negative control. A negative control has similar conditions with the test system with the absence of the enzyme or cells. However, for reusability study, a positive control was prepared, which formulated with immobilized cells of *Rhodococcus* UKMP-5M in phosphate buffer and the substrate (5mM acrylonitrile) was only added after the reaction was terminated.

The purpose of implementing this type control is to observe the ability of the non-exposed immobilized cells to be reused. From the result, it can be clearly seen that the ability of the non-exposed immobilized cells to convert the acrylonitrile to ammonia was poorer than the exposed immobilized cells of *Rhodococcus* UKMP-5M. The basis of this trend is the exposed immobilized cells has been adapted or acclimatized in the similar environment and subsequently gives good response and ability to utilize nitrile as one of the nutrient source.

Conclusion

In this present study, the immobilized cells of *Rhodococcus* UKMP-5M were found to have good performance in terms of tolerance towards higher concentration of substrate as well as reusability although the nitrile-hydrolyzing enzymes activity of immobilized cells was lower than the resting cells. Factor that contributed to the good performance may due to the extracellular structure of *Rhodococcus*

UKMP-5M. The presence of these extracellular structure which attached onto the PUF surfaces may encourage the production of exopolysaccharide which one of the components in the bacterial biofilm (Quek *et al.*, 2005). The role of bacterial biofilm in the detoxification of recalcitrant substances is due to the high microbial biomass and capability to immobilize compounds as well as aided in the gene transfer among bio film organism (Ram Singh *et al.*, 2006).

Another significant outcome of this present study is the development of the new method in preparing the optimum immobilized cells using polyurethane foam. This method is relatively cheap and involves simple preparation.

Furthermore, the properties of polyurethane foams have made the immobilized cells easy to handle and reused as well as high durability of the entrapped cells were observed. With all these advantages, it can be concluded that the optimized immobilization of growing cells of *Rhodococcus* UKMP-5M in polyurethane foam can serve as a potential biocatalyst in bioremediation of nitrile and also as an alternative of other established method to be applied in bioremediation study.

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