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# Disrupting *Rhodococcus* sp: a competent method for genomics and proteomics

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## ABSTRACT

Gram positive actinomycete known as *Rhodococcus*, is known to be highly resistant towards cell lysing. Due to the complex nature of its cell envelope, effective root studies on this bacterium require laborious protocol modifications. Hence, in search of an effective method to disrupt *Rhodococcus*, present study aimed at identifying the most effective method applicable for both genomics and proteomics studies on this novel bacterium. Techniques such as grinding under liquid nitrogen, ultrasonication, glass bead milling, and homogenization were applied in this comparative study. The quality of DNA yielded was analysed spectrophotometrically and the efficacy of intracellular metabolites were evaluated based on enzyme productions. As *Rhodococcus* has been widely utilised for the production of various enzymes, the ability to degrade both toxic and non-toxic components were represented based on activity of phenol hydroxylase and lipase from respective intracellular crude. Grinding under liquid nitrogen showed the best DNA yield with high quality and purity. Other than that, protein lysate of *Rhodococcus* further enhanced the capacity of liquid nitrogen grinding to preserve the extracted enzyme effectively. Grinding in 20 min and 40 min yielded the highest phenol hydroxylase and lipase specific activity, respectively.

**Keywords:** Glass bead milling, homogenizer, liquid nitrogen, sonication, *Rhodococcus*

## INTRODUCTION

Microorganisms are the most common source of industrial enzymes. They produce enzyme inside their cells and may secrete the enzyme for action outside the cell. Actinobacteria areone of well-known bacterial phyla that produce important secondary metabolites. Actinobacteria are Gram-positive bacteria with high guanine and cytosine contents. The genus *Rhodococcus* belongs to the suborder *Corynebacterineae*, which is a distinctive lineage within this phylum. *Rhodococcus* exhibits many valuable properties especially in bioremediation process. This genus draws significant interest to investigate their uniqueness and capability. The ability of *Rhodococci* to utilize and be tolerant to many substrates depends on its physiological basis.

The cell envelops of *Rhodococci*are dominated by the presence of mycolic acid, a large branched chain fatty acids. It also accommodates diverse non-covalently associated components such as channel-forming porin proteins, free lipids, lipoglycans, lipoproteins and capsules or polysaccharides. The presence of these components is the defining features of the genus *Rhodococcus* and they appear as the limitation points to study the intracellular compound. Disruption of cells isthe first critical step in molecular biology, enzymology and biochemical investigations. It is considered as an important step in downstream process in order to isolate intracellular products. Several works have dealt with the comparison of different cell disintegration techniques for isolation of DNA. Protein extraction methods can vary widely in reproducibility and in representation of the total proteome, yet there are limited data that compare the protein isolation methods. The methodological comparison of protein isolation methods is the first critical step in proteomic studies. However, the cell disruption procedures suitable for *Rhodococcus* have been scarcely studied. Therefore, in the present study, four physical methods namely grinding under liquid nitrogen, ultrasonication, glass bead milling, and homogenisation were applied to the *Rhodococcus*sp. cells. The effectiveness of each of the techniques was evaluated through the quantity and quality of released intracellular metabolites namely DNA and proteins that could be used in genomics and proteomics studies.

## MATERIALS AND METHODS

**Microorganism Preparation:** Rhodococcus sp. from the Culture Collection Unit of UNISEL was used as the specimen for this study. An overnight culture of Rhodococcussp. in nutrient broth with an optical density ranging from 0.7 to 0.8 was used as a starter culture. This culture was subsequently cultivated into respective production media for further evaluation.

**Production Medium:** Production medium of 100 mL was inoculated with 10% (v/v) inoculum in 250 mL conical flask. Later, the inoculated cultures were left agitated at 160 rpm in an incubator shaker (Jeio Tech SI-600R, Korea) at for 24 hours at 30°C. Below are respective compositions of the production media:

Medium 1: Nutrient broth.

Medium 2: Culture medium with pH 7.5 composed of (g/L): $KH_2PO_4$, 0.2; $K_2HPO_4$, 0.4; $MgSO_4$, 0.1; NaCl, 1; $FeSO_4H_2O$, 0.01; $MnSO_4$, 0.01; and $Na_2MoO_4.2H_2O$, 0.01 [6]. Phenol (analytical grade) was used as a sole source of carbon and filter sterilised phenol solution was added directly to the medium at a concentration of 500 mg/L.

Medium 3: Culture medium with pH 7 composed of (g/L): $(NH_4)_2SO_4$,,1.0; $K_2HPO_4$,0.9; $KH_2PO_4$,0.6; MgSO4.7H20, 0.02; yeast extract, 0.01; and olive oil, 1.0. This medium was used as a cultivation medium.

**Mechanical Disruptions:** Upon centrifugation at 14,000 rpm for 15min, the supernatant of the respective cultures was removed while the cell pellets were washed twice using 0.05 M phosphate buffer (pH 7). Later, 2 mL of buffer was added into the yielded cell pellet before subjecting for mechanical disruptions.

**Grinding under liquid nitrogen:** A scoop of liquid nitrogen was added into falcon tube containing cell pellet for 5min and was allowed to freeze. Later, the frozen cell was transferred into pre-cooled mortar in an ice box and ground for 10 min continuously using pestle.

**Glass bead milling:** 0.5 g of glass bead from Sigma Aldrich (size: 425–600 µm) was added to the falcon tube with the sample. The sample was vortexed continuously for 10 min at maximum speed modified from Ramanan*et al*, 2008.

**Sonication:** Sonication was done on the cell suspension in an ice bath for 2 min intermittently with 30s cooling for total 10min cell lysing.

**Homogenisation:** The collected sample was placed in a beaker containing glass. The impeller of the homogeniser was placed in the sample and left agitated to lyse the cell for 10min.

**Genomic: Quantitation and Quality Assessment of DNA:**

**DNA extraction:** A DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to extract the DNA from *Rhodococcus* culture O.D 0.7. Four different mechanical disruptions techniques (grinding under liquid nitrogen, glass bead milling, sonication and homogenisation) were applied before proceeding to extraction procedure. The DNA concentrations and quality were tested with a biophotometer (Eppendoft, United Kingdom). In order to compare the efficiency of the extracted DNA, the sample was subjected to 1% agarose gel electrophoresis at 90V for 45 min to view the band densities formed.

**Proteomic: Extraction and enzymes assessment on protein lysates**

**1. Phenol hydroxylase assay:** Crude cell extract from the grown cell in medium 2 was used to assay the phenol hydroxylase activity. The enzyme activity of phenol hydroxylase (EC. 1.14.13.7) was measured. Changes in absorption at 340 nm were monitored on BioMate 3 UV-Vis Spectrophotometer, Thermoscientific, USA. The activity was based on the oxidation of NADH in the presence of phenol. One unit (U) of phenol hydroxylase activity was defined as the amount of enzyme catalysing the oxidation of 1 µmol NADH per min.

**2. Lipase assay:** Cell extracts from medium 3 obtained from various mechanical disruption methods were subjected for lipase assay. Lipase activity was assayed according to the method used by Pinsirodom&Parkin, 2001. The activity was based on the liberation of *p*-nitrophenol from *p*-nitrophenolpalmitate by lipase enzyme. One unit (U) of lipase activity was defined as the amount of 1mMol *p*-nitrophenol liberated per minute by the respective catalyst. Specific enzyme activities were reported as µmol/min/mg protein. All assays were performed in triplicate.

**3. Bradford protein assay:** Crude from both production mediawas subjected to Bradford protein analysis. Absorption was measured at 595 nm with a BioMate 3 UV-Vis Spectrophotometer, Thermoscientific, USA. The total protein concentration was determined by calibration with bovine serum albumin standard according to Bradford, 1976.

**4. SDS-PAGE:** Extracted protein lysates were separated using BioRad electrophoresis device. 12.5% (w/v) polyacrylamide gel was used as resolving gel with4% (w/v) stacking gel. Electrophoresis was performed at 70 V for 30 min and increased to 180 V for 1 h. The gel was stained using Coomasie Brilliant Blue G-250 solution, BioRad, USA.

## RESULTS AND DISCUSSION

**Genomic: Quantitation and Quality Assessment of DNA:** The main idea of this study was to evaluate the hypothesis that different physical methods on *Rhodococcus* would yield different DNA recovery and quality. In search of the most effective physical disruption of *Rhodococcus* cell wall, a comparative analysis was done (Table 1).

**Table.1.Nucleic acid yield obtained by four disruption methods from *Rhodococcus*sp.**

| Methods | DNA yield (µg/ml) | 260/280 | 260/230 |
|---|---|---|---|
| Grinding under liquid nitrogen | 74.3 ± 0.37 | 1.91± 0.18 | 1.87 ± 0.16 |
| Glass bead milling | 74.0 ± 0.1 | 1.8 ± 0.01 | 1.8 ± 0 |
| Sonication | 43.4 ± 2.7 | 1.5 ± 0.10 | 1 ± 0 |
| Homogenization | 0.1 ± 0 | 0 | 0 |

**Values are mean ± standard deviation (n=3)**

As seen above, it was obvious that liquid nitrogen produced the highest DNA recovery with the value of 74.3 ± 0.37 µg/mL. Glass bead milling competitively resulted almost the same recovery with value of 74.0 ± 0.1 µg/mL, followed by sonication and homogenisation. As per theory, pure DNA has $OD_{260/280}$ratio of ~1.8 where lower valuesindicate that the extracted nucleic acid solution contains protein or phenol contaminants. From the data above, only grinding under liquid nitrogen and glass bead milling methods resulted in pure amount of DNA yield, indicating the potential of the methods to yield reliable DNA fragments for further molecular studies. Liquid nitrogen freezedthe cells and minimised DNA degradation when the samples were ground.The extracted genomes DNA were further subjected for agarose gel electrophoresis as shown in Figure 1. Microbial genomic analysis serves as a basic step to understand and detect inner workings of a subjected microbe. Capacity of a microbe can be explored in detail through various molecular techniques such as polymerase chain reaction (PCR), amplification, genome sequencing, restriction enzyme digestion, and transformation.

An effective DNA isolation procedure is determined based on yield, simplicity, speed, reproducibility, cost-effectiveness, speed, and subsequent manipulations. Although various methods for DNA extraction have been reported in the literature, the efficiency of DNA recovery differs among tested specimens as their cell wall composition differs by nature. The unique cell wall composition of *Rhodococcus* species, layers of arabinogalacton, peptidoglycan and mycolic acids or best known as high waxy lipid tends to be great barrier fortowards cell lysing. Figure 1 clearly shows that band in lane 2 contained DNA samples from liquid nitrogen with the highest intensity and observable DNA migration visibility, proving the high concentration of DNA amounted from the extracted nucleic acid solution. Lane 3, 4 and 5 shows DNA yield bands from glass bead milling, sonicator and homogenizer. On the other hand, the other two methods were not as good especially using homogeniser because there were no detectable amounts of DNA and smeared DNA by sonication. Degradation of the isolated DNA after the sonication process was most probably due to the impact of high vibration intensity. In sonication method, the outcome was not satisfied and the noise of the apparatus was intolerable. Finding from this experiment suggested various physical approaches to extracting the DNA, without the use of chemical enzymes. Use of liquid nitrogen was proven possible method as a cost-effective, fast, and high-yield method of extracting DNA from *Rhodococcus* sp. due to lack of access to most of expensive enzymes.

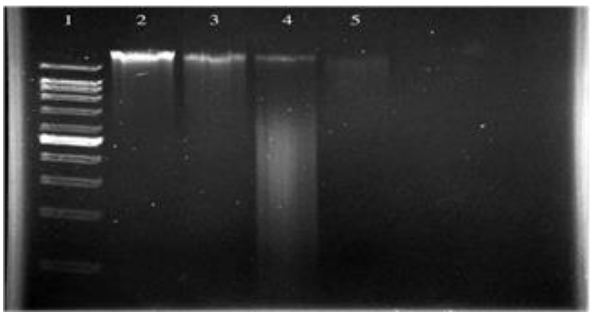


**Figure.1.Gel image of extracted DNA for quantity and quality assessmentLane 1: 1kb ladder marker, Lane 2: liquidnitrogen; Lane 3: glass bead milling; Lane 4: sonicator and Lane 5: homogenizer**

**Table.2.Analysis of total protein content (mg/ml) and specific enzyme activity (u/mg) on tested two media (medium 2 and medium 3)**

| Method | Total protein content (mg/ml) | | Specific enzyme activity (U/mg) | |
|---|---|---|---|---|
| | Medium 2 | Medium 3 | Phenol hydroxylase | Lipase |
| **Grinding under liquid nitrogen** | 1.571±0.065 | 0.854±0.024 | 43.047±1.298 | 0.048±0.001 |
| **Sonication** | 1.416±0.027 | 0.808±0.024 | 55.288±1.548 | 0.058±0.004 |
| **Glass bead milling** | 0.291±0.008 | 0.206±0.010 | 33.199±1.130 | 0.018±0.006 |
| **Homogenization** | 0 | 0 | 0 | 0 |

**Proteomics:** Extraction and Enzymes Assessment on Protein Lysates

Various physical disruption methods to penetrate the thick-layered *Rhodococcus* sp. membrane for intracellular metabolite particular enzyme were analyzed using two production media with different carbon sources. Medium 2 contained phenol as the carbon source, while medium 3 used olive oil indicating different level induced for mycolic acid, the additional strength of cell membrane complex. Table 2 shows the results obtained from the two media. Figure 2(a) and (b) describes the protein separation of the enzyme crude obtained using SDS-PAGE.

**Optimization of Grinding Time Required for Enzyme Production on Rhodococcussp:** It was clear that although the extracted protein content proportionally increased by grinding time, the analyzed enzymes still differed in their specificity. As shown in Figure 4, phenol hydroxylase showed higher specificity at 20min of grinding time while lipase was actively expressed at 40 min of grinding time. Basically, Gram positive bacteria have been recognized to be resistant towards cell lysing due to their multilayered peptidoglycan structure in their cell wall. However, the production of mycolic acid, a long fatty chain (C30-C90) by *Rhodococcus* sp. that eventually incorporates with the peptidoglycan-arabinogalacton complex of the cell wall appears to be an additional barrier from cell lysis. A review has reported that *Rhodococcus* sp. adapts the mycolic acid production based on the carbon source used in the production medium. As shown in Table 2, the protein extraction from medium 3 resulted in lower extraction compared to medium 2 for every tested method. This result indicated that the level of mycolic acid production in medium 3 tended to be induced actively by olive oil compared to medium 2. This observation showing high resistance of *Rhodococcus* sp. from cell lysis due to the toughness of the cell wall comprised of mycolic acid. Four mechanical disruption has been applied to initiate the extraction of enzymes. Bead milling, a quite expensive method also applied but it considered as the inferior technique applied to protein isolation of *Rhodococcus*. Both method, namely grinding under liquid nitrogen and sonication showed a significant overall yield of an intracellular enzyme. Although grinding under liquid nitrogen was a tedious procedure, it was an inexpensive technique compared to sonication.

From the results, higher protein extraction was obtained from grinding under liquid nitrogen, but sonication resulted in mildly higher specific enzyme recovery than liquid nitrogen. These resultsshowed that the liquid nitrogen grinding method was able to overcome the layered barrier of *Rhodoccocus* sp. cell membrane better than ultrasound energy through sonication. However a controversial opinion states that upon insufficient cooling in sonication probe, there would be a significant degradation of tested enzyme. Figure 2(a) and (b) clearly describe significant bands representing phenol hydroxylase and lipase enzyme from the protein extract obtained from liquid nitrogen grinding and eventually fades from sonication, glass bead milling and homogenization. Although grinding under liquid nitrogen yielded the highest protein extraction and relatively significant amount of enzymes in both media, another remarkable question rises, particularly on the optimum grinding time required for the highest enzyme production. Experiment on grinding time classifies the two tested enzymes from the aspect on ease of enzyme recovery based on optimum grinding duration.

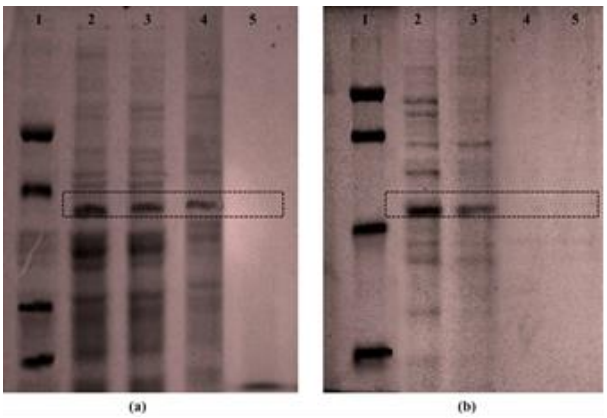


**Figure.2.Protein extraction assessments using various mechanical disruptions (a) Phenol hydroxylase (b)**

**Lipase.** ----- are the intended size of band for the analysed enzyme.

Lane.1.ladder marker**:** 101, 468 kDa; 58,654 kDa, 41; 489 kDa; 27,691kDa. Lane 2: liquid nitrogen; Lane 3: sonication; Lane 4: glass bead milling; Lane 5: homogenization.

**Optimization of Grinding Time Required for Enzyme Production on *Rhodococcussp*:** The specific activity of phenol hydroxylase was higher at 20min of grinding time while lipase was actively expressed at 40 min of grinding time (Fig. 3). This result explained that cell membrane higher barrier developed by *Rhodococcus* sp. cell in medium 3 compared to medium 2 requiring higher impact of intracellular extraction. On the other hand, towards 60 min of grinding time, both enzymes showed gradual falls in their specificity, indicating degradation of the enzyme. As the time increased, the pressure might generate heat energy and shock thus damaging the extracted enzyme as time prolonged. This finding significantly proved that the method of grinding under liquid nitrogen was a more reliable method for both genomic and proteomic analyses in compared to other mechanical disruption methods.

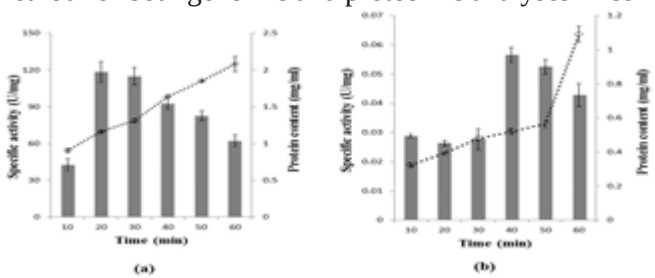


**Figure.3.Analysis of phenol hydroxylase(a) and lipase(b) using different grinding time under liquid nitrogen**

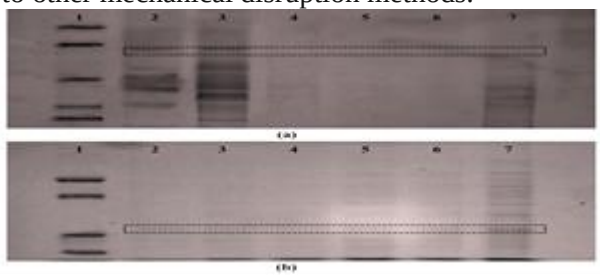

**Figure.4.Protein separation on different grinding time (a) Protein for phenol hydroxylase and (b) Protein forlipase.**

## CONCLUSION

The data presented in this paper demonstrate that four physical disruption methods have been applied on *Rhodococcus*sp cells in order to determine theeffective method to isolate intracellular products. By comparing with ultrasonication, glass bead milling, and homogenization, grinding in liquid nitrogen is the bestapproachas it released good quality and quantity of DNA and proteins that could be used in genomics and proteomics studies.

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