Enhanced Biomass Production of *Rhodococcus* UKMP-5M as Potential Biological Tool for Cyanide Bioremediation

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Abstract: Despite its toxicity, cyanide has been utilized in many industrial processes leading to the generation of voluminous effluents which necessitated treatment prior to discharge into the environment. However, the existing physical and chemical methods for cyanide-containing wastewater removal are incompetent due to reasons such as cost, generation of secondary pollutants and ineffective decontamination of cyanide complexes. Therefore, the development of biological method which can support complete detoxification at low costs is desired for treatment of cyanide-containing wastewater. *Rhodococcus* UKMP-5M exhibited the potential to detoxify cyanide due the presence of nitrile (organic cyanide) catabolism in its genome sequence. Supplementing the medium with glucose and L-proline at 2 % (v/v) inoculum resulted in enhanced biomass production of *Rhodococcus* UKMP-5M by 93 % in comparison to sole nutrient broth medium. In addition, the optimized culture conditions at temperature 30 °C and pH value of 6.6 yielded maximized production of biomass for the detoxification cyanide. These findings are highly significant since they accommodated the tropical temperature of Malaysia and the alkaline nature of cyanide-containing wastewater which should be of great interest from an environmental and economic point of views.

Keywords: Biomass; environmental factors; growth factors; optimization; *Rhodococcus*

1. Introduction

Rhodococcus species have remarkable ability to degrade many pollutants besides producing biosurfactants or emulsifiers with beneficial applications. They are increasingly becoming more valuable in the field of bioremediation and biotechnology. This is due to their indigenous property in contaminated sites and thus making them suitable to be used as inocula for bioremediation (Hernandez et al., 2019). The metabolic diversity of *Rhodococcus* is more versatile than the pseudomonads with great persistent in the environment even though they often demonstrate slow growth. In addition, catabolite repression is absent in *Rhododcoccus* suggesting that pollutants like hydrocarbons, phenols and nitriles (organic cyanide) would be degraded even in the presence of more easily assimilable carbon sources (Kuyukina & Ivshina, 2019). Thus, it is only reasonable to capitalize on these locally isolated *Rhodococcus* strains in cyanide bioremediation. Malaysia, with its rapid growth in industrial sector could greatly benefit from this study since the utilization of biological methods in wastewater treatment is cost effective and most importantly environmentally friendly. Hence, in the present study, the culture conditions for optimal growth of *Rhodococcus* UKMP-5M were identified in order to produce maximum biomass to be used as resting cells for the biodetoxification of cyanide in the future study.

2. Materials and Methods

2.1 Preparation of seed culture

Rhodococcus UKMP-5M was kindly supplied by the Culture Collection Unit, Institute of Bio-IT Selangor, Universiti Selangor (Unisel). The inoculum was prepared in 50 mL medium containing 8 g/L nutrient broth. A loop full of culture of *Rhodococcus* UKMP-5M maintained routinely on agar plate from glycerol stock was transferred aseptically into medium and left to shake at 30 °C for 24 hours at an agitation of 160 rpm. The experiment was terminated when the optical density of the seed culture reached the range between 0.900 and 1.000.

2.2 Optimization of nutritional and physical parameter

The production medium consisting of 8 g/L nutrient broth was prepared in triplicate with 50 mL medium in 250 mL Erlenmeyer flask each. Bacterial suspension amounting to 1 mL (2 % v/v) from 24 hours old seed culture (0.900 to 1.000 at 600 nm) was inoculated into the production vessels. Control experiment was established in the absence of the cultures. Table 1 shows the physical variables that were tested in order to maximize the growth of *Rhodococcus* UKMP-5M for the biodetoxification of cyanide.

Table 1: Factors tested to obtain highest growth rate of *Rhodococcus* UKMP-5M

Affecting Factors	Variables
pH	5, 6, 6.6, 7, 8 and 9
Carbon source	Glucose, fructose, sucrose, glycerol and starch
Nitrogen source	Urea, peptone, L-alanine, L-proline and ammonium sulphate
Temperature (°C)	20, 25, 30, 35 and 40
Percentage of inoculum (% v/v)	1, 2, 3, 4 and 5

2.3 Determination of cell density and dry cell weight

The optical density in triplicate was measured using the spectrophotometer at a wavelength of 600 nm against distilled water as the reference. The determination of dry cell weight followed the protocols established Nallapan Maniyam et al. (2013).

2.4 Statistical Analysis

All experiments were carried out in triplicate and the data were analysed by using SPSS version 17.0. Comparison between groups was performed by using Duncan analysis (values with different letters in superscript are significantly different). A one-way ANOVA test (95% confidence interval) was used to evaluate differences between groups and $p < 0.05$ was considered statistically significant.

3. Results and Discussion

In order to design an effective bioremediation strategy, identification of the pH optimal for growth would be essential. The effect of initial pH on the growth of *Rhodococcus* UKMP-5M was examined at 30 °C using stepwise addition of either 0.1 M HCl or 0.1 M NaOH. The strain proliferated rather well at a relatively wide pH range from 6 to 9 with maximal growth attained at pH 6.6, the pH of the medium without any alteration resulting in 4.3222 g/L dry cell weight. Growth dramatically decreased outside this pH range although the bacterium remained viable with a biomass of 0.2556 and 0.2311 g/L respectively. This may be due to lowered stability of plasma membrane coupled with inhibited enzyme membrane as well as proteins transport (Shukor et al., 2009). It was rather apparent from Table 2 that the alkaline conditions appeared to encourage the cultivation of this isolate since it could tolerate higher pH considerably as compared to that of acidic conditions, a distinct characteristic of an actinomycete (Moradkhani et al., 2018). It was also observed that *Rhodococcus* UKMP-5M exhibited highest growth at near neutral pH which was acknowledged by the fact that the cytoplasm in most bacteria has a pH level of 7.

At optimum temperature, all aspects of cell metabolism function at their finest whereby the cells were able to rapidly increase in size leading to efficient enzymatic systems. It was found that the ideal temperature for the cultivation of *Rhodococcus* UKMP-5M was at 30 °C as supported by previous reports (Moradkhani et al., 2018), a feature of many bacteria isolated from environmental habitats, generating an amount of 6.3222 g/L dry cell weight. Malaysia with temperature ranging from 28 °C to 33 °C could greatly benefit from these findings since most actual cyanide bioremediation sites were operated at ambient temperature which coincided with the optimal growth of *Rhodococcus* UKMP-5M. The strain proliferated reasonably well at 25 and 35 °C which corresponded to a biomass of 2.9889 and 2.5889 g/L, respectively as shown in Table 2. *Rhodococcus* UKMP-5M survived better in the temperature range between 25 °C and 35 °C implying the typical characteristics of a mesophile (Moradkhani et al., 2018). The growth reduced rather substantially at 20 and 40 °C resulting in merely 0.5922 and 0.1033 g/L dry cell weight, respectively. The movement of molecules decelerated as the temperature was lowered suggesting the inability of enzymes to mediate in chemical reactions and eventually all activities were brought to a halt due to the viscosity of the cell interior. Meanwhile, at higher temperature, enzymes started to denature and the total affect was detrimental to cellular growth (Chalidah et al., 2018).

Rhodococcus UKMP-5M grew well in hexoses, glucose and fructose with 7.444 g/L and 6.2778 g/L dry cell weight respectively whereby the bacterium readily used glucose for an efficient growth as shown in Table 2. Similar findings were observed with *Burkholderia cepacia* Strain C-3 in cyanide biodegradation (Adjei & Ohta, 2000). This was mainly due to the fact that glucose was more effortlessly metabolized to promote the most rapid growth and promised the easier way to ferment as compared to the other types of carbon source. Sucrose and starch promoted considerable growth, 4.9444 g/L and 4.6333 g/L dry cell weight respectively, whereas the cultivation of *Rhodococcus* UKMP-5M in glycerol was rather poor with 3.1556 g/L yield of biomass. The maximum biomass production occurred when 0.8 % of glucose was used resulting in a total biomass of 8.0000 g/L.

Meanwhile, the cultivation of *Rhodococcus* UKMP-5M favoured 1.0 % of L-proline and it grew fairly well in L-alanine, producing 8.3222 g/L and 7.8222 g/L dry cell weight of biomass respectively. However, organic nitrogen sources, in this case, urea and peptone, did not support the growth of this bacterium, generating an average biomass of 3.9389 g/L. The utilization of ammonium sulphate was not promising either contributing to 4.0444 g/L dry cell weight. Some bacteria could use nitrate or nitrite salts as source of nitrogen, however,

most require amino acids, peptides, peptones or proteins. In this case, *Rhodococcus* UKMP-5M selectively consumed L-proline, an amino acid, which stimulated the growth of this bacterium significantly ($p < 0.05$).

A 2 % v/v of inoculum supported the highest growth of this particular isolate as portrayed in Figure 4.7 yielding to 8.3222 g/L dry cell weight. Increasing the concentration of seed culture beyond 2 % in the cultivation vessel found to be disadvantageous for the propagation of *Rhodococcus* UKMP-5M. This could be possibly due to the increased viscosity in the medium because of higher inoculum concentration, which probably limited efficient mass transfer.

Table 2: Effect of different culture conditions on the growth of *Rhodococcus* UKMP-5M

Factor	Dry Cell Wight (g/L)	Optical Density at 600 nm
pH		
5	$0.2556^{e} \pm 0.036$	$0.348^{e} \pm 0.02$
6	$2.6000^{d} \pm 0.027$	$1.240^{d} \pm 0.01$
6.6	$4.3222^{a} \pm 0.019$	$1.616^{a} \pm 0.00$
7	$3.9222^{b} \pm 0.301$	$1.466^{b} \pm 0.01$
8	$3.2889^{c} \pm 0.121$	$1.373^{c} \pm 0.01$
9	$2.3889^{d} \pm 0.164$	$1.184^{d} \pm 0.01$
10	$0.2311^{e} \pm 0.088$	$0.251^{e} \pm 0.00$
Temperature (°C)		
20	$0.5922^{d} \pm 0.0136$	$0.480^{d} \pm 0.002$
25	$2.9889^{b} \pm 0.0215$	$1.338^{b} \pm 0.000$
30	$6.3222^{a} \pm 0.0016$	$1.849^{a} \pm 0.001$
35	$2.5889^{c} \pm 0.0011$	$0.992^{c} \pm 0.000$
40	$0.1033^{e} \pm 0.0119$	$0.229^{e} \pm 0.001$
Carbon source		
Glucose	$7.4444^{a} \pm 0.0119$	$1.947^{a} \pm 0.000$
Fructose	$6.2778^{b} \pm 0.0015$	$1.795^{b} \pm 0.000$
Sucrose	$4.9444^{c} \pm 0.0213$	$1.701^{c} \pm 0.000$
Glycerol	$3.1556^{e} \pm 0.0114$	$0.633^{e} \pm 0.000$
Starch	$4.6333^{d} \pm 0.0228$	$1.677^{d} \pm 0.003$
Nitrogen source		
Urea	$2.9667^{e} \pm 0.0016$	$0.588^{e} \pm 0.000$
Peptone	$4.9111^{c} \pm 0.0015$	$0.961^{c} \pm 0.000$
L-alanine	$7.8222^{b} \pm 0.0023$	$2.032^{b} \pm 0.000$
L-proline	$8.3222^{a} \pm 0.0011$	$2.116^{a} \pm 0.000$
Ammonium sulphate	$4.0444^{d} \pm 0.0223$	$0.856^{d} \pm 0.002$
% v/v inoculum		
1	$5.2556^{b} \pm 0.0018$	$1.772^{b} \pm 0.000$
2	$8.3222^{a} \pm 0.0011$	$2.116^{a} \pm 0.000$
3	$5.0556^{b} \pm 0.0014$	$1.697^{b} \pm 0.000$
4	$4.5556^{c} \pm 0.0021$	$1.604^{c} \pm 0.000$
5	$3.8889^{d} \pm 0.0119$	$1.474^{d} \pm 0.000$

4. Conclusion

The discoveries from the present study indicated that the optimum temperature and pH for maximized growth of *Rhodococcus* UKMP-5M was 30 °C and alkaline pH values, respectively which are beneficial for actual use in the biodetoxification of cyanide. This is because cyanide-containing wastewater is alkaline in pH and the optimum temperature is fitting for tropical Malaysian climate with temperature ranging from 28 °C to 33 °C. In addition, the supplementation of glucose and L-proline supported the growth of *Rhodococcus* UKMP-5M substantially resulting in an icrease of biomass by 93 %.

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