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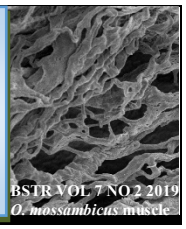

Characterization of an Acrylamide-degrading Bacterium Isolated from Hydrocarbon Sludge

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ABSTRACT

A major source of acrylamide in soil comes from herbicide formulation that contained polyacrylamide that slowly decomposes to acrylamide. Research in acrylamide biodegradation by microbe as a tool for its bioremediation is slowly gaining attention globally. In this research, a hydrocarbon-degrading *Pseudomonas* sp. strain Dr Y Kertih isolated from petroleum sludge was able to grow on acrylamide. The results show that 1% (w/v) glucose supplied with acrylamide (as the only nitrogen source) was the best carbon source for the growth of acrylamide-degrading bacterium. The isolate was also able to use diesel as a carbon source. The bacterium shows an optimal growth at 300 mg/L acrylamide, pH between pH 6.5 and 7.5 and temperature between 25 and 30 °C. The isolate was able to grow on amides such as acetamide and 2-chloroacetamide, but their growth was inhibited by toxic heavy metals such as mercury, cadmium and chromium. Growth kinetic studies using the Haldane model for growth indicated substrate toxicity at higher concentrations on acrylamide. The maximum growth rate (μmax) was 0.267 h^{-1} while the saturation constant or half velocity constant K_s and inhibition constant K_i, were 0.182 and 0.25 g/L, respectively. Thus, the bacterium holds great potential as a candidate to remediate acrylamide.

INTRODUCTION

Acrylamide is among the carcinogenic and neurotoxic compounds [1], it is found in carbohydrate-rich foods that are processed at high temperatures through a pathway called Maillard reaction [2]. Maillard reaction is the main pathway for acrylamide formation, occurs between reducing sugars and amino acids [3]. Conversely, other carbonyl compounds likewise contribute to the formation of acrylamide [4].

In Sweden and Norway, nearby streams became contaminated with acrylamide, causing cows and fish death [5]. The major application of acrylamide is for the production of polyacrylamide (PAM), a polymer used in the adhesive, plastic and printing industry and in the treatment of drinking water [5]. Generally, production of a commercial polyacrylamide is contaminated by its toxic monomer, acrylamide and thus the boundless application of this harmful substance has affected our food chain system. In agriculture, acrylamide contamination of soil comes from the herbicide Roundup formulation that contained 30% polyacrylamide [6]. Therefore, there is a need to overcome this problem focusing on remediating acylamide using the biological method.

Microorganisms that have been reported as capable of utilizing acrylamide include the yeast *Rhodotorula* sp. [7], bacteria *Enterobacter aerogenes* [8], *Pseudomonas* sp. [9] *Burkholderia* sp. [10], *Bacillus cereus* [9], an Antarctic bacterium [9] and the fungi *Aspergillus oryzae* [11]. *Pseudomonas* is a special strain which can degrade acrylamide, reduce molybdate to molybdenum blue [6,9] SDS and degrade diesel [12,13]. Examples of *Pseudomonas* sp. that can degrade acrylamide are *Pseudomonas stutzeri* [14] and *Pseudomonas chlororaphis* [15]. *Pseudomonas* sp. has also been suggested as heavy metal remover [16–18]. Here we describe the isolation and characterization of a new *Pseudomonas* strain that capable of degrading acrylamide. We also describe the growth kinetics of this strain on acrylamide.

MATERIALS AND METHODS

All chemical reagents were abundantly prepared and employed in the analysis in their forms that were not further purified, and the materials used herein were all purchased from Baker (Phillipsburg, U.S.A.) and British Drug House (BDH), UK. Acrylamide stock solution was prepared by dissolution in water to a concentration of 1mg/ml. Working concentrations were prepared via further dilution of stock solution in water thereby forming 100, 50, 20, 10, 5 and 1 µg/ml. all experiments conducted were in triplicates unless otherwise stated.

Growth and maintenance of *Pseudomonas* sp. strain Dr Y Kertih

The bacterium was previously isolated from sludge that had been contaminated with crude oil in Kertih Malaysia [19]. The bacterium was grown in 45 ml of acrylamide enrichment medium in a 100 ml volumetric flask and the culture was incubated at 25 °C on an incubator shaker (Certomat R, USA) at 150 rpm for 48 hours. Basalt salt medium (BSM) was used to isolate the strains with constituents including 0.5 g acrylamide g/L, glucose 10 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 6.8 g/L, $FeSO_4 \cdot H_2O$ 0.005 g/L and 10 mL of H_3BO_3 0.05 g/mL, $ZnCl_2$ 0.03 g/L, $CoCl_2 \cdot 6H_2O$ 0.003 g/mL, $Cu(CH_3COO)_2 \cdot H_2O$ 0.01g 0.002 g of $FeCl_2 \cdot 6H_2O$ [6]. The pH of the media was adjusted to 7.5. Acrylamide was the sole nitrogen source and the sterilization was carried out using polytetrafluoroethylene (PTFE) syringe filter (0.45 µm). One ml of sample was transferred before serially diluted up to 10^7. Aliquots (50 µL) of each dilution were spread on a mineral medium agar plate (supplemented with acrylamide) and incubated for 3 days (at RT) until pure cultures were obtained.

Monitoring Acrylamide Degradation using HPLC

The concentration of acrylamide was determined on an Agilent 1100 series HPLC. Twenty microliters (20 µl) of the test sample was injected into a Rheodyne™ sample injector and the separation cultivated on a segment (Microsorb MV100-5 C18, 4.6 x 250 mm, 5 µm; Alltech Associates, Illinois, USA) and a mobile phase was utilized at a stream pace of 1 ml/min. The sample was eluted with ultrapure water (mobile phase) and detection was carried out with UV detector (196 nm) [20].

Kinetic Studies

Growth rates profiles of several biomasses could be utilized in finding the kinetic factors from batch experiments [21]. A plot of bacterial dry weight (X) against time can be used to obtain specific growth rate coefficient (μ) value at each original diesel concentration. A nonlinear curve could be obtained at the point when these values were plotted against substrate concentrations and modeling can be prepared to determine some kinetic constants example the maximum growth rate (μ_{max}), half-saturation constant (K_s) and inhibition constant (K_i). Kinetic coefficients above correlated to the cell growth were gotten through non-linear regression exploration using the software CurveExpert Professional (Version 1.6).

Statistical Analysis

All the information was evaluated utilizing Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Values are means ± SD. One-way analysis of variance (with post hoc analysis by Tukey's test) or Student's t-test was used to equate among groups (Miller and Miller, 2004). P-value of < 0.05 was considered was significant.

RESULTS

Effects of Initial pH and Temperature on Growth

The effect of initial pH on the growth of this strain was analysed at room temperature using 0.05 M phosphate buffer (pH 5.7 to 8.5). The growth measurement was carried out after 48 h of incubation. Optimum pH was detected at pH 6.5 - 7.5 with no significant difference ($p>0.05$) between the pHs (**Fig. 1**). Growth declined dramatically outside of this range.

The effect of temperature on the growth of acrylamide degrading bacteria shows an optimal growth at 25 and 30°C with no significant difference at $p<0.05$ between the two temperatures (**Fig. 2**). A dramatic drop was seen at a temperature lower than 25°C and higher than 30°C.

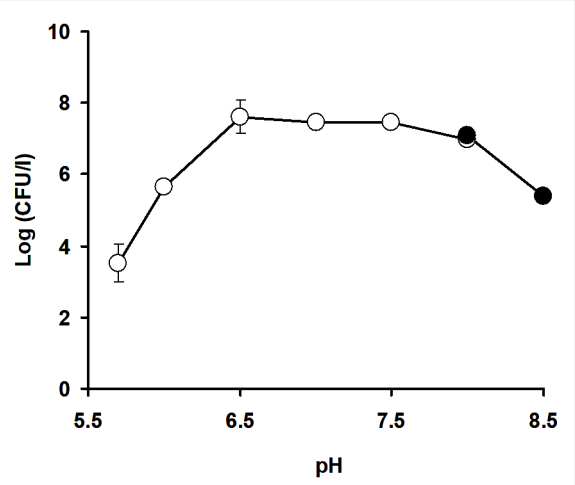

Fig. 1. Effect of pH on acrylamide degradation and growth of *Pseudomonas* sp. strain Dr.Y Kertih. An overlapping buffer system (50 mM) of phosphate (○) and Tris (●) were used. Each data point represents the mean ± standard deviation of three replicates.

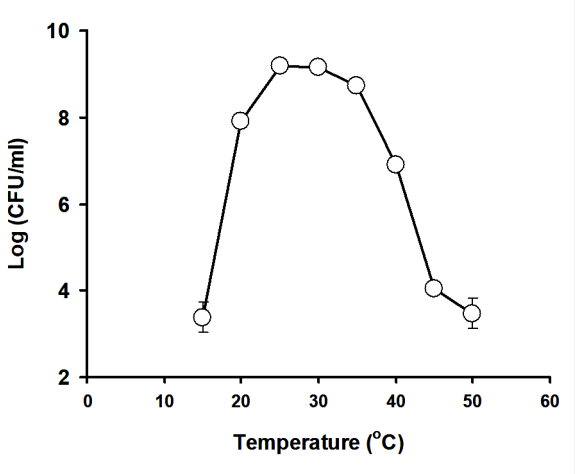

Fig. 2. Effect of temperature on acrylamide degradation and growth of *Pseudomonas* sp. Dr.Y Kertih. Microbial growth (○) was determined using the colony-forming unit (CFU). Each data point represents the mean ± standard deviation of three replicates.

Effects of Carbon Sources on Growth

The effects of an initial concentration of 1.0% (w/v) of various organic carbon sources such as fructose, glucose, lactose, maltose, mannitol, citric acid and diesel on acrylamide degradation were carefully studied. The results revealed that all the carbon sources enhanced the cellular growth compared to control ($p<0.05$) and glucose gave the maximum cellular growth after 72 h of incubation (**Fig. 3**). Growth on diesel as a carbon source and ammonium sulphate as the nitrogen source gave better cellular growth at log CFU/ml of 9.02 compared to 7.35 in this study.

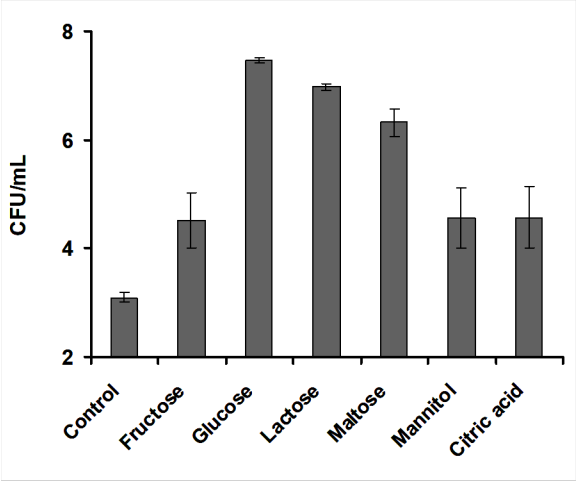

Fig. 3. The effect of carbon sources on degradation of 0.5 g/L acrylamide and bacterial growth of *Pseudomonas* sp. Dr.Y. Kertih No carbon source was used for the control. The represents the mean ± standard deviation and n=3.

Effect of Acrylamide Concentration on Growth

In this current study, acrylamide concentrations of ranging between 200 to 1000 mg/L were used to establish the optimal concentration of acrylamide for the growth of the strain. **Fig. 4** shows that the highest growth was achieved at an acrylamide concentration of 300 mg/L and the growth was absolutely inhibited at 900 mg/L.

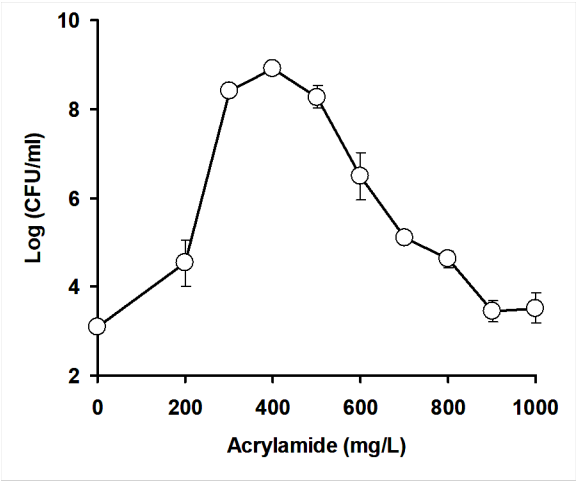

Fig. 4. Effect of different acrylamide concentrations on the growth of *Pseudomonas* sp. Dr. Y Kertih. Microbial growth was determined using the colony-forming unit (CFU). Each data point represents the mean ± standard deviation n=3.

Effect of Different Amides on Growth

Fig 5 shows the effect of different amides on the growth of this strain. This bacterium was able to grow on acrylamide, acetamide and 2-chloroacetamide but not able to grow on methacrylamide, propionamide and nicotinamide. The highest growth was obtained with acetamide followed by acrylamide and 2-chloroacetamide with significant difference in terms of cellular growth ($p<0.05$). For the control experiment, no amide was included in flask.

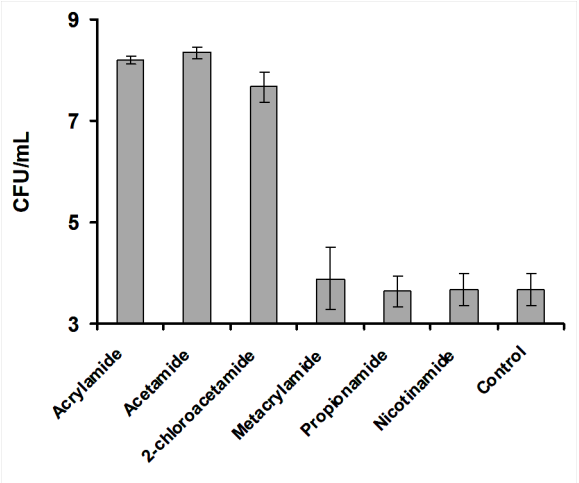

Fig. 5. Effect of different types of amides on acrylamide degradation by *Pseudomonas* sp. Dr.Y Kertih. Microbial growth was determined using the colony-forming unit (CFU). Each data point represents the mean ± standard deviation n=3.

Effect of Heavy Metals on the Growth and Degradation of Acrylamide

Among the major restricting factor for bioremediation is the occurrence of heavy metals at the pollution site, this is due to fact that many microbes could not tolerate high heavy metals concentrations and hence lose their capability to degrade target compounds. 2 ppm of heavy metals (nickel (Ni), copper (Cu), lead (Pb), cadmium (Cd), chromium (Cr), silver (Ag), zinc (Zn), and mercury (Hg)) were tested and it shows strong inhibition towards acrylamide degradation. The strongest inhibition was exhibited by Hg, Cr and Cd while Ag, Cu and Pb exhibited moderate inhibition (**Fig. 6**). No inhibition was detected with Zn since it showed no significant difference with control ($p>0.05$) in term of cell growth.

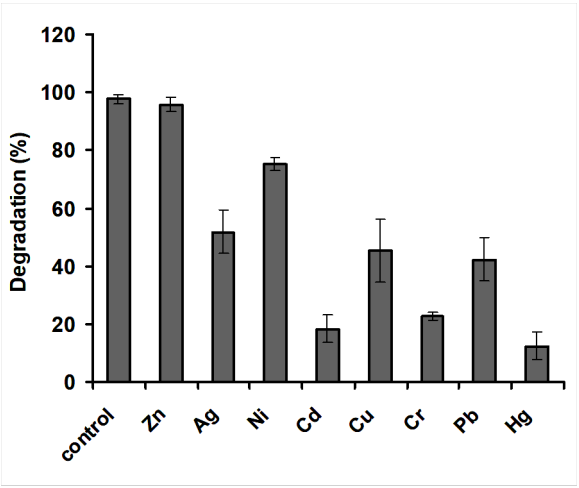

Fig. 6. The effect of heavy metals on acrylamide degradation by *Pseudomonas* sp. Dr.Y Kertih. Each data point represents the mean ± standard deviation of three replicates.

Growth Kinetics

The correlation coefficient value for the substrate inhibition of Haldane and Monod model were 0.96 and 0.71 respectively. Haldane model shows a better fit for this study (**Fig. 7**). The value

of specific growth rate μ increases with the increasing of substrate concentration until it reached a peak value before decreases. The maximum growth rate- μmax was 0.267 h^{-1} while the saturation constant or half velocity constant K_s and inhibition constant K_i, were 0.182 and 0.25 g/L, respectively.

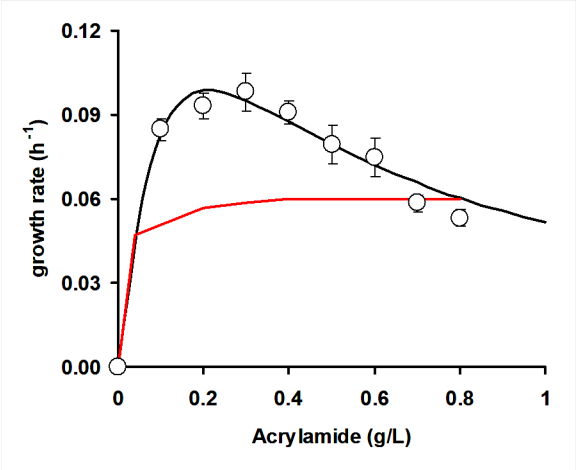

Fig. 7. Growth kinetics of strain Dr.Y Kertih on acrylamide**.** Each data point represents the mean ± standard deviation of three replicates. Haldane (solid black line), Monod (solid red line) and experimental data (○).

DISCUSSION

For the effect of initial pH, the result obtained in this work is in agreement with several previous works. Optimum pH of about 7.0 is reported by several bacteria such as *Pseudomonas* sp. MCI3434 [22], for *Rhodococcus* sp. [23] and the yeast *Rhodotorula* sp. Rahim *et al.* [7] and *Pseudonocardia thermophilic* [24]. Tropical soils usually reveal lower due to active metabolic activity, such as the formation of organic acid and production of carbon dioxide may account for the low pH. Consequently, pH-controlling substances need to be supplemented to achieve close to neutrality in order to optimize remediation [25].

Temperature is one of the important factors affecting the biodegradation. The isolated bacterium shows an optimal growth similar to other acrylamide-degrading microorganisms such as *Pseudomonas chlororaphis, Pseudomonas aeruginosa* and *Pseudomonas stutzeri* at 26, 28 and 30°C, respectively. A temperature of 30°C was reported as the best temperature for the growth of *Rhodococcus rodochrous* and *Rhodococcus* sp. [14,15,26] [27] and [23] whereas for *Helicobacter pylori*, found in the human gut, the optimum temperature is at 37°C [28]. Meanwhile, thermoactive bacteria require higher temperature for their optimum growth. *Pseudonocardia thermophilic* and *Brevibacillus borstelensis* BCS-1 for instance grows optimally at 50°C and 55°C respectively [24,29]. The only one reported acrylamide-degrading bacterium that degrades acrylamide optimally at the temperature of 15°C(cold-loving) is the *Pseudomonas* sp. strain DRYJ7 [30]. This isolate in this study is a right indigenous bacterium that could be engaged in soil bioremediation of acrylamide in tropical countries such as Malaysia with a temperature of around 35°C.

In general, the addition of carbon sources in the minimal salt medium increases bacterial growth on acrylamide. The carbon source most reported as the best is glucose including this strain. An additional benefit of using this bacterium is its ability to utilize diesel as a carbon source indicating multi xenobiotic degradation capability. Currently, this is the first reported acrylamide-degrading bacterium with diesel degradation capability. The results indicated that acrylamide was inhibitory to growth on diesel. *Bacillus clausii*, *Burkholderia* sp. [7], *Rhodococcus rhodochrous* [31]. *Bacillus cereus* [6], and *Pseudomonas* sp. [30] requires glucose at concentrations ranging from 0.5 to 2.0% (w/v). Other carbon sources have been reported. For example, the eukaryote *Aspergillus oryzae* KBN1010 grows optimally on sucrose at 3% (w/v) [11]. Salad oil was used by *pseudomonas aeruginosa* [32] and soluble starch was used by *Pseudonocardia thermophilic* [24] to enhance the degradation of acrylamide.

This study shows that *Pseudomonas* sp. strain Dr.Y Kertih can tolerate up to 600 mg/L acrylamide with optimum growth at 300 mg/L. TheWith nitrate and sucrose as nitrogen and carbon sources respectively a fungus *A. oryzae* could degrade around 100 mg /L acrylamide concentrations and that concentration is regarded as lower concentration, [11]. However, acrylamide degradation in this microbe is probably not a major assimilatory pathway. *Pseudomonas stutzeri and Pseudomonas* sp. strain DRYJ7 were reported to show an optimum acrylamide concentration that supports growth at 440 and 500 mg/L, respectively [9,31,33] reported that *Ralstonia eutropha* TDM-3 and *Ralstonia eutropha* AUM-01 can utilize up to 780-1990 mg/L acrylamide as the sole carbon and nitrogen source.

This study shows that *Pseudomonas* sp. strain Dr.Y Kertih was able to use simple aliphatic amides as reported by Skouloubris *et al.* [28]. Instead of several short-chain amides, it can also degrade 2-chloroacetamide, an amide compound that cannot be utilized by *Pseudomonas* sp. strain DRYJ7 [9] and *Bacillus cereus* strain DRY135 [13]. Both acetamide and 2-chloroacetamide are two carbon atom molecules which are probably a major assimilatory pathway for amide degradation in this microorganism. Even though acrylamide and propionamide are similar three-carbon atom molecules, the number of double bonds in acrylamide is higher than propionamide making it a polyunsaturated (less stable) compound and easy to be attacked [34,35]. Hence, short or less stable amide compound are easily utilized by *Pseudomonas* sp. strain Dr.Y Kertih.

The degradation of acrylamide is strongly influenced by heavy metals as reported by Bååth [36] with more inhibition occurred in the presence of Cd, Cr and Hg. Currently, little data is available from the literature regarding the effect of heavy metals on acrylamide degradation. Due to limited discussion available in microbial tolerance towards heavy metal, this study holds significant importance for bioremediation applications in future. The kinetics of the bacterium growth was analysed centered on the substrate inhibition. Monod model is habitually used when the substrate is not inhibiting the growth while the Haldane model pronounced growth under substrate inhibition environments. The inhibition models are as follows;

$$\mu_{\max}\frac{S}{K_s+S} \qquad [1]$$

$$\mu_{\max}\frac{S}{S+K_s+\frac{S^2}{K_i}} \qquad [2]$$

where, μ, μmax, S, K_s and K_i, are the specific growth rate (h^{-1}), maximum specific growth rate (h^{-1}), substrate concentration (g/L), half-saturation constant (g/L), inhibition constant (g/L), respectively. There is limited data on acrylamide degradation and utilization kinetics in the literature. The results suggest that acrylamide is toxic to the growth of bacteria and the mechanism

of inhibition is probably through a general attack on thiol group of proteins [37].

CONCLUSION

A new acrylamide-degrading *Pseudomonas* sp. strain Dr.Y Kertih previously isolated from petroleum sludge is reported. Glucose was determined as the best carbon source and amides such as 2-chloroacetamide supported growth while the other strain was not able to grow on this type of amide. Toxic heavy metals such as mercury, chromium, and cadmium inhibited growth on acrylamide similar to other research. Growth kinetic studies indicate that acrylamide is toxic and retarded growth. Future works include the use of this bacterium as an autochthonous microorganism in remediating acrylamide-spiked agriculture soils. In overall, this bacterium holds great potential for bioremediation of acrylamide, especially in agriculture soils.

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