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Characterisation of an Acrylamide-Degrading Bacterium and Its Degradation Pathway

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ABSTRACT

Widespread use of acrylamide by the industry led to the environmental pollution that results from the indiscriminate discharge of acrylamide. The presence of acrylamide in the environment is a major threat due to its neurotoxic, carcinogenic and teratogenic properties. In this work bacterial isolate identified as *Burkholderia* sp. strain AQ5-13 is capable of degrading acrylamide as both its carbon and nitrogen sources were screen based on the capability of the bacteria to grow on solid basal salt media that contain 500 mg/L acrylamide as sole carbon and nitrogen sources without supplementation of additional carbon or nitrogen sources. Bacteria grow curve study was carried out by measuring the absorbance value at 600 nm. *Burkholderia* sp. strain AQ5-13 showed a complete life cycle in five days of incubation with no lag phase identified at the earlier growth phase. Optimum conditions for acrylamide degradation was conducted at different initial pH and incubation temperature. Growth optimisation of the bacteria was measured by plate counting in CFU after 48 h of incubation in 250 mL basal salt media that contain 500 mg/L acrylamide. *Burkholderia* sp. strain AQ5-13 showed optimum growth in acidic media at pH 5.7 (5.57 log 10 CFU/mL) and optimum growth at 30°C (6.84 log 10 CFU/mL) compared to other temperature ranges. Quantitative monitoring of acrylamide degradation was performed using HPLC. *Burkholderia* sp. strain AQ5-13 could degrade 14% of 500 mg/L acrylamide as its carbon and nitrogen sources after 72 h of incubation at 5 min of retention time. The appearance of acrylic acid peak as an intermediate was not detected, possibly because all the acrylic acid produced had been consumed immediately by the bacterium. The result from this study showed that bacteria *Burkholderia* sp. strain AQ5-13 has a good potential that can be applied in the bioremediation of waste containing acrylamide.

INTRODUCTION

Acrylamide are widely used in the industry as a synthetic vinyl monomer that can be utilised in numerous products. Due to this, the demand for acrylamide production keeps on increasing year by year. This has led to the indiscriminate discharge of acrylamide causing the contamination of soil and water [1]. In 2019, it is expected that the global polyacrylamide market size is going to reach USD 6.91 billion. The demand for polyacrylamide is mainly prompted by the growing demand of water treatment and petroleum industry [2]. Acrylamide is classified as a carcinogen by the International Agency for Research on Cancer (IARC). It is highly toxic to human, plant and animal [3–7]. It has been reported that the significant portion of polyacrylamide is known to contain acrylamide as contaminant and degradation products.

Because of this, the need to understand acrylamide degradation is vital as it can lower the potential of acrylamide as a pollutant that contaminates in the environment. The removal of acrylamide from polluted areas has been widely applied using different physical, chemical and biological methods. Bioremediation of acrylamide to treat pollutant and contaminant using bacteria have been widely accepted as it is proven to be effective, reliable and cost effective as it allows complete mineralisation of contaminant [8–10].

In recent years, the number of studies on microbial degradation has increased due to its sustainability ways of cleaning up contaminated areas [1,11,12]. Degradation of acrylamide monomer depends on the nature of microbes that are able to utilise acrylamide as its energy sources. This process is regulated by the action of enzyme amidase that degrades acrylamide monomer to acrylic acid and ammonia. Acrylic acid as an intermediate was detected in several acrylamide-degrading bacteria of *Pseudomonas* sp. strain DRYJ7 and *Pseudomonas stutzeri* [11,13]. One of the most challenging aspects of bioremediation is in isolating bacterial strains that are able to use acrylamide as both carbon and nitrogen sources.

MATERIALS AND METHODS

Bacterial strains

Mesophilic bacteria *Burkholderia* sp. strain AQ5-13 was isolated from Kedah Malaysia and deposited in Genbank under the Accession No.KX792234.1. [14]. The sampling site were chosen based on previous history of glyphosate as acrylamide is used in glyphosate formulation as pesticide additive and as soil treatment agent in agriculture [15]

Growth and maintenance of acrylamide-degrading bacterium

Burkholderia sp. strain AQ5-13 was grown and maintained according to the modified medium of Ciskanik et al. The media compose of: 10 g of glucose, 0.5 g of $MgSO_4.7H_2O$, 0.05 g of $FeSO_4.2H_2O$, 500 mg/l acrylamide and 1 ml of the following trace elements; $ZnSO_4 \cdot 7H_2O$, 0.34 µM; $MnCl_2 \cdot 4H_2O$, 0.15 µM; H_3BO_3 4.85 µM; $CoCl_2 \cdot 6H_2O$, 0.84 µM; $CuCl_2 \cdot 2H_2O$, 0.05 µM;$NiCl_2 \cdot 6H_2O$, 0.08 µM and $Na_2MoO_4 \cdot 2H_2O$, 0.123 µM [16] The isolate were maintained in the above described medium at 25° C for 48 h under shaking condition (150 rpm). Maintenance of the isolate were done in 500 mg/l acrylamide basal medium. Subcultures were inoculated into solid media that were prepared as described in the above with the addition of 20 g of bacteriological agar (g/L) and poured into sterile petri dish and incubated at 25° C for 48 h until growth is significant and stored at 4°C for further use.

Determination of acrylamide utilisation rate by bacterial optical density measurements at 600 nm (OD600)

Burkholderia sp. strain AQ5-13 was then grown in 100 mL basal salts media that contains 500 mg/l acrylamide as both carbon and nitrogen source. In this study, quantification was measured every day for 7 days. The bacterial cell was centrifuge at 10,000 rpm for 10 min. The supernatant was discarded and resuspended in distilled water. The absorbance value was then measured at 600 nm using UV spectrophotometer.

Characterisation of acrylamide-degrading bacteria

Preliminary study on the characteristic of acrylamide-degrading bacteria such as pH and temperature were identified to determine the optimum condition for the growth of acrylamide-degrading bacteria. 100 mL of a sterile liquid basal salt medium that contained acrylamide as both the carbon and nitrogen sources were prepared at different initial pHs (pH 5.7, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0) and temperatures (20, 25, 30, 35 and 40 ^oC) in 250 ml conical flasks. About 1 mL of bacterial resting cell ($OD600_{nm}$=1.0) were pipetted into the medium and was incubated at 25° C for 48 h on a 150 rpm rotary shaker. The medium was prepared as the described above.

After 48 h of incubation, the bacterial count in CFU/ml was carried out to identify the optimum condition for the growth of acrylamide-degrading bacteria. The CFU method was conducted on a solid basal salt medium that contained 500 mg/L of sterile acrylamide as both the carbon and nitrogen sources with the initial pH was same as the pH for the growth media. Then, 1 mL of sample was diluted in 9 mL of sterile phosphate buffered saline at pH 7.4 at up to 5 times dilutions. Then, 0.4 mL of the sample from the last three dilutions was pipetted and spread on a plate. The 1× sterile phosphate buffered saline pH 7.4 were prepared according to the [17]. It contained (g/L) 8.0 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4 and 0.24 g KH_2PO_4.

Monitoring of acrylamide degradation using HPLC method

Acrylamide degradation was monitored on an HPLC system (Sykam, Classic Series, Germany) consisted of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. 20 µL of sample was injected into S 5200 auto sampler and separation was performed on a column (C18, 4.6 × 250 mm). The sample was eluted with filtered ultra-pure water (Elix® Advantage pure water, type 2, US) particulates size >0.22 µm at a flow rate of 1 mL/min. Detection was performed by monitoring the absorbance at λ= 196 nm.

Acrylamide degradation was quantified by observing the reduction in the acrylamide concentration during the experimental period. One millimeter of the sample from culture media was removed and filtered using 0.45 µm PTFE syringe filter to remove the cell from the sample [18]. The sample prepared were then stored at 4°C to ensure all the bacteria no longer survive and the degradation stops up to the hour sample were taken. At the same time, 1 mL of the sample was taken for plate count in colony forming unit (CFU/mL). The acrylamide standard curve was prepared by dissolving acrylamide in 100% ultra-pure water. Under this condition, the retention time of acrylamide was determined at 5 min.

RESULTS AND DISCUSSION

Bacterial growth study

The growth kinetic of *Burkholderia* sp. strain AQ5-13 was then moniter to gain in depth understanding of the bacteria isolate using 600nm absorbance value. This isolate was grown in minimal salts media that contains acrylamide as both carbon and nitrogen source.

Based on **Fig. 1** *Burkholderia* sp. strain AQ5-13 showed complete life cycle in 5 days of incubation. At day 0 (0.04) to day 4 (0.266), there is an immediate increase in the cell number of the bacteria is observed. This stage is called the log phase where there is a dramatic increase in the cell number of the bacteria. At this stage, the bacteria grow rapidly by utilizing acrylamide as both their carbon and nitrogen sources for growth. From this result, it is observed that there is no lag phase in the bacteria growth.

This, show that that the bacteria isolate is able to directly use acrylamide as both its carbon and nitrogen sources without adapting to it. The lag phase is the condition where the bacteria need to synthesize necessary enzyme to break down its new food sources [18]. After 5 days of incubation, a decline in bacterial cell started was observed at 0.272 as there are no longer sources of nutrients for their growth. This result showed that *Burkholderia* sp. strain AQ5-13 can use acrylamide as their carbon and nitrogen source.

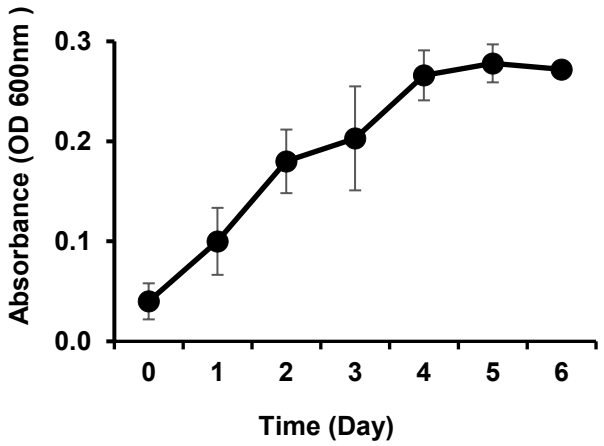

Fig 1. The absorbance reading at wavelength 600 nm of isolate *Burkholderia* sp. strain AQ5-13. The error bars represent the mean ± standard deviation of three replicates.

As previously describe by Wen et al. two bacterial strain identified as *Bacillus cereus* EU439437 and *Bacillus flexu* DQ837543 that can degrade acrylamide as both its carbon and nitrogen sources after 96 h cultivation was reported [19]. More than that, Lakshmikandan et al. reported *Stenotrophomonas acidaminiphila* MSU12 that could degrade 30 mM acrylamide as both nitrogen and carbon sources using the same method. His result also indicates that the bacterial cell reached death phase completely after 48 h of incubation [20].

Effect of initial pH on growth

The study of the effects of initial pH on the growth of *Burkholderia* sp. strain AQ5-13 was carried out at room temperature by altering the initial pH of the media. The purpose of this experiment is to optimise the pH for bacterial growth for the purpose of designing effective bioremediation strategy. The measurement of growth was carried out after 48 h of incubation in colony forming unit (CFU).

Based on **Fig. 2** result, this bacterium is able to grow at relatively wide pH range, from 5.7 to 7.5 with optimal growth at pH 5.7. As the pH increase the growth of the bacteria drop. At pH 8.0 to 9.5 there is no growth of the bacteria. From this, we can conclude that *Burkholderia* sp. strain AQ5-13 grow at acidic pH rather than alkaline pH. Alkaline pH seems to retard growth while growing at pH less than 5.5 was not attempted as a pH of less than 5.0 can cause coagulation of the mineral salts media.

This result is in agreement with the previous study as reported by Bedade and Singhal where the optimum pH for the growth of *Arthrobacter* sp. DBV1 is at 6.0 to 7.0[21]. In addition, Postec et al. reported the optimum pH for the growth of hyperthermophilic archaeon *Thermococcus hydrothermalis* is pH 6.0 in continuous culture [22]. While the previous study mostly reported that the optimum growth was at neutral or nearly neutral pH as reported on *Pseudomonas* sp. DRY J7 which was optimum at pH 7.5 to 8.5 [11], *Stenotrophomonas acidaminiphila* MSU12 at pH 7.2 [20], and *Rhodococcus* sp. at pH 7.0[23]. This maybe because every microorganism has its own optimal pH for its growth and metabolism. Their cell can tolerate a range of pH because their all have their own mechanism to regulate their cytoplasm pH.

In this study the optimum pH for *Burkholderia* sp. strain AQ5-13 was identified at pH 5.7 as *Burkholderia* sp. strain AQ5-13 was isolated from agriculture soil sample that uses glyphosate as the herbicide. As glyphosate was an acidic molecule, this has changed the pH of the soil to slightly acidic thus lead to the growth of only acidic bacteria. Similarly, as reported by [24] where he reported isolation of eight different strain isolated from Yanahaya mine, Okayama, Japan where all isolate were identified as acidophilic.

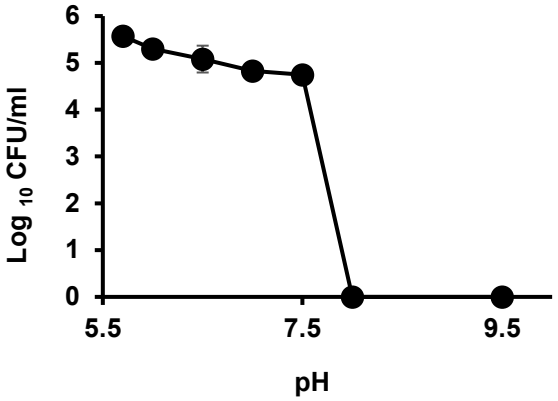

Fig 2. Effects of pH on the growth of *Burkholderia* sp. strain AQ5-13 in acrylamide media.

Effect of incubation temperature on growth

The study on the effects of incubation temperature on the growth of *Burkholderia* sp. strain AQ5-13 was also carried out by setting the initial pH at 5.7 (optimum pH) and the media was incubated at a different temperature. The study of optima temperature is useful as optimum bacteria growth enhance a better bioremediation.

Based on **Fig. 3**, a bell-shaped curve was obtained where the optimum growth of *Burkholderia* sp. strain AQ5-13 occurred at 30°C (6.837 log 10 CFU/mL). At the temperature lower than 30°C there is no significance difference of the growth pattern. Both temperature 20°C and 25°C result in the same growth pattern 5.58 log $_{10}$ CFU/mL. While as temperature increase to 40°C, a dramatic decrease in the growth pattern is observed at 5.5 log $_{10}$ CFU/mL. This result is similar with previous study that reported most acrylamide-degrading bacteria are mesophiles with the range of 30-35°C with the growth decreasing at higher temperature [12,25]. Lakshmikandan et al. and Wampler et al. Also reported to incubate *Stenotrophomonas acidaminiphila* MSU12 at 32°C, and *Rhodopseudomonas palustris* at 30°C [20,26]. In contrast bacterial isolated from cold region show positive growth optimum at lower temperature with the growth decreasing as temperature increase. Syed et al. reported *Pseudomonas* sp. strain DRYJ7 growth was optimum at temperature 15°C [27]

The study of optimum temperature is important elements in bioremediation as temperature plays a very important role in the composition, organization, and function of biological membrane. Syed et al. suggested that the changes in temperature give effect to the outer membrane protein and lipopolysaccharide of *Pseudomonas aeruginosa* [27]. As the temperature increase, a protein known as porin will modulate its channel size thus result in increase in the fluidity of the lipid bilayer and leakiness of the membrane.

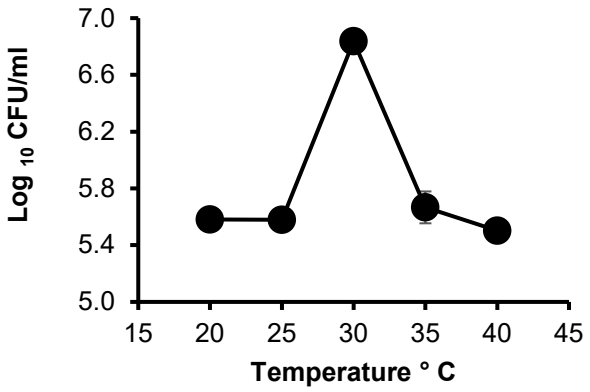

Fig 3. Effects of incubation temperature on the growth of *Burkholderia* sp. strain AQ5-13 in acrylamide media.

Based on this study the optimum temperature for *Burkholderia* sp. strain AQ5-13 was identified at 30°C. This is significance as *Burkholderia* sp. strain AQ5-13 was isolated from mesophilic area in Kedah, Malaysia. At this temperature point, the porin in the bacterial membrane was functioning at its best stabilizing the membrane transport machinery [28]

Acrylamide degradation pathways

The determination of acrylamide degradations pathways was carried out using HPLC by observing the decrease in the acrylamide peak area value in milliabsorption units per second (mAU.s) and the emerging of new peak indicate acrylic acid as intermediates. The retention time of acrylamide was observed at 5 min by running the standard at 196 nm for 20 min.

The result in **Fig. 4** shows that, at 0 h of incubation, the acrylamide peak appears at 5.53 min with the peak area value of 2668.251 (mAU.s). After 72 h of incubation, the peak area value appears at 5.32 min with the peak area value decrease to 2382.615 (mAU.s). The decrease in the peak area value indicates the degradation of acrylamide by the *Burkholderia* sp. strain AQ5-13 after 72 h of incubation.

Shanker et al. reported complete disappearance of acrylamide peak after 5 days of incubation with the emerging of new peak identified as acrylic acid just after one day of incubation [7]. This new peak area value is reported to increase as the incubation time is longer. From this, we can conclude that as acrylamide degradation happen, the accumulation of acrylic acid as intermediates is increasing. Previously, Acrylic acid as an intermediate was detected in some of the acrylamide-degrading bacteria in *Pseudomonas* sp. strain DRYJ7 [29], *Pseudomonas stutzeri* [30]. In addition [20,21,26] all reported the emerging of acrylic acid as intermediated in acrylamide degradation. But in this result, the appearance of acrylic acid peak was not observed even after 72 h of incubation.

Acrylic acid as a metabolite was estimated at 4 min retention time from previous experiment [11]. This finding is significant with Buranasilp and Charoenpanich, 2011 where quick disappearance of acrylic acid after 24 h of incubation is observed and the appearance of other subsequent metabolites indicate the possibility of the biodegradative potential of acrylic acid to be use as bacterial carbon source [29,31].

The result from this study indicates a significant relationship between the incubation time and the decrease in the peak area value of acrylamide is due to the degradation of acrylamide by *Burkholderia* sp. strain AQ5-13. From the result, at 0% degradation the retention times was 5.53 min and after 14% degradation, the retention time was observed at 5.32 min. The change in retention time denotes the formation of intermediates indicating acrylamide degradation [1].

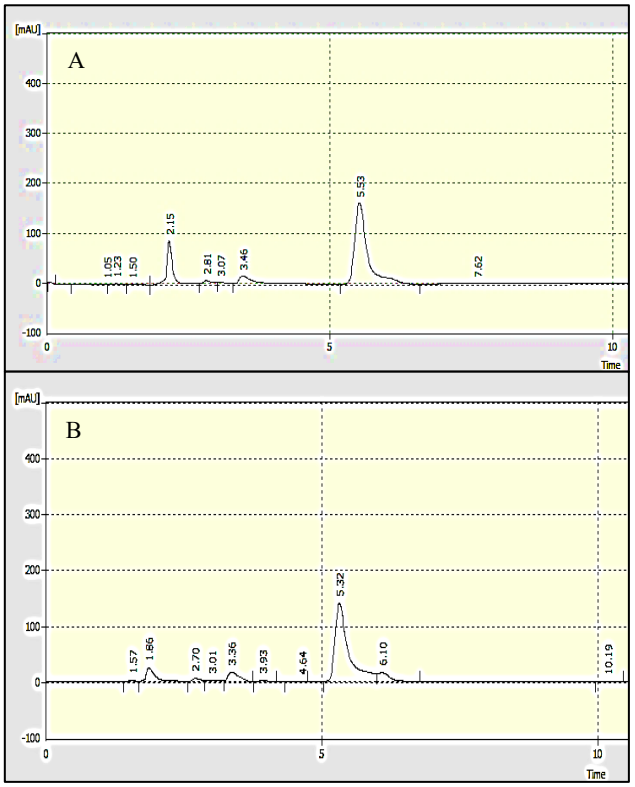

Fig 4. Chromatogram of acrylamide degradation and in liquid culture at 0 h (a) and after 72 h of incubation (b). Acrylamide shows a retention time of 7.10 minutes whilst acrylic acid shows a retention time of 3.57 minutes

CONCLUSION

In conclusion, *Burkholderia* sp. strain AQ5-13 isolated from glyphosate contaminated soil are able to utilize acrylamide as both carbon and nitrogen sources. The optimum initial pH for acrylamide degradation by *Burkholderia* sp. strain AQ5-13 were determined to be acidic at pH 5.7. While the optimum temperature for acrylamide degradation by *Burkholderia* sp. strain AQ5-13 were determined to be at 30°C. At its optimum conditions, *Burkholderia* sp. strain AQ5-13 was identified as able to degrade 14% of 500 mg/L acrylamide as its carbon and nitrogen sources after 72 h of incubation. The appearance of acrylic acid as an intermediate was not detected, possibly because all of the acrylic acid produced had been consumed immediately by the bacterium. For future work, this bacterium can be immobilized to increase the efficiency of the bacteria to degrade acrylamide as sole carbon and nitrogen sources as many studies reported that immobilized cells are more efficient in bioremediation of acrylamide.

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