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Isolation and Growth Characterization of an Acrylamide-degrading *Bacillus* sp. strain UPM2021n Isolated from the Juru River

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ABSTRACT

Polyacrylamide, in which acrylamide is the primary component, degrades back into acrylamide over time. Major amounts of acrylamide can be found in soil. The bioremediation strategy of using microbes to break down acrylamide is gaining popularity in many parts of the world. Several acrylamide-degrading bacteria have been isolated from sediment from the Juru River's bank. The best isolate was a bacterium identified tentatively as *Bacillus* sp. strain UPM2021n based on cultural, colony morphology and biochemical tests. According to early studies, ideal growth parameters included a pH range of 6.5 and 7.0 and a temperature range of 25 to 35 degrees Celsius. Both glucose and sucrose performed at a similar level in supporting the growth of this bacterium on acrylamide as the sole nitrogen source. The highest growth occurs in between 300 and 500 mg/L of acrylamide, resulting in a growth of nearly 7.7 log CFU/mL with a nett growth of about 4 Log CFU/mL as compared to the control. Growth was nearly tolerated at the highest concentrations tested, which was 1500 mg/L and growth completely ceased at higher concentrations Toxic heavy metals tested such as mercury, copper, chromium, and cadmium showed that mercury strongly hampered growth on acrylamide whilst other metal ions such as copper, lead, cadmium, and chromium showed from 30 to 60% inhibition. The relatively high tolerance of acrylamide makes this bacterium suitable for remediation of soil contaminated with acrylamide whilst its sensitivity to heavy metals chiefly mercury means metal-chelating or sequestering compounds must be added to soil contaminated with both acrylamide and heavy metals.

INTRODUCTION

About 12 kilometres southeast of Butterworth is the approximate location of the Juru River. The downstream length of the Juru River, which begins in the Bukit Minyak region and flows westward into the South China Sea, was estimated to be 7.95 kilometres. Two more rivers—the Permatang Rawa River and the Rambai River—make up the Juru River upstream. About 144.9 kilometres from Ipoh, on its route to Alor Setar, the Juru River crosses the North-South Highway. Kuala Sungai Juru's salvation lies in the Juru River since the majority of Bukit Minyak's residents rely on the fishing industry for their livelihoods. In addition, the Bukit Minyak region is well-known for the cockle-breeding sector (industrialisation) [1–3]. Before the advent of modern industry, the Juru River was in pristine condition, with water that was as pure as glass. However, beginning in the 1970s, industrial and housing constructions, as well as human settlements, began to take place along the riverbanks of the Juru River, drastically altering the natural terrain. The Juru River is so polluted with industrial pollution [4] that it is dubbed one of Asia's most polluted rivers along with the Citarum River in Indonesia [5].

Some foods include the potentially harmful chemical compound acrylamide, and it is widely used in industrial processes including those for making paper, pigments, and plastics. Acrylamide has been linked to cancer in humans and is thus classified as a carcinogen. A carcinogenic and neurotoxic substance called acrylamide can be produced through the Maillard reaction during high-temperature cooking. The Maillard

reaction can produce acrylamide, which has been linked to increased levels of cancer in high-carbohydrate diets. The Maillard process takes place when sugars and amino acids are mixed. A high concentration of acrylamide is produced by this process [6]. Additionally, it has the potential to cause damage to the nervous system, making it a neurotoxin. Liver and kidney damage, as well as toxicity to reproduction and development, have all been associated with acrylamide. Inhalation, ingestion, and skin contact are all potential routes of exposure to acrylamide. Inhaling acrylamide can lead to respiratory problems and irritation of the eyes, nose, and throat. Ingestion of acrylamide can lead to sickness, vomiting, and abdominal pain. When acrylamide comes into contact with the skin, it can cause swelling, redness, and itching. The DNA of persons who have been exposed to acrylamide has been proven to mutate, therefore it is also known to cause genetic damage. Toxic acrylamide in nearby waterways killed cows and fish in Sweden and Norway. Polyacrylamide (PAM), which is produced from acrylamide, has several applications in the adhesive, plastic, printing, and water treatment industries. Toxic acrylamide monomer has been found in commercially used polyacrylamides, which has a major impact on the integrity of our food supply in 2005. Roundup herbicide, which includes 30 percent polyacrylamide, is a major contributor to acrylamide pollution. Remediation of acylamide is required to fix this problem [7].

Acrylamide has been designated as a Group 2A carcinogen by the International Agency for Research on Cancer, indicating that it is most likely harmful to people. Acrylamide has also been designated as a Group 2B carcinogen by the World Health Organization, indicating that it may cause cancer in humans. The US Environmental Protection Agency has also classed acrylamide as a reproductive and developmental hazard [8]. Acrylamide is a poisonous chemical that has been linked to several serious health problems in humans, including cancer, birth defects, and damage to the central nervous system. Histological abnormalities in the seminiferous tubules are another result of acrylamide's impact on the reproductive systems of male rats. Acrylamide is a carcinogen that can be absorbed through the lungs or the skin and induce a burning sensation or rash. Signs of a breakdown in the nervous system include profuse perspiration, drowsiness, and shaking in the tongue [9]. Because acrylamide is very water soluble, it may be absorbed through the respiratory tract, digestive system, placental barrier, and skin. As a result, it is critical to limit acrylamide exposure as much as possible. Despite the fact that acrylamide is rapidly metabolized and eliminated after exposure, it poses a risk to both workers and consumers [10–12]. Acrylamide adducts to haemoglobin may be tested to estimate how much acrylamide the average worker is exposed to on the job. The study discovered that 41 workers at an acrylamide facility had increased levels of neurotoxicity using haemoglobin adducts as a biomarker. Workers' haemoglobin adduct levels increased at a Chinese acrylamide facility, showing that they were exposed to exceptionally high doses of acrylamide [13]. Igisu et al. [14] reported on grouting operations at a depth of 2.5 meters caused pollution levels in the well to rise to as high as 400 mg acrylamide per liter. Five people who drank the contaminated water were found to have exhibited symptoms of truncal ataxia and disorientation due to acrylamide toxicity. By far the most often found microorganisms capable of digesting acrylamide are bacteria [15–24]. The status of the Juru River means that the waters contained unique xenobiotics-degraders [25] including potential acrylamide degraders. In this study, we report a potent acrylamide-degrading bacterium from the sediments of this river. The identification and characterization of the acrylamide-degrading bacterium are described here.

MATERIALS AND METHODS

Experiments were conducted in triplicates. All of the materials utilized in this investigation were of analytical grade unless otherwise specified.

Growth and maintenance of acrylamide-degrading bacterium

Sediment soil samples were taken (5 cm deep from topsoil) from sediment from the Juru River's bank, Penang, Malaysia in 2021. One gram of the sediment was sterically transferred into a 100 mL container and sterile tap water was added. The whole container was mixed several times. A 0.1 mL aliquot of the suspension was pipetted and spread onto Minimal Salts Medium agar supplemented with 1% glucose as the carbon source and 0.5 g/L of acrylamide as the sole nitrogen source. From this, several intense and unique colonies were added into 50 mL of acrylamide enrichment medium in a 100 mL volumetric flask and the culture was incubated at 150 rpm for 72 h at 25 °C on an incubator shaker (Certomat R, USA).

Minimal salt medium (MSM) for growth was supplemented with 0.5 g acrylamide g/L as the sole nitrogen source, glucose 10 g/L as the carbon source, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, KH_2PO_4 6.8 g/L (buffering species and source of phosphorous), $FeSO_4 \cdot H_2O$ 0.005 g/L and 0.1 mL of trace elements [7]. Bacteriological agar (Oxoid) was added to the final concentration of 1.5% (w/v). The presence of phosphate in the medium acts as an inorganic buffer system, maintaining a pH range that spans from 5.8 to 7.8 as well as the source of phosphorous. Acrylamide was the only source of nitrogen that was employed for the sterilisation process, and PTFE syringe filters with a pore size of 0.45 microns were used. In order to determine the number of bacteria present, samples of one millilitre each were successively diluted in sterile tap water and plated on nutrient agar.

Morphological, physiological and biochemical characterization of the Mo-reducing bacterium

Characterization of the bacterium was done via biochemical and phenotypical methods. These include the shape of the colony, size of the colony and colour of nutrient agar. Gram staining, bacterial motility, oxidase test (24 h), beta-galactosidase, catalase production (24 h), ornithine decarboxylase and other standard tests were carried out according to the Bergey's Manual of Determinative Bacteriology [26]. The results were interpreted via the ABIS online system [27].

Statistical Analysis

Between-group comparisons were made using either a one-way analysis of variance (with post hoc analysis by Tukey's test) or the Student's t-test. Results are presented as the mean standard deviation (SD) of three independent experiments. Any value below 0.05 was considered to be non-trivial.

RESULTS AND DISCUSSION

Acrylamide is a chemical compound used in many industrial processes, including those used to make paper, pigments, and plastics. It is also present in some foods. Exposure to acrylamide has been linked to an increased risk of cancer in humans, according to research. When food is cooked at high temperatures, a chemical reaction called the Maillard reaction takes place, and the byproduct is acrylamide, which is both carcinogenic and neurotoxic. High carbohydrate eaters have been linked to increased levels of acrylamide, a byproduct of the Maillard reaction. The Maillard reaction is a chemical reaction that occurs when sugars and amino acids are combined. The production of

acrylamide is greatly increased during this procedure [6]. It is also considered a toxin because of the possible harm it could cause to the nervous system. The toxicity of acrylamide has been studied, and it has been linked to reproductive and developmental toxicity as well as damage to the liver and kidneys. Inhalation, ingestion, and skin contact are all potential routes of exposure to acrylamide. If you breathe in acrylamide, it can irritate your lungs and make it hard to breathe. Acrylamide is toxic and should be avoided at all costs. Puffiness, redness, and itching are some of the inflammatory effects of acrylamide on the skin. In addition to being known to cause mutations in the DNA of exposed individuals, acrylamide is also known to cause genetic damage. Toxic acrylamide in drinking water killed cattle and fish in Sweden and Norway. Polyacrylamide (PAM) is an acrylamide byproduct that has many applications. It is used in a wide range of industries, from printing to water purification to adhesives and plastics. As of 2005, it has been discovered that commercially used polyacrylamides contain toxic acrylamide monomers, which has serious implications for the security of our food supply. A large percentage of acrylamide pollution comes from the use of the herbicide Roundup, which is 30% polyacrylamide.

Remediation of acrylamides is required to fix the problem [7]. With its classification as a Group 2A carcinogen by the International Agency for Research on Cancer, acrylamide is considered to be extremely dangerous to human health. Acrylic acid has been labelled as a Group 2B carcinogen by the World Health Organization, meaning it has the potential to cause cancer in humans. The United States Environmental Protection Agency has identified acrylamide as a chemical that may be harmful to reproduction and development [8]. In humans, acrylamide exposure has been linked to an increased risk of cancer, congenital malformations, and central nervous system dysfunction. Histological abnormalities in the seminiferous tubules of male rats have also been linked to acrylamide exposure. Because of its carcinogenic properties, acrylamide can be irritating whether it is inhaled or absorbed through the skin. Signs of a nervous system malfunction include profuse sweating, extreme tiredness, and trembling in the tongue [9]. Acrylamide is easily absorbed through the skin, lungs, bloodstream, and even the placenta because of its high solubility in water. Therefore, minimizing exposure to acrylamide is essential. Although acrylamide is rapidly metabolized and eliminated from the body, it still poses a risk to workers and consumers who are exposed to it [10, 12].

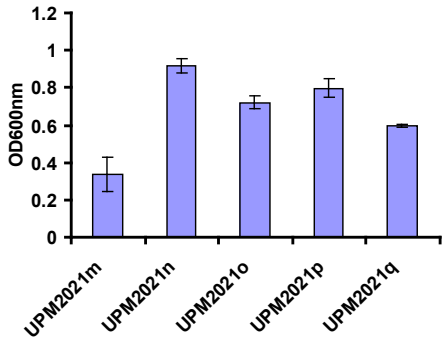

Fig. 1. Screening of acrylamide-degrading bacteria using MSM media supplemented with 1% glucose, 0.5 g/L acrylamide as the sole nitrogen source. Data is mean ± standard deviation of triplicate.

Strain UPM2021n was a Gram-positive, rod-shaped bacterium. The results of morphological and various biochemical tests are presented in (**Table 1**) using the ABIS online software [27]. Gave three suggestions for the bacterial identity with the highest similarity or homology (90%) and accuracy (100%) as *Bacillus subtilis*. However, more work in the future especially a polyphasic approach including molecular identification technique is needed to identify this species further. However, at this juncture, the bacterium is tentatively identified as *Bacillus* sp. strain UPM2021n. Previously, two degraders from this genus have been isolated, identified and characterized [28,29]. As opposed to acrylamide, polyacrylamide degraders are less numerous with only a few reports on the isolation of degraders for this polymer [19,30–32].

Table 1. Biochemical tests for *Bacillus* sp. strain UPM2021n.

		Acid production from	
Gram positive staining	+		
Motility	+	N-Acetyl-D-Glucosamine	d
Hemolysis	+	L-Arabinose	+
Growth at 45 ºC	+	Cellobiose	+
Growth at 65 ºC	–	Fructose	+
Growth at pH 5.7	+	D-Glucose	+
Growth on 7% NaCl media	+	Glycerol	+
Anaerobic growth	–	Glycogen	+
Casein hydrolysis	+	meso-Inositol	+
Esculin hydrolysis	+	Lactose	d
Gelatin hydrolysis	+	Mannitol	+
Starch hydrolysis	+	D-Mannose	+
Tyrosine degradation	d	Maltose	+
Beta-galactosidase (ONPG)	+	Melezitose	–
Catalase	+	Melibiose	d
Oxidase	d	Raffinose	+
Urease	–	Rhamnose	–
Arginine dehydrolase (ADH)	–	Ribose	+
Lysine decarboxylase (LDC)	–	Salicin	+
Ornithine decarboxylase (ODC)	–	Sorbitol	+
Citrate utilization	+	Sucrose	+
Egg-yolk reaction	–	Starch	+
Nitrates reduction	+	Trehalose	+
Voges-Proskauer test (VP)	+	D-Xylose	+

Note: + positive result,– negative result, d indeterminate result

Effects of Initial pH and Temperature on Growth

Bacterial growth was studied in response to an initial pH range of 5.7 to 8. After being incubated for 72 h, the growth was determined. The best pH range, according to ANOVA, is between 6.5 and 7.0, with no significant difference ($p>0.05$) of values within this range (pos Hoc Tukey test). When temperatures were outside this range, cellular development was drastically stunted (**Fig. 2**). Temperature also played an important role in acrylamide development (**Fig. 3**), with the best growth on acrylamide achieved between 25 and 35 °C with no significant difference ($p>0.05$) of values within this range as analyzed by ANOVA (pos Hoc Tukey test).

The range of pH is within the range to prior studies which have shown that bacteria that break down acrylamide generally prefer a pH of around 7.0 [15–24]. Additionally, organic acid and carbon dioxide production in tropical soils often leads to acidic soils, thus pH-regulating chemicals should be applied to reach close to neutrality for ideal remediation [33]. Temperature plays a significant part in acrylamide degradation by bacteria, with an optimal temperature of around 30 ºC reported for many acrylamide-degrading microorganisms [15–24,34–40].

On the contrary, thermoactive bacteria need a higher temperature to achieve optimum growth, such as *Pseudonocardia thermophilic* and *Brevibacillus borstelensis* BCS-1 which require temperatures of 50 °C [41,42].

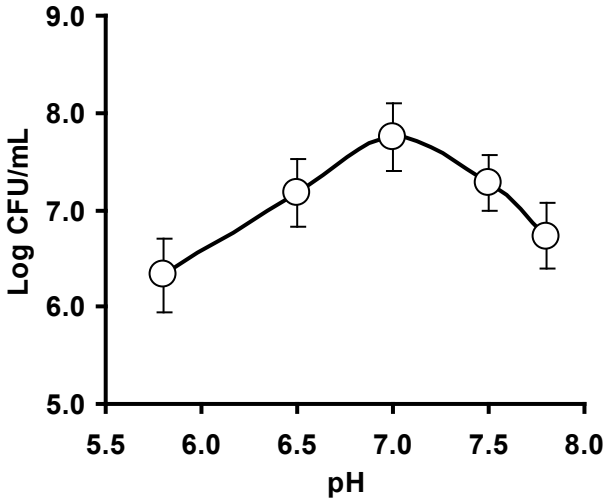

Fig. 2. Growth of *Bacillus* sp. strain UPM2021n at various pH. Each data point represents the mean ± SD.

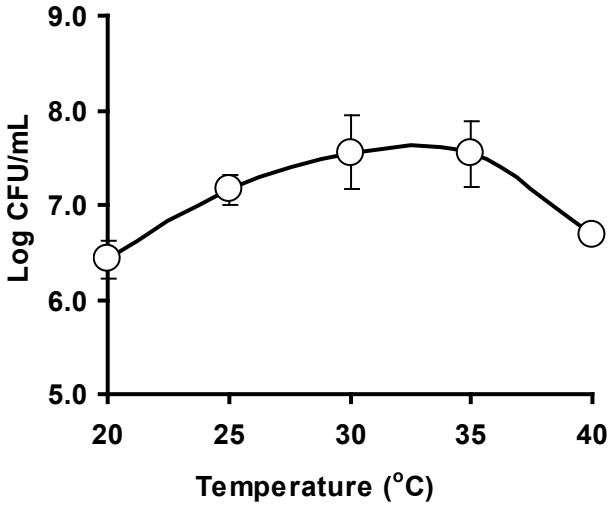

Fig. 3. Growth of *Bacillus* sp. strain UPM2021n at various temperatures. Each data point represents the mean ± SD.

Effects of Carbon Sources on Growth

Bacterial growth on acrylamide was studied in detail in relation to the addition of several organic carbon sources at a 1% (w/v) starting concentration. These included fructose, glucose, lactose, maltose, mannitol and citric acid. After 72 hours of incubation, the greatest growth was shown on either glucose or sucrose; growth on glucose was not substantially superior to sucrose ($p>0.05$), while it did achieve a higher growth rate than the other carbon sources and the control (7.2 log CFU/mL). Using ANOVA, we found that none of the other carbon sources substantially accelerated cell growth beyond the control ($p>0.05$) (**Fig. 4**). Most acrylamide-degraders employ acrylamide as their primary nitrogen source, therefore readily assimilable carbon sources must be supplied when growing bacteria on acrylamide in a low-salt medium [15–24,34–40]. This bacterium shares the consensus that glucose is the best carbon source [15–24]. *Bacillus clausii* and *Burkholderia* sp. [43], *Rhodococcus rhodochrous* [44], *Bacillus cereus* [7] and *Pseudomonas* sp. [45] require glucose at concentrations ranging from 0.5 to 2.0% (w/v) for optimal growth. The high growth reported is due to acrylamide only contributing as a nitrogen source instead as both carbon and nitrogen sources [46]. Other than simple carbon sources, complex carbon sources such as starch was used by *Pseudonocardia thermophilic* [42] whilst salad oil was the sole carbon source by *Pseudomonas aeruginosa* [47]. Acrylic acid, the byproduct of acrylamide breakdown, may be digested by many different types of bacteria via the Kreb's cycle. In aerobic acrylate-using bacteria, for instance, the metabolic pathway involves hydroxylation to beta-hydroxypropionate, followed by oxidation to carbon dioxide [40].

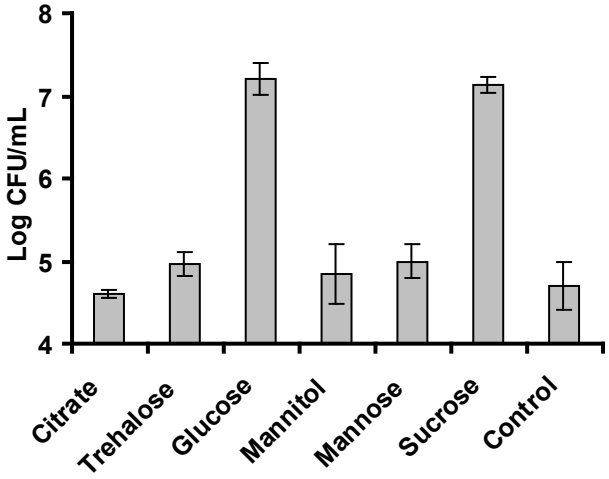

Fig. 4. Growth of *Bacillus* sp. strain UPM2021n at various carbon sources and 0.5 g/L acrylamide. The error bars represents the mean ± SD and n=3.

Effect of Acrylamide Concentration on Growth

Acrylamide concentrations up to 2500 mg/L were studied while using a single nitrogen source. Between 300 and 500 mg/L of acrylamide, the most development takes place, leading to nearly 7.7 log CFU/mL of development and a net development of about 4 Log CFU/mL in comparison to the control. Maximum concentrations of 1500 mg/L were almost tolerable for growth, while higher concentrations completely halted growth (**Fig. 5**). The results of this research show that the bacterium responsible for degrading acrylamide can withstand acrylamide concentrations of up to 1500 mg/L. Tolerance thresholds as low as 100 mg/L were overcome by the fungus *A. oryzae*, which utilized nitrate and sucrose as nitrogen and carbon sources, respectively, to degrade acrylamide.

We consider this sum to be negligible [48]. [49] 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 while *Pseudomonas stutzeri* and *Pseudomonas* sp. strain DRYJ7 require between 440 and 500 mg/L, respectively, for optimal growth [44,50]. The highest tolerant and degrader so far is *Cupriavidus oxalaticus*, which can degrade up to 60 mM or 4260 mg/L acrylamide [20]. *Bacillus* sp. strain UPM2021n grew more slowly as acrylamide concentrations were increased during experimentation with growth conditions.

The lag time was between 1 and 3 days as the acrylamide concentration increased from 100 to 1500 mg/L. In the presence of 1500 mg/L of acrylamide, all development ceased. As

acrylamide concentrations rose, the maximal growth rate also fell, suggesting an upward trend in toxicity (Data not shown). Above 1000 mg/L, acrylamide is toxic to the growth of most microorganisms [15-24,34-40]. Some microbes are able to thrive in these high concentrations because they produce the enzyme amidase [20,36,37,39,51-54]. It was found in this study that the lag period is significantly increased when growth occurs at very high acrylamide concentration. Primary growth models, such as the modified Gompertz or logistics model, or even other available models, can be used to obtain crucial growth parameters like the specific growth rate, the maximum growth rate, and the lag period [22,55]. The obtained specific growth rate is a valuable parameter for use in secondary models like the Monod, Haldane, Teissier (Tessier), Yano, Aiba, etc. [21,22].

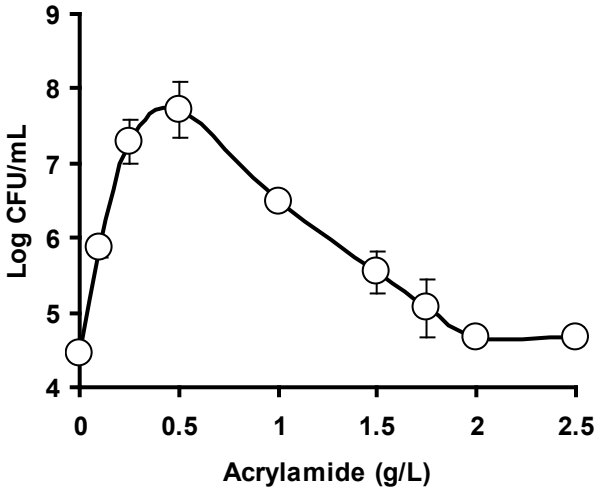

Fig. 5. Growth of *Bacillus* sp. strain UPM2021n at various concentrations of acrylamide. Each data point represents the mean ± SD n=3.

Effect of Heavy Metals on the Growth and Degradation of Acrylamide

An important factor that makes bioremediation more difficult is the presence of heavy metals at the polluted site. This is due to the fact that many bacterial species cannot survive in environments with elevated levels of heavy metals, and thus lose their degradative capabilities in the presence of these contaminants. An analysis determined that the concentration of heavy metals (copper (Cu), lead (Pb), cadmium (Cd), chromium (Cr), and mercury (Hg) at 2 parts per million shows variable inhibition to the growth of the bacterium on acrylamide. Lead was the most tolerant of the metals tested, with an IC50 of 2 ppm, while copper, cadmium, and chromium all showed inhibition of 30–50% at this concentration (**Fig. 6**). The presence of heavy metals has a major impact on the degradation of acrylamide, with mercury being especially inhibitive [16-19,21-24].

The impact of heavy metals on the degradation of acrylamide and other xenobiotics is poorly documented in the published literature. The results of this study will have a major impact on future bioremediation applications due to the dearth of literature on the topic of microbial tolerance to heavy metals. Research using metal inhibition models is crucial due to the prevalence of heavy metals in industrially contaminated rivers, but it is underrepresented in the narrative. A lot of studies is being put into how bacteria can adapt to and thrive in extremely hostile environments. Toxic metals' impact on *Pseudomonas* sp. and *Bacillus* sp. degradation rates of monoaromatic hydrocarbons was successfully determined using the Andrews model [56]. There is a strong correlation between heavy metal binding to the sulfhydryl group present in many enzyme-active sites and the inhibition of enzyme activity [57]. When thinking about the issue of heavy metals inhibiting biodegradation, a few things should be kept in mind. Biodegradation in the presence of a toxic metal can be enhanced by inoculation with bacteria that are resistant to the metal. [58]. In order to maximize the efficiency of acrylamide degradation, it is best to use a combination of a primary bacterial degrader and a metal-resistant bacterium. Soil microcosm experiments show that introducing 2,4-D-degrading bacteria and a cadmium-resistant *Pseudomonas* H1 strain (which accumulates cadmium in the cell) to soil contaminated with both cadmium (60 mg total cadmium/kg) and 2,4-D (500 mg/kg) improves the efficiency with which the xenobiotic is broken down. Calcium carbonate, manganese oxide, cement, phosphate, and magnesium hydroxide are all examples of treatment additives that can reduce metal bioavailability and mobility, making it easier to clean up metal contamination [59]. Instead, you could try incorporating clay minerals as a replacement strategy.

As a result of their ability to bind to and remove metals, clay minerals have been shown to be beneficial in reducing both metal bioavailability and metal-induced toxicity. For instance, adding kaolinite (1-20%) or montmorillonite (1-5%) to cadmium-containing agar medium used by yeasts, bacteria, and an actinomycete decreased the toxicity of the cadmium [60]. The toxicity of 150 mg total cadmium/L to *Streptomyces bottropensis* was also found to be mitigated by a combination of 3 percent bentonite and vermiculite. However, kaolinite required a higher concentration (6 percent vs. 3 percent) and provided less overall protection than the other clays [61], despite the possibility that it could lessen cadmium toxicity.

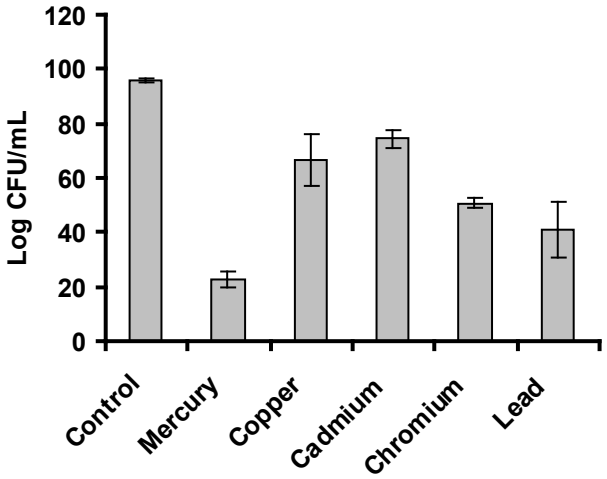

Fig. 6. The effect of heavy metals on acrylamide degradation by *Bacillus* sp. strain UPM2021n. Each data point represents the mean ± SD.

CONCLUSION

From the sediment of one of Malaysia's most polluted rivers, we were able to isolate several acrylamide-degrading bacteria. Based on results from cultural, colony morphology, and biochemical analyses, the best isolate was tentatively labelled as *Bacillus* sp. strain UPM2021n. pH values between 6.5 and 7.0 and temperatures between 25 and 35 degrees Celsius were found to be optimal for growth. The growth of this bacterium on acrylamide alone was supported similarly by both glucose and sucrose. Between 300 and 500 mg/L of acrylamide, the most

development takes place, leading to nearly 7.7 log CFU/mL of development and a net development of about 4 Log CFU/mL in comparison to the control. Maximum concentrations of 1500 mg/L were almost tolerable for growth, while higher concentrations completely halted growth. When tested against other toxic heavy metals like mercury, copper, chromium, and cadmium, mercury was found to significantly inhibit acrylamide growth, while other metal ions like copper, lead, cadmium, and chromium showed 30%-60% inhibition. This bacterium's high tolerance for acrylamide makes it useful for cleaning up acrylamide-contaminated soil. However, the bacterium is sensitive to heavy metals. Soil contaminated with acrylamide and heavy metals, most notably mercury, necessitates the addition of metal-chelating or sequestering compounds.

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