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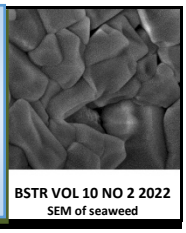

Isolation and Growth Characterization of an Acrylamide-degrading *E. cloacae* strain UPM2021a Isolated from a Paddy Field

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ABSTRACT

Acrylamide is a suspected carcinogen and a global pollutant. The presence of acrylamide in the soil is a major source of this chemical. Microbe-mediated acrylamide breakdown as a bioremediation technique is gaining popularity across the world. Several bacteria capable of digesting acrylamide have been identified in paddy field soils. The best isolate was a bacterium identified tentatively as *E. cloacae* strain UPM2021a based on cultural, colony morphology and biochemical tests. According to early studies, ideal growth parameters included a pH range of 6.5 and 7.5 and a temperature range of 25 to 35 degrees Celsius. Acrylamide dosages of up to 2500 mg/L were explored as a single nitrogen supply. The greatest growth occurs between 300 and 1000 mg/L of acrylamide, resulting in an approximate nett growth of 3 log CFU/mL when compared to the control. Growth was practically tolerated at 1700 mg/L, and growth stopped entirely at concentrations above 2000 mg/L. Toxic heavy metals such as mercury, copper, chromium, and cadmium hampered acrylamide development with mercury being the strongest inhibitor whilst other metal ions such as copper, cadmium, and chromium show from 30 to 50% inhibition whilst lead was the least inhibiting. The relatively high tolerant 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

Acrylamide is a potentially hazardous chemical component that is found in some foods. It is also frequently employed in industrial processes, such as the production of paper, pigments, and plastics, among other things. Acrylamide is a recognized carcinogen, which simply means that it has the potential to cause cancer in human beings. The Maillard reaction, which takes place when food is cooked at high temperatures, can result in the production of acrylamide, a chemical that is both cancer-causing and neurotoxic. The Maillard reaction is what can lead to the creation of acrylamide, and consuming a lot of carbs can be a contributing factor. When sugars and amino acids are combined, a chemical reaction known as the Maillard reaction takes place. This technique results in the production of extremely high concentrations of acrylamide [1]. In addition to this, there is the possibility that it will harm the neurological system, which is what makes it neurotoxic. Acrylamide has been linked to several negative health effects, including toxicity to reproduction and development, as well as harm to the kidneys and liver. Acrylamide can be absorbed into the body through the lungs, the digestive tract, or the skin.

These are the three primary routes of exposure. Inhaling acrylamide can irritate the eyes, nose, and throat in addition to difficulties with the respiratory system. Ingestion of acrylamide can result in symptoms like nausea, vomiting, and stomach discomfort. Acrylamide can cause irritation, redness, and itching when it comes into contact with the skin. Acrylamide is known to cause genetic damage since there is evidence that it may induce mutations in the DNA of people who are exposed to it. As a result, acrylamide is known to cause genetic damage. Cows and

fish in Sweden and Norway both perished after coming into contact with acrylamide that was present in surrounding streams. Acrylamide is the chemical of choice when it comes to producing polyacrylamide (PAM), a substance that finds use in the adhesive, plastic, printing, and water treatment sectors, among others. 2005 was a year in which the widespread use of commercial polyacrylamides, which are commonly contaminated by acrylamide's deadly monomer, had a major and negative effect on the security of our nation's food supply. The use of the herbicide Roundup, which contains thirty percent polyacrylamide, contributes significantly to the problem of pollution. Acylamide will need to go through a remediation process in order for this problem to be fixed [2].

Acrylamide has been given the designation of a Group 2A carcinogen by the International Agency for Research on Cancer, which indicates that it is most likely harmful to people. Acrylamide has also been labeled as a Group 2B carcinogen, which indicates that the World Health Organization considers it to be a substance that has the capability of causing cancer in people. The Environmental Protection Agency in the United States has identified acrylamide as a substance that poses a risk to both reproduction and development [3]. Acrylamide is a toxic substance that poses a risk to human health and has been linked to an increased risk of developing cancer, as well as problems with reproduction, development, and the neurological system. The effects of acrylamide on the reproductive systems of male rats are shown to result in histological abnormalities in the seminiferous tubules of the rats. It is possible for acrylamide to cause a burning feeling or a rash if it is breathed in or absorbed through the skin.

A neurological breakdown can be identified by several symptoms, including excessive sweating, fatigue, and shaking in the tongue [4]. Due to the fact that acrylamide is highly soluble in water, it has the potential to be absorbed through the skin, the respiratory tract, the digestive system, and even the placental barrier. As a consequence of this, it is extremely important to restrict one's exposure to acrylamide to the greatest extent feasible. Even while acrylamide is quickly digested and removed after exposure, it nevertheless poses a danger to those who deal with it [5–7]. It is possible to determine how much acrylamide an ordinary worker is exposed to on the job by testing the acrylamide adducts that haemoglobin forms with acrylamide. According to the results of the study, which used haemoglobin adducts as a biomarker, 41 employees working at an acrylamide plant exhibited elevated levels of neurotoxicity. At a Chinese acrylamide plant, worker haemoglobin adduct levels rose, indicating that they were subjected to abnormally high dosages of the chemical [8]. Igisu et al. [9] A grouting operation that took place at a depth of 2.5 meters was the source of the pollution that led to an acrylamide concentration in the well that reached up to 400 mg acrylamide/L. The results of the investigation showed that five of the volunteers who drank the contaminated water had symptoms of truncal ataxia and disorientation as a result of acrylamide poisoning. Bacteria are the most prevalent type of microbe that has been found to be capable of breaking down acrylamide [10–19]. The identification and characterization of another acrylamide-degrading bacterium is described here.

MATERIALS AND METHODS

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

Growth and maintenance of acrylamide-degrading bacterium

In the year 2021, soil samples were collected from a paddy field located in Kepala Batas, Penang, Malaysia. The samples were obtained at a depth of five centimetres below the topsoil. One gram of the soil sample was dispersed across the sterile water from the tap. A 0.1 mL aliquot of the soil solution was pipetted and put onto agar that had been supplemented with 1 percent glucose as the carbon source and 0.5 g/L of acrylamide as the only nitrogen source. The medium was Minimal Salts Medium. Following this, several distinct and potent colonies were transferred into 50 millilitres of acrylamide enrichment medium contained within a 100-milliliter volumetric flask. The flask was then placed on an incubator shaker and subjected to a temperature of 25 degrees Celsius for a period of 72 hours (Certomat R, USA). The minimal salt medium (MSM) that was used for development was supplemented with 0.5 g acrylamide g/L as the only 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 [2]. The presence of phosphate in the medium functions as a buffer system, keeping the pH within the range of 5.8 to 7.8 all the time. During the sterilization procedure, the only source of nitrogen that was used was acrylamide, and the PTFE syringe filters that were utilized had a pore size of 0.45 microns. Samples of one millilitre each were sequentially diluted in sterile tap water and then plated on nutrient agar in order to assess the number of bacteria that were present.

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

Methods from the biochemical and phenotypic realms were utilized in the process of characterizing the bacterium. On nutritional agar, these features include the morphology of the colony, its size, and its color. Gram staining, bacterial motility, an oxidase test that lasted for 24 hours, beta-galactosidase, catalase production that lasted for 24 hours, ornithine decarboxylase, and other regular tests were carried out [20]. This was done in accordance with Bergey's Manual of Determinative Bacteriology. The ABIS online system was used to do the interpretation of the results [21].

Statistical Analysis

Comparisons between the groups were made using either a one-way analysis of variance (followed by post hoc analysis using Tukey's test) or a Student's t-test. The values represent the means plus the standard deviation (SD) of the experiment performed three times. A p-value of less than 0.05 was taken to indicate statistical significance.

RESULTS AND DISCUSSION

On MSM agar, several colonies were shown to have developed, and acrylamide and glucose were the only sources of nitrogen and carbon, respectively. During the portion of the experiment in which acrylamide served as the only source of carbon and nitrogen, no bacterial colonies were generated.

Many of the isolated degraders are only able to use acrylamide as their sole source of nitrogen, whereas the bacteria that are capable of using acrylamide as their only supply of carbon are rather uncommon. In order to move forward with the study, the best isolate from the preliminary test was selected using OD600nm as the criterion. This allowed the study to move forward.

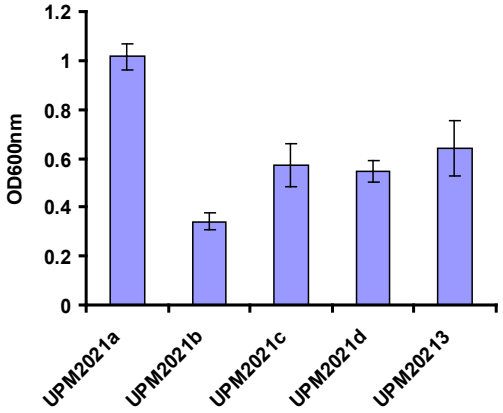

Fig. 1. Screening of acrylamide-degrading bacteria using MSM media supplemented with 1% glucose, 0.5 g/L acrylamide as the sole nitrogen source. Data is mean ± standard deviation of triplicate.

Bacterium of the strain UPM2021a has the characteristics of being Gram-negative, motile, short-rod shaped, and a facultative anaerobe. Using the ABIS online software, we were able to make a partial identification of the bacterium based on its culture, morphology, and a number of different biochemical assays (**Table 1**) [21]. Gave three suggestions for the bacterial identity with the highest similarity or homology (90%) and accuracy (100%) as *Enterobacter cloacae*. 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 *E. cloacae* strain UPM2021a. Previously, two degraders from this species have been isolated [11,22]. As opposed to acrylamide, polyacrylamide degraders are less numerous with only a few reports on the isolation of degraders for this polymer [14,23–25].

Table 1. Biochemical tests for *Enterobacter cloacae* strain UPM2021a.

Motility	+	Acid production from:	
Pigment	–	Alpha-Methyl-D-Glucoside	+
Catalase production (24 h)	+	D-Adonitol	+
Oxidase (24 h)	–	L-Arabinose	+
ONPG (beta-galactosidase)	+	Cellobiose	+
Arginine dihydrolase (ADH)	+	Dulcitol	+
Lysine decarboxylase (LDC)	–	Glycerol	+
Ornithine decarboxylase (ODC)	+	D-Glucose	+
Nitrates reduction	+	myo-Inositol	+
Methyl red	–	Lactose	+
Voges-Proskauer (VP)	+	Maltose	+
Indole production	–	D-Mannitol	+
Hydrogen sulfide (H2S)	–	D-Mannose	+
Acetate utilization	+	Melibiose	+
Malonate utilization	+	Mucate	+
Citrate utilization (Simmons)	+	Raffinose	+
Tartrate (Jordans)	+	L-Rhamnose	+
Esculin hydrolysis	+	Salicin	+
Gelatin hydrolysis	–	D-Sorbitol	+
Urea hydrolysis	+	Sucrose	+
Deoxyribonuclease	–	Trehalose	+
Lipase (corn oil)	–	D-Xylose	+
Phenylalanine deaminase	–		
Growth on KCN medium	+		

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

Growth on acrylamide at various pH and temperature

The influence of the initial pH on the growth of bacteria was studied between 5.7 and 8 on the pH scale. The calculation of a growth rate came after a time of incubation that lasted for a total of forty-eight hours. According to the results of the analysis of variance (ANOVA), the optimal pH range was discovered to be between 6.5 and 7.5, and there was not a significant difference ($p>0.05$) in the values that were found within this range. The ANOVA study also found that the values that were found did not differ significantly from one another (pos Hoc Tukey test). The pace of cellular growth significantly slowed down when it was exposed to conditions that deviated from these norms (**Fig. 2**). Temperature also played a crucial part in the development of acrylamide (**Fig. 3**).

The optimum growth on acrylamide was attained between 25 and 35 °C, and there was no significant difference ($p>0.05$) in the data within this range when examined using ANOVA (pos Hoc Tukey test). Previous research [10–19] has demonstrated that bacteria that degrade acrylamide normally favor an environment with a pH of around 7.0. The pH range that was found to be acceptable falls within this range. In addition, the synthesis of organic acid and carbon dioxide in tropical soils frequently results in acidic soils; hence, pH-regulating chemicals should be added in order to bring the soils as close as possible to neutrality in order to achieve optimal remediation [26].

Temperature has a key role in the process of acrylamide degradation by bacteria. An ideal temperature of around 30 degrees Celsius has been observed for a wide variety of microorganisms that degrade acrylamide [10–19,22,27–32]. 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 [33,34].

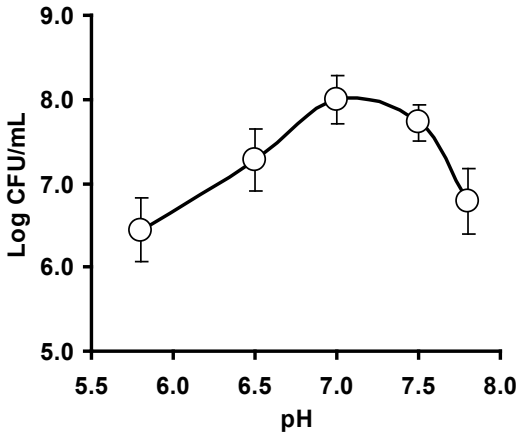

Fig. 2. Growth of *Enterobacter cloacae* strain UPM2021a at various pH. Each data point represents the mean ± SD.

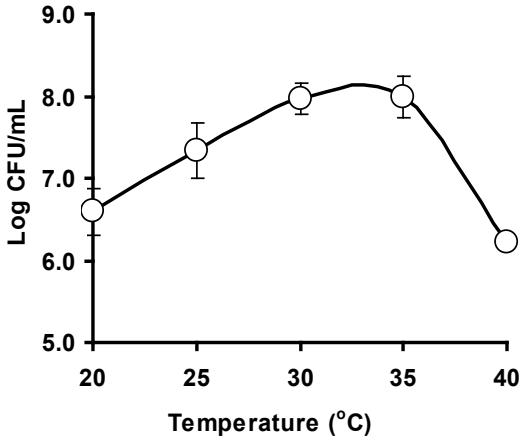

Fig. 3. Growth of *Enterobacter cloacae* strain UPM2021a at various temperatures. Each data point represents the mean ± SD.

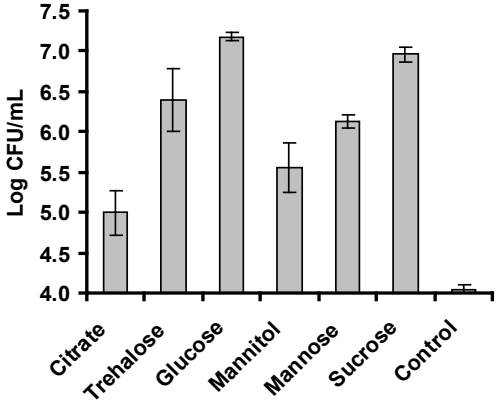

Fig. 4. Growth of *Enterobacter cloacae* strain UPM2021a at various carbon sources and 0.5 g/L acrylamide. The error bars represents the mean ± SD and n=3.

Growth on acrylamide at various carbon Sources

The effects of an initial concentration of 1.0 percent (w/v) of a number of organic carbon sources, including fructose, glucose, lactose, maltose, mannitol, citric acid, and diesel on the growth of bacteria on acrylamide were comprehensively examined. The growth on glucose was somewhat better than the growth on sucrose, with the former obtaining a growth of 7.18 log CFU/mL, surpassing the other carbon sources and the control. After 72 hours of incubation, glucose and sucrose exhibited similar greatest growth. The results demonstrated that the growth of the cells was stimulated by any source of carbon as compared to the control group (**Fig. 4**). Because the majority of acrylamide-degrading bacteria use acrylamide as their only source of nitrogen, easily assimilable carbon sources must be supplemented for bacterial growth on acrylamide when the medium is low in salt [10–19,22,27–32].

Carbon sources are very important for bacterial growth on acrylamide when the medium is low in salt. This bacterium subscribes to the widely held belief that glucose is the superior source of carbon. [10–19]. *Bacillus clausii* and *Burkholderia* sp. [35], *Rhodococcus rhodochrous* [36], *Bacillus cereus* [2] and *Pseudomonas* sp. [37] 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 of as both carbon and nitrogen sources [38]. Other than simple carbon sources, complex carbon sources such as starch were used by *Pseudonocardia thermophilic* [34] whereas salad oil was the only source of carbon that *Pseudomonas aeruginosa* [39] had access to. In most cases, the decomposition of acrylamide results in the formation of acrylic acid, which may be metabolized by a wide variety of bacteria via the Kreb's cycle. For instance, it was shown that the metabolism of acrylate in aerobic bacteria that use acrylate proceeds via hydroxylation to -hydroxypropionate, which is subsequently oxidized to carbon dioxide [32]. This was confirmed to be the case in these bacteria.

Growth on acrylamide at various acrylamide concentrations

Acrylamide dosages as high as 2500 mg/L were tested when supplied by a single source of nitrogen. The maximum growth is observed between concentrations of 300 and 1000 mg/L of acrylamide. This results in a growth of around 7.0 log CFU/mL and a net growth of approximately 3 Log CFU/mL in comparison to the control. At the maximum dosages that were tested, which was 1700 mg/L, growth was practically tolerated, but growth entirely stopped at 2000 mg/L and above (**Fig. 5**). This study shows that the acrylamide-degrading bacterium is resistant to acrylamide concentrations of up to 1700 mg/L The lowest tolerant microorganism is the fungus *A. oryzae*, which was able to breakdown acrylamide concentrations of roughly 100 mg/L by using nitrate and sucrose as nitrogen and carbon sources, respectively. This amount is regarded to be minimal [40]. [41] 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 [36,42].

The highest tolerant and degrader so far is *Cupriavidus oxalaticus*, which can degrade up to 60 mM or 4260 mg/L acrylamide [15]. The lag time that was observed during the development of *Enterobacter cloacae* strain UPM2021a at varying doses of acrylamide was shown to increase when the acrylamide concentrations were elevated. The lag time varied from one day to three days depending on the concentration of acrylamide, which was increased from 100 mg/L to 1500 mg/L. At a concentration of 1500 mg/L, there was no sign of any growth. As the amounts of acrylamide were raised, there was also a drop in the maximum growth, which indicated an overall rise in the trend of toxicity (Data not shown). Acrylamide is hazardous to the development of many microorganisms, and at concentrations of 1000 mg/L or more, growth will often be stopped [10–19,22,27–32]. The presence of the enzyme amidase in particular microorganisms makes it possible for them to

continue growing at these significantly higher concentrations [15,28,29,31,43–46].

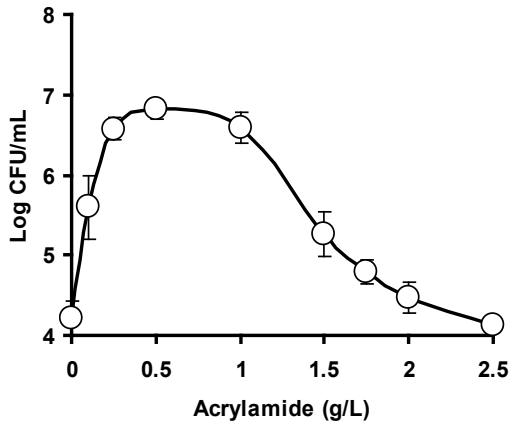

Fig. 5. Growth of *Enterobacter cloacae* strain UPM2021a at various concentrations of acrylamide. Each data point represents the mean ± SD n=3.

One discovery that is particularly noteworthy is that the lag period is lengthened when development is carried out at a very high concentration of acrylamide, as was seen in this work. Utilizing primary growth models like modified Gompertz or logistics, or even other models that are accessible is one way to acquire essential growth characteristics like specific growth rate, maximum growth rate, and lag duration [17,47].

Various primary growth models include other options. The particular growth rate that was determined is a valuable parameter that may be further modeled using secondary models such as Monod, Haldane, Teissier (Tessier), Yano, and Aiba, amongst others [16,17].

Growth on acrylamide in the presence of various heavy metals

The presence of heavy metals at a polluted site is one of the most important factors that make bioremediation more challenging. This is due to the fact that many different types of bacteria are unable to survive in environments with high concentrations of heavy metals, and as a consequence, they lose the capacity to degrade the chemicals that they are intended to break down. According to the findings of an investigation, a concentration of heavy metals consisting of copper (Cu), lead (Pb), cadmium (Cd), chromium (Cr), and mercury (Hg) at 2 parts per million exhibits varying hindrance to the development of the bacteria when it is fed acrylamide. Mercury at a concentration of 2 parts per million induced roughly 80 percent inhibition, whereas other metal ions such as copper, cadmium, and chromium showed inhibition ranging from 30 to 50 percent; lead was the most tolerant of the metals (**Fig. 6**).

Heavy metals have a significant impact on the process by which acrylamide is broken down, and the presence of mercury has been shown to have a more profoundly inhibiting effect on this process than the presence of other metals [11–14,16–19]. The amount of evidence that is currently available in the published literature about the impact that heavy metals have on the degradation of acrylamide and other xenobiotics is quite limited. The findings of this study will have a substantial influence on future applications of bioremediation since there is a dearth of literature on the topic of microbial tolerance to heavy metals. Research utilizing metal inhibition models is essential, but it is underrepresented in the narrative despite the prevalence of heavy metals in rivers that have been damaged by industry. This is despite the fact that these models can effectively reduce the number of metals in water. An extensive study on the ability of bacteria to live in and spread in very hazardous environments is now being carried out. The Andrews model was successfully used to determine the effect of toxic metals on the rates of degradation of monoaromatic hydrocarbons by *Pseudomonas* sp. and *Bacillus* sp. [48].

Inhibition of enzyme activity is likely caused by heavy metals binding to the sulfhydryl group found frequently in enzyme-active sites [49]. There are a few things to consider when it comes to heavy metals impeding biodegradation. Inoculation of metal-resistant bacteria can reduce bioavailable metal concentrations, improving biodegradation in the presence of a hazardous metal. [50]. Combining a primary bacterial degrader with a metal-resistant bacterium can improve acrylamide breakdown efficiency. An example is shown in a soil microcosm experiment study, where a cadmium-resistant *Pseudomonas* H1, which accumulates cadmium in the cell, and 2,4-D-degrading bacteria were introduced to soil contaminated with both cadmium (60 mg total cadmium/kg) and 2,4-D (500 mg/kg) resulting in a better degradation efficiency of the xenobiotic.

Treatment additives such calcium carbonate, manganese oxide, cement, phosphate, and magnesium hydroxide can help lower the bioavailability and mobility of metals, making it easier to clear up metal contamination [51]. Including clay minerals is an alternative method that can be used. Clay minerals have demonstrated use in lowering metal bioavailability as well as the toxicity that is brought on by the presence of metals. When kaolinite (1-20 percent) or montmorillonite (1-5 percent) was added to a cadmium-containing agar medium for use by yeasts, bacteria, and an actinomycete, for example, the toxicity of cadmium was decreased [52]. In a study with the same objective, it was shown that adding 3 percent bentonite and vermiculite to a solution containing 150 mg total cadmium/L lowered the toxicity of the cadmium to *Streptomyces bottropensis*. Although kaolinite had the potential to lessen the toxicity of cadmium, it required a higher concentration than the other clays (six percent as opposed to three percent) and provided less overall protection [53].

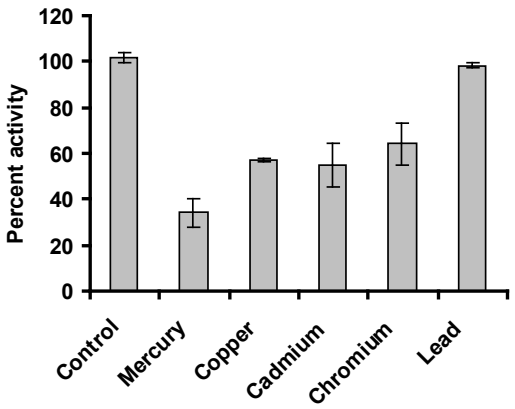

Fig. 6. The effect of heavy metals on acrylamide degradation by *Enterobacter cloacae* strain UPM2021a. Each data point represents the mean ± SD.

CONCLUSION

In our laboratory, research has been conducted to investigate the acrylamide-degrading capabilities of the *Enterobacter cloacae* strain known as UPM2021a. This strain was isolated from an area that had rice paddy. The preliminary research that was conducted found that the ideal circumstances for development included a pH range of 6.5 to 7.5 and a temperature range of 25 to 35 degrees Celsius. These were the parameters that were found to be best for development. Concentrations of acrylamide in the range of 2500 mg/L and higher were investigated and tested as part of a single nitrogen supply. The maximum rate of growth is seen between concentrations of 300 and 1000 mg/L of acrylamide, which corresponds to an estimated net increase of 3 log CFU/mL. This is when compared to the control. The development of the organism was virtually tolerated at concentrations of 1700 mg/L, but it was entirely stopped at concentrations of 2,000 mg/L and above. Researchers discovered that the formation of acrylamide may be stopped by using toxic heavy metals such as mercury, copper, chromium, and cadmium. It was discovered that mercury was the most effective inhibitor, whereas other metal ions such as copper, cadmium, and chromium showed inhibition ranging from 30 to 50 percent. Mercury was determined to be the most effective inhibitor. Among the heavy elements that were examined, lead was shown to be the least inhibiting. As part of the ongoing investigation, a test utilizing a two-level factorial design will be carried out in order to identify essential characteristics that contribute to increased development. After these parameters have been determined, they will be utilized in an experiment that is based on RSM in order to achieve more growth on acrylamide. In addition, an investigation of the degradation products using high-performance liquid chromatography (HPLC) is now being carried out. In order to replicate the evolution of the bacteria over time in response to different quantities of acrylamide, both primary and secondary models are being employed. The use of these bacteria, in particular in agricultural soils, presents a sizeable window of opportunity for the technique known as bioremediation, which is utilized in the removal of acrylamide.

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