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Isolation and Growth Characterization of *Staphylococcus* sp. strain Amr-15 on Acrylamide as the Sole Nitrogen Source

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ABSTRACT

Some of agricultural pesticides contained polyacrylamide as a dispersant and wetting agent. Polyacrylamide is slowly degraded to acrylamide, a suspected carcinogen and a neurotoxicant that has been documented to cause deaths of ruminants exposed to this compound. An acrylamide-degrading bacterium has been isolated from polluted soils in Egypt. The bacterium was identified partially as *Staphylococcus* sp. strain Amr-15 based on biochemical tests. At room temperature, the effect of the initial pH on bacterial growth shows that the optimum pH range was discovered to be between 7.0 and 7.5. The optimal growing temperature at pH 7.5 ranged from 25 to 40 degrees Celsius. In a series of experiments using a 1.0 percent (w/v) starting concentration of various organic carbon sources, it was determined that both glucose and sucrose supported the greatest amount of cellular growth on acrylamide. Acrylamide dosages of up to 2000 mg/L were explored as a single nitrogen supply. The greatest growth occurred between 300 and 1000 mg/L of acrylamide, resulting in a nearly 7.0 log CFU/mL increase with a nett growth of near 3 log CFU/mL when compared to the control. Growth was practically tolerated at the highest dosage tested, 2000 mg/L, and growth stopped entirely at 2500 mg/L. Mercury at 2 ppm caused 82% of inhibition whilst other metal ions such as copper, cadmium, lead and chromium show from 30 to 50% inhibition. The concentration of acrylamide and the time it took for this bacterium to start growing show an inverse relationship. A lag time of 1-3 days was found as the content of acrylamide was raised from 100 to 1000 mg/L while growth was abolished at 1500 mg/L. The maximal growth rate increased as acrylamide concentrations increased, indicating an overall trend of increased toxicity.

INTRODUCTION

Acrylamide is the primary component that goes into the construction of polyacrylamide, which eventually degrades back into acrylamide. Acrylamide is one of the primary factors responsible for the occurrence of the chemical in soil. The utilization of a microbe-mediated acrylamide breakdown as a bioremediation strategy has been gaining interest all around the world in a manner that may be described as slow but steady. The Maillard process takes place in the presence of sugars and amino acids working together. This technique results in the formation of a substantial quantity of acrylamide [1]. In addition to this, it is neurotoxic, which denotes that there is the possibility that it will cause harm to the nervous system. In addition to the harm it causes to the liver and kidneys, acrylamide has also been linked to toxicity in the reproductive and developmental systems. Acrylamide can be absorbed into the body through the lungs, the digestive tract, or the skin. These are the three possible routes of exposure.

Acrylamide can irritate the eyes, nose, and throat, in addition to causing difficulties with the respiratory system, if it is inhaled. Ingestion of acrylamide can result in symptoms such as nausea, vomiting, and discomfort in the abdominal region. It is possible for acrylamide to produce irritation, redness, and itching on the skin if it comes into contact with the skin. Acrylamide is

known to be harmful to genetic material since it has been proven to produce changes in the DNA of persons who have been exposed to it, and these mutations have the potential to be passed down from one generation to the next. Both cattle and fish in Sweden and Norway perished as a result of being poisoned by acrylamide, which was discovered in streams in the surrounding area. The primary application for acrylamide is in the production of polyacrylamide, also known as PAM. PAM is a substance that has a wide range of uses across a variety of industries, including those that deal with printing, plastics, adhesives, and the treatment of drinking water.

Acrylamide's primary use is in this production process. In 2005, one of the most important aspects that went into deciding how safe our food supply was the amount of risk created by the usage of polyacrylamides that were available for purchase on the market. These polyacrylamides are commonly contaminated with the deadly monomer of acrylamide. The widespread use of the herbicide Roundup, which contains polyacrylamide in a concentration of thirty percent, is the primary contributor to acrylamide pollution. Acrylamide will need to go through a process of remediation in order for us to be successful in resolving this issue [2]. Acrylamide has been designated as a Group 2A carcinogen by the International Agency for Research on Cancer, which suggests that there is a high probability that exposure to it will result in cancer in people.

Acrylamide is one of the compounds that the World Health Organization has identified as having the potential to cause cancer in people by assigning it the classification as a Group 2B carcinogen. This classification shows that acrylamide has this potential. In addition, the United States Environmental Protection Agency has classified acrylamide as a substance that poses a threat to both reproduction and development [3]. Acrylamide is a dangerous substance that is a potential risk to human health since it has been associated with a number of negative health consequences, some of which include cancer, toxicity to the reproductive and developmental systems, and impairment of the neurological system. Acrylamide is known to have negative effects on the reproductive systems of male rats, one of which is the development of histological abnormalities in the seminiferous tubules. If acrylamide is breathed in or absorbed through the skin, it may cause a rash or a burning feeling.

Acrylamide may also cause an allergic reaction. A multitude of signs, such as increased sweating, sleepiness, and shaking in the tongue, can be used to diagnose a breakdown in the neurological system [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]. By analyzing the acrylamide adducts that are produced when haemoglobin is combined with acrylamide, it is able to calculate the amount of acrylamide that an average worker is subjected to while on the job. The findings of the research, which employed haemoglobin adducts as a biomarker, revealed that 41 people working at an acrylamide facility displayed higher levels of neurotoxicity. The research was conducted using haemoglobin adducts. The amount of haemoglobin adducts in the blood of employees at an acrylamide facility in China grew, which is an indication that the workers were subjected to exceptionally high doses of acrylamide [8]. 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. Intoxication with acrylamide was shown to be the cause of truncal ataxia and disorientation in five of the study's participants [9], all of whom had consumed the tainted water. Bacteria are the most prevalent type of microbe that has been found to be capable of breaking down acrylamide [10–19]. In this article, the finding and characterisation of yet another bacterium capable of digesting acrylamide are detailed.

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 2014, soil samples were collected from the grounds of a contaminated location in the Egyptian city of Sadat City. The samples were collected at a depth of five cm into the topsoil. One gram of the soil sample was evenly distributed among the sterile water that was drawn from the faucet. A 0.1 mL aliquot of the soil solution was transferred using a pipette onto agar that had been supplemented with 1 percent glucose as the carbon source and 0.5 g/L of acrylamide as the sole nitrogen source. The agar was then incubated at 37 degrees Celsius for 24 hours. The medium used has minimal amounts of salt in it. After that, the most prominent of these colonies were transferred to a volumetric flask that contained 100 mL and then 50 mL of acrylamide enrichment media, respectively.

After that, the flask was put in an incubator shaker, and it was kept at a temperature of 25 degrees Celsius for a total of 48 hours (Certomat R, USA). The minimal salt medium (MSM) used for growth 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 serves as a buffer, ensuring that the pH level remains in a consistent range that extends from 5.8 to 7.8. The only source of nitrogen that was used in the process of sterilization was acrylamide, and the PTFE syringe filters that were utilized had a pore size of 0.45 micron. 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 really present.

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

Methods drawn from biochemistry and phenotyping were utilized in the process of characterizing the bacteria. On the nutrient agar plate, these features may be determined by looking at the size of the colony as well as its form and color. In line with the Bergey's Manual of Determinative Bacteriology, the Gram staining, bacterial motility, oxidase test (24 hours), beta-galactosidase, catalase production (24 hours), ornithine decarboxylase, and other standard tests were carried out [20]. The results were interpreted via the ABIS online system [21].

Statistical Analysis

One-way analysis of variance (with post hoc analysis by Tukey's test) or Student's t-test was used to compare between groups. Values are means ± standard deviation (SD) of triplicate experiments. P-value of < 0.05 was considered was significant.

RESULTS AND DISCUSSION

Strain Amr-16 has the characteristics of being Gram-negative, motile, short-rod shaped, and a facultative anaerobe. The bacterium was identified by using the Bergey's Manual of Determinative Bacteriology [20] and the ABIS online program [21] in conjunction with the findings of several culture, morphological, and various biochemical tests (**Table 1**). The software came up with a few different suggestions for the identification of the bacterium, all of which had similarities with Staphylococcus aureus subsp. aureus. The score for similarity was 88.6 percent, the score for probability was 37.2 percent, and the score for matrix integrity was 100 percent.

At this point in time, it is not feasible to correctly categorize people with regard to the species to which they belong. However, further research in the future, particularly molecular identification approaches that are based on comparisons of the 16srRNA gene, will be necessary in order to accurately identify this species. On the other hand, it is believed that the bacterium in question is of the strain *Staphylococcus* sp. Amr-15 at this point in time. It has been shown that certain strains of this species, notably *S. aureus*, are able to manufacture multiple amidases [22], which may assist the bacterium in growing on acrylamide.

Table 1. Biochemical tests for *Staphylococcus* sp. strain Amr-15.

Diameter > 5 mm in 48 hours	+	Acid production from	
Carotenoid pigment production	+		
Aerobic growth	+	Arabinose	–
Anaerobic growth	+	Cellobiose	–
Growth at 15 ºC	+	Fructose	d
Growth at 45 ºC	+	Fucose	–
Hemolysis	+	Galactose	+
Growth on 10% NaCl agar	+	Glucose	+
Growth on 15% NaCl agar	+	Glycerol	+
Nitrates reduction	+	Lactose	+
Acetoin production (VP)	+	Maltose	+
Alkaline phosphatase (PAL)	+	Mannitol	+
Arginine dihydrolase (ADH)	+	Mannose	+
Urease	+	Melezitose	–
Hyaluronidase	–	Raffinose	–
Growth on $(NH_4)_2SO_4$	+	Ribose	+
Coagulase-rabbit plasma	–	Salicin	–
Clumping factor	–	Sucrose	+
Fibrinolysin	–	Trehalose	+
Deoxyribonuclease agar	+	Turanose	–
Heat-stable nuclease	+	Xylitol	–
Beta-glucosidase (esculinase)	–	Xylose	–
Beta-glucuronidase	+	L-Lactic acid production	+
Beta-galactosidase (ONPG)	+	D-Lactic acid production	+
Catalase	d		
Oxidase	–		

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

Effects of Initial pH and Temperature on Growth

Between pH 5.7 and pH 8, the effect of the initial pH on the growth of bacteria was investigated. Following a period of incubation lasting for forty-eight hours, a growth rate was calculated. The results of an ANOVA study showed that the optimal pH range for cellular growth was between 7.0 and 7.5, with a significant drop in growth occurring outside of this range (**Fig. 1**). Temperature also played an important role in acrylamide growth (**Fig. 2**), with the best growth on acrylamide achieved between 30 and 40 °C. The range of pH is within the range of prior studies which have shown that bacteria that break down acrylamide generally prefer a pH of around 7.0 [10–19].

In addition, the creation of organic acid and carbon dioxide in tropical soils frequently results in acidic soils; hence, pH-regulating chemicals should be administered in order to bring the soils as close to neutrality as possible for optimal restoration [23]. Temperature plays a significant part in acrylamide degradation by bacteria, with an optimal temperature of around 30 ºC reported for acrylamide-degrading microorganisms [10–19]. 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 [24,25].

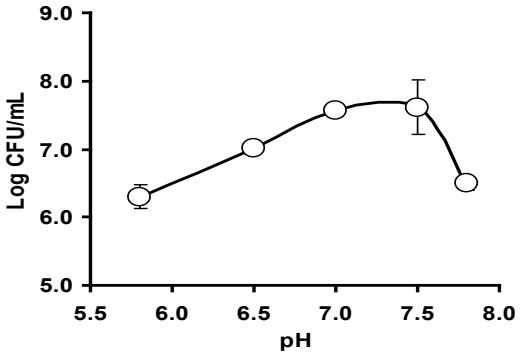

Fig. 1. Growth of *Staphylococcus* sp. strain Amr-15 at various pH. Each data point represents the mean ± SD.

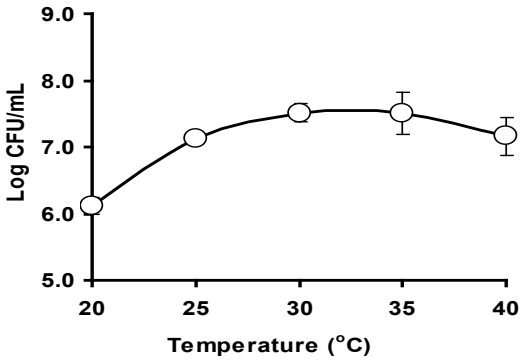

Fig. 2. Growth of *Staphylococcus* sp. strain Amr-15 at various temperatures. Each data point represents the mean ± SD.

Effects of Carbon Sources on Growth

In this in-depth study, the effects of an initial concentration of 1.0 percent (w/v) of a variety of organic carbon sources, such as fructose, glucose, lactose, maltose, mannitol, citric acid, and diesel, on the growth of bacteria on acrylamide were investigated. These organic carbon sources included fructose, glucose, lactose, maltose, and mannitol. The growth on glucose was somewhat superior to the growth on sucrose, with the former reaching a growth of 7.49 log CFU/mL, which was higher than the growth on any of the other carbon sources as well as the control. After an incubation period of 72 hours, the maximum growth was seen in glucose and sucrose, respectively. When compared to the control group, the findings revealed that the proliferation of the cells was enhanced by any supply of carbon (**Fig. 3**).

When a low-salt medium is used, carbon sources are extremely crucial for bacterial development on acrylamide. This is because the majority of acrylamide-degraders require acrylamide as their only source of nitrogen, and easily assimilable carbon sources must be added. This bacterium shares the consensus that glucose is the best carbon source [10–19]. *Bacillus clausii* and *Burkholderia* sp. [26], *Rhodococcus rhodochrous* [27], *Bacillus cereus* [2] and *Pseudomonas* sp. [28] 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 [29]. Other than simple carbon sources, complex carbon sources such as starch was used by

Pseudonocardia thermophilic [25] whilst salad oil was the sole carbon source by *pseudomonas aeruginosa* [30]. In the majority of instances, the breakdown of acrylamide will result in the creation of acrylic acid. Acrylic acid is a compound that can be digested by a broad range of bacteria via the Kreb's cycle. For instance, it has been demonstrated that the metabolism of acrylate in aerobic bacteria that use acrylate goes via hydroxylation to beta-hydroxypropionate, which is then oxidized to create carbon dioxide. These bacteria use acrylate as a source of energy. These bacteria are able to incorporate acrylate into their metabolic processes. [31].

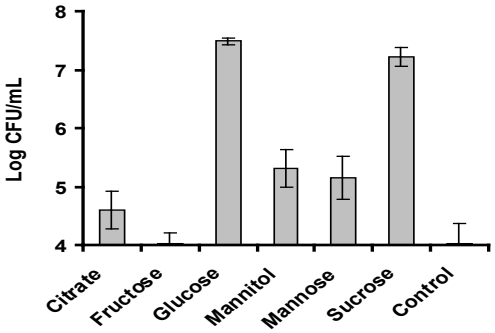

Fig. 3. Growth of *Staphylococcus* sp. strain Amr-15 at various carbon sources and 0.5 g/L acrylamide. The error bars represents the mean ± SD and n=3.

Effect of Acrylamide Concentration on Growth

As a single nitrogen supply, acrylamide doses up to 2000 mg/L were investigated. The highest growth occurs in between 300 and 1000 mg/L of acrylamide, resulting in a growth of near 7.0 log CFU/mL with a nett growth of near 3 Log CFU/mL as compared to control. Growth was nearly tolerated at the highest concentrations tested, which is 2000 mg/L and growth completely ceased at 2500 mg/L (**Fig. 4**). This study shows that the acrylamide-degrading bacterium is resistant to acrylamide concentrations of up to 1500 mg/L The *A. oryzae* fungus 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 [32]. [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 while *Pseudomonas stutzeri* and *Pseudomonas* sp. strain DRYJ7 require between 440 and 500 mg/L, respectively, for optimal growth [27,34].

The highest tolerant and degrader so far is *Cupriavidus oxalaticus*, which can degrade up to 60 mM or 4260 mg/L acrylamide [15]. Heavy metals have a considerable influence on the breakdown of acrylamide, with stronger inhibition occurring in the presence of mercury, copper, and silver than in the presence of other metals [11–14,16–19]. There is a scarcity of evidence available in the published literature about the influence of heavy metals on the breakdown of acrylamide and other xenobiotics. Because of the scarcity of literature on the issue of microbial tolerance to heavy metals, the findings of this study will have a significant impact on future bioremediation applications.

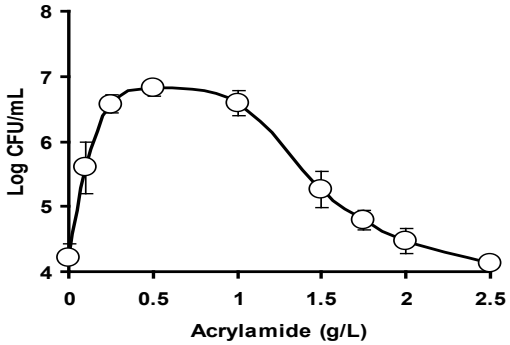

Fig. 4. Growth of *Staphylococcus* sp. strain Amr-15 at various concentrations of acrylamide. Each data point represents the mean ± SD n=3.

Effect of Heavy Metals on the Growth and Degradation of Acrylamide

One of the most critical aspects that contribute to the difficulty of bioremediation at a contaminated site is the presence of heavy metals in the environment. This is because many different types of bacteria are unable to survive in environments with high concentrations of heavy metals, and as a result, they lose the ability to degrade the chemicals that they are intended to break down. This is because heavy metals prevent the bacteria from producing the enzymes necessary for their survival. 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 growth of the bacteria when it is fed acrylamide. The heavy metals in question were copper, lead, cadmium, and mercury. At a concentration of 2 parts per million, mercury was able to induce inhibition of 82 percent, but other metal ions, such as copper, cadmium, lead, and chromium, only exhibited between 30 to 50 percent inhibition (**Fig. 5**).

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 contaminated by industry. This is despite the fact that these models can effectively reduce the harmful effects of metals. An extensive study on the ability of bacteria to live in and spread in very hazardous environments is now being carried out. It was possible to assess the influence of toxic metals on the rates of monoaromatic hydrocarbon degradation caused by Pseudomonas species and Bacillus species by the use of the Andrews model, which was carried out effectively [35]. It is believed that the binding of heavy metals to the sulfhydryl group, which is present in enzyme active sites rather often, is what causes enzyme activity to be inhibited. [36]. When it comes to heavy metals preventing biodegradation, there are a few aspects that need to be taken into consideration. Inoculation with bacteria that are resistant to metals can lower the amounts of bioavailable metals, which increases the rate of biodegradation even in the presence of dangerous metals [37]. Combining a main bacterial degrader with a metal-resistant bacterium can lead to an increase in the rate at which acrylamide is broken down into its component molecules.

A cadmium-resistant *Pseudomonas* H1, which accumulates cadmium in the cell, and 2,4-D-degrading bacteria were introduced to a soil that was contaminated with both cadmium (60 mg total cadmium/kg) and 2,4-D (500 mg/kg), which resulted in a better degradation efficiency of the xenobiotic. This is shown as an example in a soil microcosm experiment study. Treatment additives including calcium carbonate, manganese oxide, cement, phosphate, and magnesium hydroxide can help lower the bioavailability and mobility of metals, making it easier to clean up metal pollution [38]. 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, cadmium toxicity was decreased. This allowed the organisms to be used [39]. 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 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 [40].

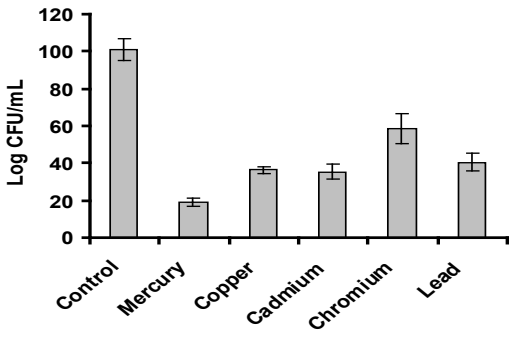

Fig. 5. The effect of heavy metals on acrylamide degradation by *Staphylococcus* sp. strain Amr-15. Each data point represents the mean ± SD.

Growth profile

When the concentration of acrylamide was raised, the lag time that was detected in the development of *Staphylococcus* sp. strain Amr-15 at various dosages of acrylamide also increased. The lag period ranged from one day to three days when the concentration of acrylamide was raised from 100 mg/L to 1500 mg/L. The lower the concentration, the shorter the lag time. There was not the slightest indication of any development at an acrylamide concentration of 1500 mg/L (**Fig. 6**). The maximal growth was also decreased indicating general toxicity increased trend as the concentrations of acrylamide increased.

Acrylamide is toxic to the growth of many microbes and levels above 1000 mg/L usually will arrest growth [11–14,16–19]. Growth at these elevated concentrations is allowable by the enzyme amidase present in certain microbes [15,41–47]. One notable observation is the lag period is prolonged when growth at very high acrylamide concentration is discovered in this study. Important growth parameters such as specific growth rate, maximum growth rate and lag period can be obtained by employing primary growth models such as modified Gompertz or logistics or even other available models [17,48]. The specific growth rate obtained is a valuable parameter that can be further modelled using secondary models such as Monod, Haldane, Teissier (Tessier), Yano, Aiba etc [16,17].

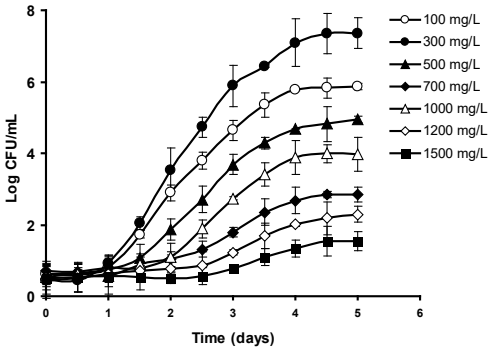

Fig. 6. The growth profile over time of *Staphylococcus* sp. strain Amr-15 at various concentrations of acrylamide. Each data point represents the mean ± SD n=3.

CONCLUSION

It has been determined that the *Staphylococcus* sp. strain Amr-15, which was once categorized as a metal reducer, is capable of degrading acrylamide, and the results of the test have been published. Initial investigations have shown that the ideal circumstances for plant growth have a pH range of 7.0 to 7.5 and a temperature range of 25 to 40 degrees Celsius. These parameters may be found in the best conditions for plant growth. As a viable single nitrogen source, acrylamide concentrations of up to 2,000 mg/L were examined. The greatest growth occurs between concentrations of 300 and 1000 mg/L of acrylamide, which results in an increase of approximately 7.0 log CFU/mL and nett growth of approximately 3 log CFU/mL in comparison to the control. The acrylamide concentration at which the largest growth occurs is between 300 and 1000 mg/L. The highest dose that was tested, which was 2000 mg/L, resulted in a growth rate that was virtually tolerable; nevertheless, at a dosage of 2500 mg/L, the growth rate was entirely halted. It has been demonstrated that the formation of acrylamide may be stopped by using toxic heavy metals such as mercury, copper, chromium, and cadmium. It was shown that mercury was the most efficient inhibitor, whereas other metal ions including copper, cadmium, lead, and chromium showed inhibition ranging from 30 to 50 percent. Mercury was determined to be the most effective inhibitor. 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 predict the development of the bacteria over time in response to different amounts of acrylamide, both primary and secondary models are being applied. The utilization of this bacterium, in particular in the soils used for agricultural purposes, provides a significant possibility for the bioremediation of this poisonous chemical.

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