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Research Article

ISOLATION AND IDENTIFICATION OF ACRYLAMIDE DEGRADING BACTERIA FROM SOIL

Nidhi Jain[1], P.J. John[2]*, Kanti Prakash Sharma[3], I. Soni[4]

[1, 2, 4]Environmental Toxicology Unit, Centre for Advanced Studies, Department of Zoology, University of Rajasthan, Jaipur, 302004. Rajasthan, India.

[3]FASC, MITS University, Lakshmangah, Sikar- 332311, Rajasthan, India.

Email: placheriljohn@yahoo.com. Mobile: +91-9414059670.

ABSTRACT: Acrylamide is an aliphatic amide, which is produced by industrial processes and during heating of food. It is neurotoxic and a suspected carcinogen. In the present study an attempt was made to isolate acrylamide degrading bacteria from soil. The optimum growth conditions and physiological characteristics for the isolated acrylamide degrading bacteria were investigated. The isolated bacterium was identified as Bacillus clausii strain 1779 based on full 16S rRNA molecular phylogeny. The bacteria can degrade 800 mg l^{-1} acrylamide after eight days of incubation with concomitant cell growth. In addition to above, it also grows optimally at a concentration of acrylamide between 500-2000 mg l^{-1} between pH 8-10 and temperature and 25 – 45 ^{0}C. Thus the isolate would be useful in the bioremediation of environment from acrylamide in alkali conditions.

Keywords: Acrylamide, bacteria, soil, molecular phylogeny, bioremediation

INTRODUCTION

Environmental pollution increases with the progress of chemical industry all over the world. Many of the pollutants from the industries are decomposed of a very low rate in the environment because of their complex nature. Bioremedation has evolved as very economical and viable process for detoxification of xenobiotics in general. Polyacrylamide will degrade to acrylamide under different environmental conditions (Smith, et.al., 1997). Acrylamide has half life from weeks to months in rivers. It is a neurotoxicant, carcinogen and teratogen and also hazardous for environment, so its bioremediation must be sought. Dietary intake of acrylamide and the risk of developing cancers of the oral cavity, pharynx, esophagus, larynx, large bowel, kidney, breast, and ovary were investigated by many research (Pelucchi, et. al., 2006), (Mucci, et. al., 2003,2004,2005,2006). High levels of acrylamide in the workplace have been shown to cause neurological damage, e.g., among workers using acrylamide polymers to clarify water in coal preparation plants (Mulloy, 1996). Acrylamide is produced industrially for use in products such as plastics and cosmetics. It is intensively used as water soluble thickeners (Sax and Lewis, 1987), in gel electrophoresis (USEPA, 1981), paper making (Sax and Lewis, 1987), printing ink stabilizers, binders for seed coating, foundary sand (Mannsville, 1993) etc. It is also used as a grouting agent in the construction of dam foundations, tunnels and sewers (Kirk-Othmer, 1979). The industrial use of polyelectrolytes based on polyacrylamide to sludge conditioning and secondary oil recovery is continuously increasing. Acrylamide subunits are readily cross linked to form polyacrylamide, which is widely used as a strengthener for pulp fiber.

Acrylamide is also formed in some foods during high-temperature cooking processes, such as frying, roasting, and baking through the Malliard reaction of asparigine and reducing sugars that are naturally present in the food (Tareke and Rydberg, 2002). Potato chips and French fries have been found to contain relatively high levels of acrylamide compared to other foods. Lower levels of acrylamide is also present in bread and cereals which are heated to a temperature of 120 ^{0}C (Mottram, et. al., 2002).

Several researchers have isolated bacteria from various environments capable of utilizing acrylamide as the sole carbon and nitrogen source (Skouloubris, et. al., 2001). Acrylamide deaminated to ammonia and acrylic acid (acrylate) during its catabolism. This process is catalyzed by amidase or amidohydrolase (Nawaz, et. al., 1994, 1998) (Shanker, et. al., 1990) (Zabaznaya, et. al., 1998). Further acrylate is hydroxylated to hydroxypropionate, which is oxidized to CO_2 or reduced to propionate (Ansede, et, al., 1999) (Wampler and Ensign, 2005).

Thus considering the acute toxicity of acrylamide and availability of a very few microorganisms those can degrade it in alkali conditions the study is planned to isolate, identify and characterize a suitable bacteria from natural source. Besides above, the amidase and its associated acyltransferase activity of can be useful for the biosynthesis of useful industrially important compounds such as hydroxamic acids etc.

MATERIALS AND METHODS

(A) Reagents and Equipment:

(i) Acrylamide- It is white odourless crystalline solid, soluble in water, ethanol, ether and chloroform.
Chemical formulae: C_3H_5NO (Wells, 1984)
Molecular weight: 71
Chemical structure:

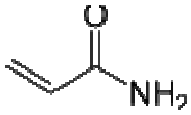

Fig 1: Acrylamide

(ii) Nutrient Agar Medium, Nitrogen Deficient Agar Medium
(iii) Spectrophotometer, Autoclave, Laminar Air flow
All chemicals used were of analytical grade and obtained from Hi Media, Merck and Sigma-Aldrich.

(B) Procedure:

Isolation of acrylamide degrading bacterium

Isolation of acrylamide degrading microorganism was successfully attempted from soil samples. Soil samples were randomally collected from the Kumarappa handmade paper industry, Sanganer, Jaipur 15-20 centimeters (cm) beneath the surface using a sterile spatula and were placed in sterile screw-capped vials. These vials were then placed in sterilized plastic bottles. Acrylamide degrading bacterium was isolated from the soil samples by enrichment culture technique with 500mg l^{-1} acrylamide as the sole nitrogen source. The medium contained (per liter) 10g of glucose, 0.5g of $MgSO_4.7H_2O$, 0.05g of $FeSO_4.2H_2O$, 500mgl^{-1} acrylamide and 1ml of the following trace elements; $ZnSO_4.7H_2O$, 0.34µM; $MnCl_2.4H_2O$, 0.15µM; H_3BO_3, 4.85µM; $CoCl_2.6H_2O$, 0.84µM; $CuCl_2.2H_2O$, 0.05µM; $NiCl_2.6H_2O$, 0.08µM and $NaMoO_4.2H_2O$, 0.123µM (Shukor, et. al., 2009). Subcultures were prepared by incubating the bacterium on a rotary shaker (200 rpm) for 48 hrs at 25^0C in 50 ml conical flasks containing the described 500mg l^{-1} acrylamide. The subcultures were inoculated into 150 ml volumes of the same medium and incubated with rotary shaking (200 rpm) for three days at 37^0C.

Identification of isolate

The selected isolates were examined for morphological (Gram's reaction, cell shape, arrangement, presence of capsule, presence of spores), biochemical characteristics (Catalase test, H_2S Production, MR-VP test, Indole Production, Carbohydrate utilization pattern: Arabinose, Glucose, Sucrose, Fructose, Galactose, Mannitol, Xyloses, Rhamnose, Raffinose, Salicin and m-Inositol) and 16S rRNA nucleotide sequence features. To identify the bacteria on the basis of 16S rRNA gene sequence features as done by (Rainey, et. al., 1996) chromosomal DNA of the isolate was isolated and the 16S rRNA gene was amplified with primers (5'-AgAgTTTgATCCTggCTCAg-3') and (5'-AgAAAggAggTgATCCAgCCA-3'). The amplified DNA fragment was separated on 1 % agarose gel, eluted from the gel and purified using a QIA quick gel extraction kit (Qiagen). The purified PCR product was sequenced by Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems). The obtained sequence was aligned through BLAST at NCBI to know its position in bacterial classification system.

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Optimization of Bacterial Growth

Optimizations of nutrient and growth conditions were carried out to obtain maximum bacterial growth. All the steps were carried out in triplicate and in 25 ml of medium. The process was optimized by keeping the isolate under standard assay conditions and by changing one parameter at a time while keeping all other parameters constant. The parameters optimized are temperature, pH, NaCl concentration, carbon sources, acrylamide concentration and amides.

Amidase Production

A loop of bacterial cells from the slant were inoculated in Nitrogen Deficient Broth Medium (NDBM) (pH-9.0) containing acrylamide as sole nitrogen source and incubated at 37 ^{0}C for 36 hrs in an incubator shaker (200 rpm). This culture was added to 50 ml. of modified nitrogen deficient broth medium (NDBM) in flask, followed by incubation at 37 ^{0}C for 48 hrs in an incubator shaker. The culture was centrifuged at 5000 g for 15 minutes. The supernatant was discarded and the cell pellets was suspended in 0.1M sodium phosphate buffer (pH -0.9) after two washings with the same buffer and the cell suspension was referred as resting cells and assayed for enzyme activity as suggested by Bhalla, et. al. (1992).

RESULTS AND DISCUSSION

Identification of the isolate

The primary identification was done according to the Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986). The isolate was found to be Gram- positive, rod shaped, spore forming bacteria. The isolate was found to be positive for catalase activity and citrate utilization. However, it was found to be negative for oxidase activity, H_2S production, MR-VP test, and indole production. Carbohydrate utilization test revealed that the isolate possess the ability to use arabinose, glucose, sucrose, fructose, galactose, mannitol, xyloses, rhamnose, raffinose, salicin and m-inositol as carbon source. Glucose was the best carbon sources for the growth of the isolate. Based on the morphological, culture and biochemical characteristics, the isolate was presumptively belongs to Bacillaceae family of the domain Bacteria and the kingdom Eubacteria.

PCR revealed an amplification of 16S rRNA gene of bacterial genome. The result of the BLAST analysis revealed that the isolated bacterium belongs to species clausii of genus Bacillus, family Bacillaceae, order Bacillales, class Bacilli and the phylum Firmicutes.

Phylogenetic tree was also constructed by using neighbour joining method at a sequence difference of 0.75 (Fig. 1) to know the genetic relatedness of the isolate with other bacteria. The obtained isolate was deposited in the MTCC at IMTECH, Chandigarh, India with the strain identification 1779 (*Bacillus clausii* 1779).

Table 1 CFU value of isolate on different pH

pH	Log CFU/ml
7.5	0.2630±0.0135
8.0	0.8826±0.0065
8.5	0.9970±0.0050
9.0	1.0436±0.0061
9.5	0.9837±0.0026
10.0	0.9573±0.0073
10.5	0.2857±0.0264

Table 2 CFU values of isolate on different temperature

Temperature^{0}C	Log CFU/ml
20	0.1941±0.0314
25	0.5946± 0.0063
30	0.8864± 0.0056
35	0.9460 ±0.00056
37	0.9852± 0.0026±
40	0.7852±0.0071
45	0.1935±0.043

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Table 3 CFU values of different carbon sources

Carbon sources	Log CFU/ml
Control	0.1841±0.0427
m-inositol	0.2731±0.0866
Glucose	0.6718±0.0161
Sucrose	0.5752±0.0292
Maltose	0.6016±0.0217
Fructose	0.5916±0
Mannitol	0.5602±0.0068
Xyloses	0.5185±0
Rhamnose	0.3905±0.449
Salicin	0.4469±0.0155

Table 4 CFU values for different amides

Amides	Log CFU/ml
Control	0.3667±0.0396
2-chloroacetamide	0.5639±0.0182
Methylacrylamide	0.8612±0.0068
Nicotinamide	0.8750±0
Acrylamide	1.0183±0.0047
Acetamide	1.040±0.0022
Propionamide	1.0413±0.0079
Urea	1.063±0.0042

Table 5 CFU values for different concentration of amides

Effect of amides mgl^{-1}	Log CFU/ml
0	0.2929±0.4320
100	0.2630±0.0135
500	0.8216±0.0099
1000	0.9509±0.0028
1500	0.9047±0.0113
2000	0.8750±0
2500	0.2547±0.241
3000	0.2547±0.2415

Effect of pH

The measurement of growth was carried out after 48 hrs of incubation. The isolated sbacteria grow well in a pH range from 8.0-10.0 at 35 ^{0}C (Fig 2). The results revealed that the isolate is alkalophilic in nature. Others bacteria *Psuedonocardia thermophilia* (Nawaz, et. al., 1998) and *Rhodococcus sp.* (Komeda, et. al., 2004) *Thermococcus hydrothermalis* (Postech, et. al., 2005) are not able to grow well in alkalophilic conditions.

Effect of temperature

The effect of incubation temperature on the growth of acrylamide degrading bacteria shows an optimum growth at 35^{0}C (fig.3). A dramatic drop in growth is seen at temperature lower than 25^{0}C and higher than 42^{0}C. Most of the acrylamide degrading bacteria are mesophiles with optimum temperature for growth in the range of 25^{0}C to 42 ^{0}C. It was reported by Kotlova, et. al., (1999), Nawaz, et. al.,(1998), Wang and Lee, (2001) that 30^{0}C is the optimum temperature for the growth of *Rhodococcus rodochrous, Rhodococcus sp*. and *Psuedomonas stutzeri* respectively. Thermophiles known to degrade acrylamide are *Psuedonocardia thermophilia* and *Brevibacillus borstelensis* BCS-1 which requires higher temperature 50^{0}C and 55^{0}C respectively.

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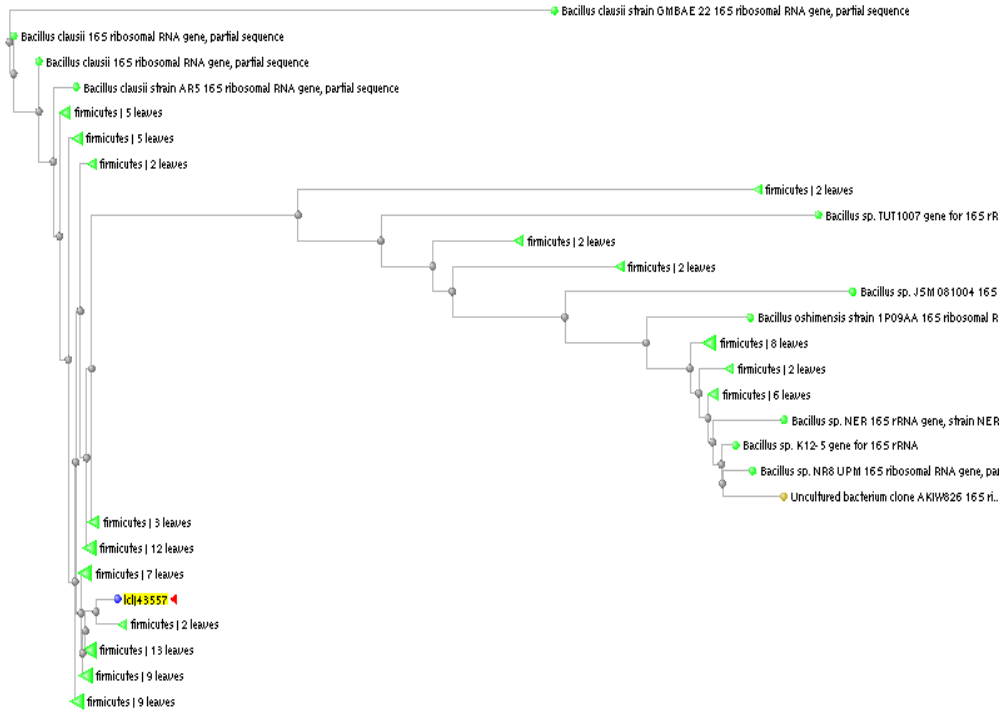

Fig.2 Phylogenetic tree showing relationship of strain 1779 to other bacillus bacteria by using the neighbour – joining method of BLAST at NCBI at a sequence difference of 0.75

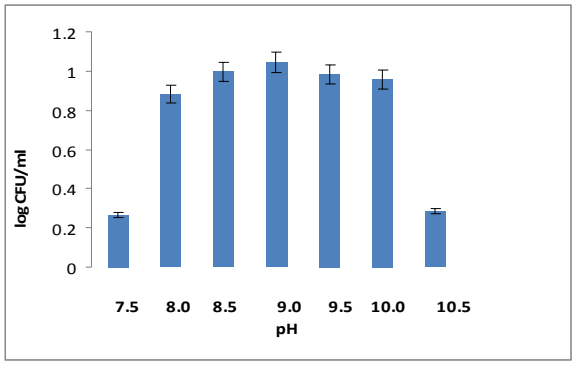

Fig.3 Graph showing effect of pH on growth of bacteria *Bacillus clausii* strain 1779

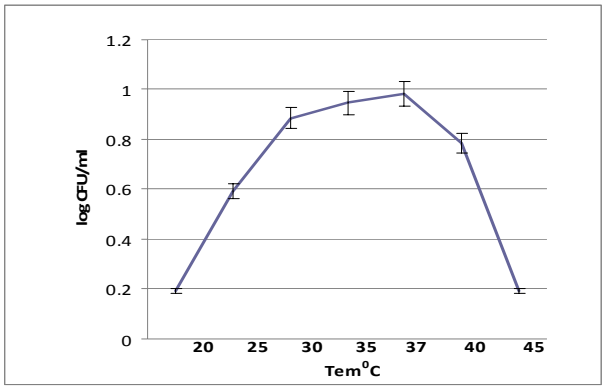

Fig.4 Graph showing effect of Temperature on growth of bacteria strain 1779.

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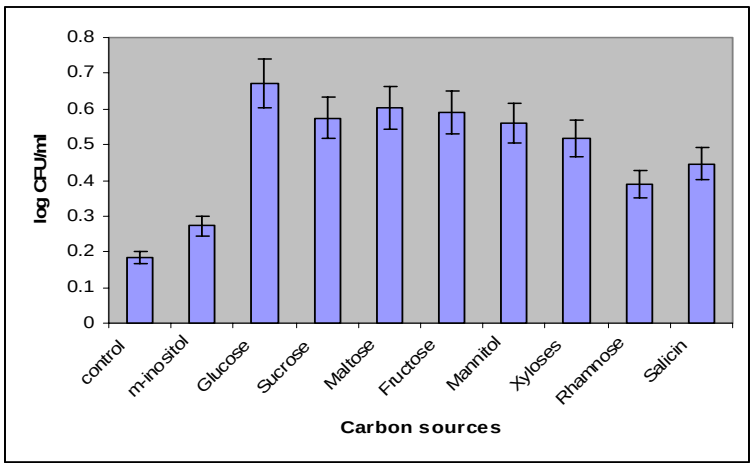

Fig.5 Graph showing effect of different carbon sources on growth of bacterial strain 1779

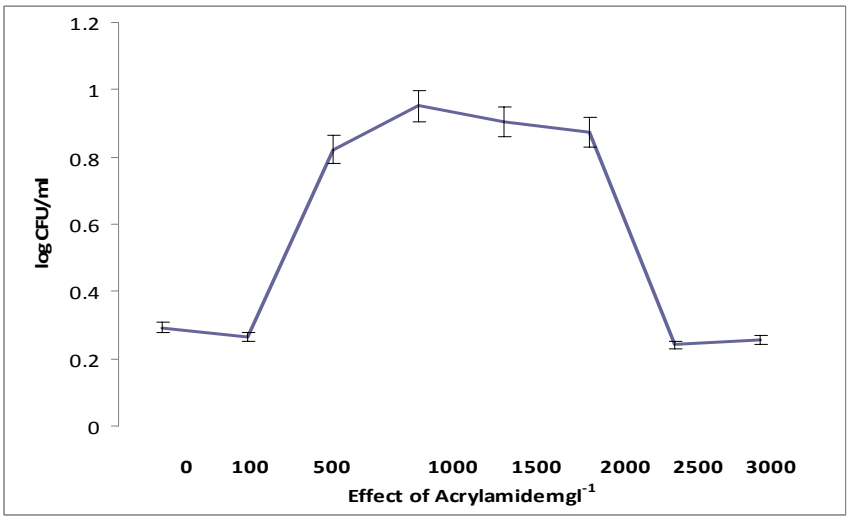

Fig.6 Graph showing effect of acrylamide concentration on growth of bacterial strain 1779

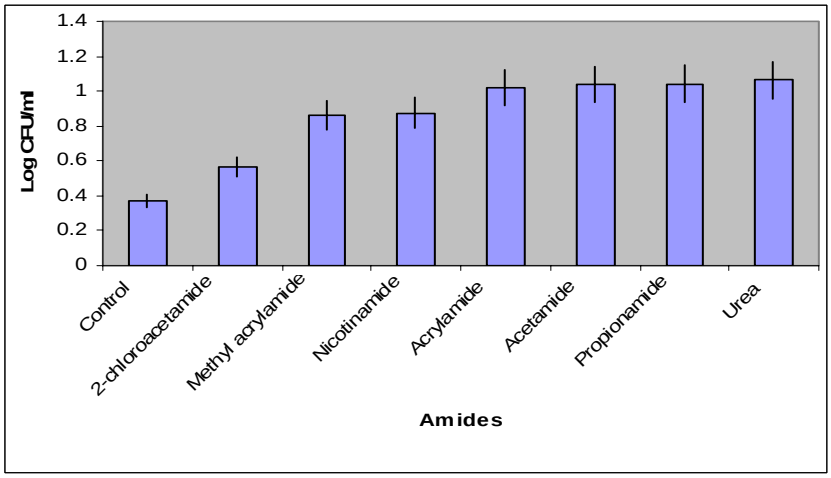

Fig.7 Graph showing effect of different amides on growth of bacteria *Bacillus clausii* strain 1779.

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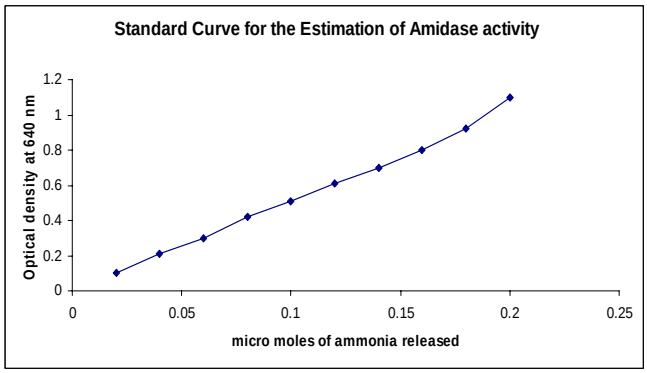

Fig. 8 Graph showing estimation of amidase activity.

The effects of carbon sources on growth of bacterium

Glucose, sucrose, fructose, maltose, mannitol, xyloses, rhamnose, salicin and m-inositol at an initial concentration of 1.0% (w/v) supported growth of this strain. Glucose is the best carbon source (fig.4). Addition of carbon sources and other nutrients increases growth rate of bacteria in minimal medium.1 to 2 % glucose appear to be the optimum source in some cases (Kotlova, et. al., 1999) (Nawaz, et. al., 1996) (Ciskanik, 1995).

Effects of various amides on growth

2-chloroacetamide, methacrylamide, nicotinamide, acrylamide, acetamide, propionamide and urea supported growth of this strain with increasing assimilative capability from 2-chloroacetamide to urea (fig.5). It was reported by Skouloubris, et. al. (1997) that amide-degrading bacteria able to use several short chain aliphatic amides aside from acrylamide.

Effect of acrylamide concentration

The isolated bacteria grow well between between 500 to 2000 mg l-1 of acrylamide concentration (fig 6). Growth is inhibited at 2000 mg l-1 and above. Probably it occurs due to the inhibitory effect of acrylamide on thiol groups of proteins. Strain 1779 could degrade 800 mg l^{-1} of acrylamide after eight days of incubation with concomitant cell growth. Growth increase up to 2 log units at day 2 before it reaches a plateau.

Amidase production

The isolate was found to have 0.4 units/ml activity against acetamide. The enzyme production was intracellular. No enzyme activity was detected in broth.

CONCLUSIONS

The study isolates identified as bacteria which degrades acrylamide. The isolated bacteria seems very promising since it is able to degrade acrylamide in alkali conditions. Moreover, it can tolerate a high concentration of acrylamide i.e. upto 2000 ppm thus seems very promising to overcome the toxicity of acrylamide in environment.

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