Research Article

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF HIGH SALINITY TOLERANT, HEAVY METAL CONTAMINANT AND ANTIBIOTICS RESISTANT AMYLOLYTIC-THERMOPHILIC *PSEUDOMONAS Sp.*

Siddharth Vats*, Dr Rajesh Kumar and Kanupriya Miglani
University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, Haryana, India.
*Corresponding author's E-mail: vatssidd@gmail.com

Accepted on: 11-04-2011; **Finalized on:** 28-09-2011.

ABSTRACT

Earth is our home and it is in danger. Pollution is threatening its sustainability and carrying capabilities. Heavy metal pollution can cause toxicity by inhibiting metabolic reactions. The Minimum Inhibitory Concentration (MIC) of the isolated samples against Nickel (Ni^{2+}), Lithium (Li^{1+}), Copper (Cu^{2+}), Manganese (Mn^{2+}), Zinc (Zn^{+2}) and Iron (Fe^{+3}) was determined in solid media. Multiple metal resistances of the isolate associated with resistance to various antibiotics were also determined. Salt tolerance was determined both in solid and liquid media. Identified halo-tolerant, metal resistant bacteria find use in the bioremediation of heavy metal contaminated sewage and waste water, and also for the digestion of the high salt rich soil grown plants. Enzymes from these microbes can be used for various bioremediation purposes. Though amylases originate from different sources (plants, animals, and microorganisms), the microbial amylases are the most abundantly produced and used in industry, due to their productivity and thermo stability. Microbe identified is *Pseudomonas lini* and show remarkable heavy metal tolerance, high salinity tolerance, antibiotic resistance and amylolytic activities.

Keywords: Heavy metal, Pollution, Halotolerant, thermostability, antibiotics resistant.

INTRODUCTION

Our earth is the only planet which has been found to support life. It supports millions of species and billion of living organisms. But population explosion has led to its uncontrolled exploitation. Man to full fill its need has established industries which twenty four in to seven, produces toxic products and cause pollutions and harm nature. Pollution of heavy metal is one of the important menaces. And this pollution is increasing with the expansion of the industries. Heavy metals pose a real serious contamination of soil and water[1]. Water effluents coming out from the industries and sewage wastes have permanent toxic effects to human and the environments. Metals like potassium, copper, nickel, zinc, manganese, ferrous and lithium are the most commonly used and the widest spread contaminant.[2] Heavy metals frequently generate strong reactive species and directly or indirectly cause gene mutation and harm living cells. Iron, copper and zinc are the metal with dual role. At trace level these metal ions are very essential but beyond a concentration they start showing negative effects. These metals are used as the Co- factors in the enzymatic reactions but their over presence can also inhibit the metabolic reactions and can cause toxicity in the living organisms. Microbes especially Bacteria are most abundant and are almost omnipresent, they found every environment hospitable. They can survive almost any kind of nutrients available to them. Some heavy metals are micronutrients but heavy metals in excess definitely toxic. These micro-organisms react and respond with different ways like biosorption to the cell wall, transport across the cell membrane, oxidation- reduction reactions, complexation, precipitations and entrapment in their extracellular capsules.[3] Enzymes are biological *catalysts* or assistants. Enzymes consist of various types of proteins that work to drive the chemical reaction required for a specific action. Enzymes can either perform a reaction or speed it up. The chemicals that are transformed with the help of enzymes are called *substrates*. In the absence of enzymes, these chemicals are called *reactants*. Biocatalysis involves enzyme catalysis in the living cells. Over 3000 enzymes have already been identified and the number is growing with research in the field of genomics and proteomics. Enzymes can be extracted from living tissues, purified and even crystallized. Though amylases originate from different sources (plants, animals, and microorganisms), the microbial amylases are the most abundantly produced and used in industry, due to their productivity and thermo stability.[4] Enzymes from bacterial and fungal sources have dominated applications in industrial sectors. Amylases are classified based on how they break down starch molecules. α-amylase (alpha-amylase) -Reduces the viscosity of starch by breaking down the bonds at random, therefore producing varied sized chains of glucose. ß-amylase (Beta-amylase) - Breaks the glucose-glucose bonds down by removing two glucose units at a time, thereby producing maltose. Amyloglucosidase (AMG) - Breaks successive bonds from the non-reducing end of the straight chain, producing glucose. The α-amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate being glucose residues linked through α-1-1, α-1-4, α-1-6, glycosidic bonds. Members of this family share a number of common characteristic properties.[5] Amylases can be divided into two categories based upon their site of action, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exoamylases act from the non-reducing end successively resulting in short end products.[6] Amylases constitute a class of industrial enzymes having approximately 25% of the enzyme market.[7,8] It is desirable that α- amylases should be active at the high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) to economize processes, therefore there has been a need for more thermophilic and thermostable α-amylases.[7] With the availability of High salinity tolerant microbe number of new possibilities for industrial processes has emerged. While the most widely used thermostable enzymes are the amylases in the starch industry.[9-11] A number of other applications are in various stages of development. The spectrum of amylase application has widened in many other fields, such as clinical, medical, and analytical chemistries, as well as their wide spread application in starch saccharification and in the textile, food, fermentation, paper, brewing and distilling industries.[12]

Sources of contamination

Water effluents coming out from the industries and sewage wastes have permanent toxic effects to human and the environments.[13] Resistance system of bacteria is made so that all kinds of Bacteria are resistant to heavy metals.[14] Nickel is the 24th most abundant element with soft and hard metal like characteristics show binding nature with sulphur, nitrogen and oxygen groups.[15] Nickel is the metal used mostly in alloy formation. The nickel content of these alloys varies widely from, as examples, 1-3% for special engineering steels, 8-14% for stainless steels, 15-40% for special engineering alloys, 40-90% nickel for special alloys for the aerospace and electronic industries. And these industries are the source of its contamination. Chromium in natural environment mostly present in hexa-valent and trivalent species.[16] Main sources of chromium contamination are leather tanning, chromium electroplating, alloy preparation and its use in the nuclear power plant.[17] Zinc serve as a co-factor in various cellular processes carried out by more than 300 enzymes.[18] Zinc is an important player in the structural stability and in transcription. It an important part and adult human weight contain 2.5 gram of zinc mostly used by the enzymes. Sources for the zinc contaminations are permitted industries, residential wastes, human wastes, corrosion and other sources. Sources for the copper contaminations are copper pesticides, vehicle brake pads, industrial copper use, soil erosion and copper in domestic water discharge. Copper-containing pesticides are widely used to control fungi, algae and roots. Big doses of copper can cause liver and kidney damage. Iron as a pollutant is released by iron and steel industries. Iron bearing water is journal orange is colour. In cities manganese pollution is increasing with the increase of traffics and most likely source is exhaust.[19]

MATERIALS AND METHODS

Materials

All the chemicals used were from Himedia and glass wares used were from Perfitt.

Sample collection

Samples are collected from the sewage water, industrial effluents disposal sites, waste water treatment plants and sites where waste clothes are treated with chemicals. Samples were collected in the sterile autoclaved plastic cap bottles. 15 samples were collected and taken for study.

Isolation of pure bacterial cultures

Different water samples were spreaded on nutrient agar and pure colonies obtained. These pure colonies were again spreaded on nutrient agar. After this, stock solutions of each bacterium were prepared and used to carry out study and to find the best bacteria among them.

Selection of thermostable microbes

Nutrient agar plates are made of pure colonies and are incubated at a temperature of 40°C for 48 hours. Those colonies which survive this temperature are selected and tested also in nutrient broth. Microbes having good growth in the solid as well as in liquid media are selected.

Selection of the high salinity tolerant microbes

To select halotolerant bacteria, pure culture were spreaded on the 1 and 2 molar NaCl containing nutrient agar plates and growth were observed incubating at 27°C after 24-48 hours. The resistant bacteria are then inoculated in 1 and 2 molar nutrient broth and best growing microbe are selected.

Selection of amylolytic microbes

Halo tolerant microbes selected are sub cultured over 2% starch enriched nutrient agar plate. After 48 hr incubation bacterial colonies were flooded with 1 % Iodine solution, to select isolates which show wide clear zone around their colonies.

Selection of heavy metals resistant microbes

Selection of the heavy metal resistant microbe is done by use of heavy metal incorporated media. Heavy metals incorporated were Nickel (Ni^{2+}), Lithium (Li^{1+}), Copper (Cu^{2+}), Manganese (Mn^{2+}), Zinc (Zn^{+2}) and Iron (Fe^{+3}). Salinity tolerant microbes are exposed to high metal concentration. Chemicals used were LiCl [Lithium chloride], $MnSO_4.H_2O$ [Manganese (II) Monohydrate], $CuSO_4.5H_2O$ [Copper (II) Sulphate Pentahydrate], $ZnSO_4.7H_2O$ [Zinc Sulphate], [Ferrous III Sulphate] $Fe.Cl_3$ and $NiSO_4.6H_2O$ [Nickle Sulphate].

Antibiotic resistance

Antibiotic resistance of the halotolerant, multiple metal resistance thermophilic microbe was determined Kirby-Bauer disc diffusion method.[20] After incubation of the microbe grown on MHA, it is considered as resistant or susceptible based on the clear zone shown or not by the antibiotics.

RESULTS AND DISCUSSION

Isolation and identification of the bacteria with heavy metal resistance

A no. of microbes were screened from the samples collected from the various effluents sites near Kurukshetra University and were screened for high salinity tolerance, multiple heavy metal resistance. Halo-tolerant Bacteria with highest salinity tolerance and multiple heavy metal resistance ability was selected. Bacteria showed (table 1 & 3) MIC for different metal ranging from 400µg/ml to 1800µg/ml. The test indicates multiple heavy metal and antibiotic resistance. Bacteria have maximum resistance to lithium, ferrous, zinc and manganese with moderate resistance to nickel and copper.

Table 1: Resistance of bacteria to heavy metals (Halotolerant, multiple heavy metal resistant Bacteria)

Resistance to	MIC
Nickel (Ni^{2+})	1400µg/ml
Lithium (Li^{1+})	2000µg/ml
Copper (Cu^{2+})	1900µg/ml
Manganese (Mn^{2+})	1800µg/ml
Iron (Fe^{+3})	1900µg/ml
Zinc (Zn^{+2})	1200µg/ml

Antibiotic sensitivity of multiple heavy metal resistant bacteria

Sensitivity of bacteria was tested against 15 antibacterial antibiotics like amoxicillin, penicillin, methicillin, doxocycline, chlorocycline, erythromycin, nitronidozole, minocycline, clindocycline, ampiciline, clindomycine, ofloxocine, rifampicine, gentamycine, vancomycine. It was resistant again vancomycine, clindomycine, amoxycilline, penicillin, methicilline, nitronidazole, minocyline and streptomycine.

Salt resistance is very important characteristic of isolated microbe and is use full in treating amylolitic waste rich in salt. This is very important in bioremediation purposes. We observed good resistance both in liquid media as well as solid media by *Pseudomoonas sp.* against NaCl. Results are notified in the table 2.

Table 2: Resistance of bacteria to salt concentration**.**

Salt Concentration (Molar)	Resistivity
0.5	+++++
1.0	+++++
1.5	+++++
2.0	+++++
2.5	+++
3.0	+

+: Growth; ++, ++++: Growth status; - : no growth

Table 3: Antibiotic Resistance of halotolerant and heavy metal resistant bacteria

Antibiotic disc of size 6mm	Disc content	Diameter of inhibition zone	Resistivity of bacteria
Vancomycine	30 mcg	6 mm	+++++
Erythromycin	15 mcg	49 mm	- - - - -
Penicillin	10 mcg	6 mm	+++++
Minocycline	30 mcg	15 mm	- -
Gentamycine	10 mcg	25 mm	- -
Rifampicin	5 mcg	12 mm	- -
Oflaxacine	5 mcg	25 mm	- -
Amoxycoline	25 mcg	6 mm	+++++
Methicilline	5 mcg	6 mm	+++++
Chloroamphinicole	30 mcg	47 mm	- - - - -
Spectinomycine	100 mcg	6 mm	+++++
Metronidazole	5 mcg	6 mm	+++++
Nitronidazole	10 mcg	20 mm	- -
Clindomycine	10 mcg	30 mm	- - -
Ampicilline	5 mcg	25 mm	- - -
Deoxycholine	10 mcg	6 mm	+++++

+: Resistance; +++, +++++: growth status; - : Susceptibility to antibiotics.

Identification of the microbe

The microbe isolated was sent to Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh India. Biochemical and morphological test were also conducted by them and also in our labs (table 4.1 to 4.4). Microbe identified is *Pseudomonas lini.*

Table 4: biochemical characterization of halo tolerant heavy metal resistant bacteria

Table 4.1: Colony morphology

Configuration	Circular
Margin	Entire
Elevation	Flat
Surface	Smooth
Color	Cream
Opacity	Opaque
Gram's reaction	Negative
Cell shape	Rod
Size	$0.8x2^{-3}$
Spore	No
Motility	No

Table 4.2: Biochemical test

MacConkey	Yes
Voges Proskauer Test	No
Citrate utilization	Yes
Dextrose (Acid production)	Yes
Starch hydrolysis	Yes
Oxidase test	Yes
Catalase test	Yes
Urea hydrolysis	No
Indole	Negative
Gelatine hydrolysis	No
H_2S production	No
Esculin hydrolysis	No
Ornithine decarboxylase	No
Lysine decarboxylase	No

Table 4.3: Utilization of different carbon and nitrogen sources

Carbon sources	Utilization	Nitrogen sources	Utilization
Maltose	Yes	Beef extract	Yes
Sucrose	Yes	Yeast extract	Yes
Galactose	Yes	Peptone	Yes
Fructose	Yes	Glutamine	Yes
Starch	Yes	Aspartic acid	Yes
Lactose	Yes	Leucine	Yes
Glycerol	Yes	Glycine	Yes

Table 4.4: Growth at different pHs and temperature:

Temperature (°C)	Growth	pH	Growth
4	No	4.0	No
10	No	5.0	Yes
15	No	6.0	Yes
20	No	7.0	Yes
25	No	8.0	Yes
30	Yes	9.0	Yes
37	Yes	10.0	Yes
40	Yes	11.0	Yes
42	No	12.0	Yes

CONCLUSION

During the present study focus was to isolate microbe which is heavy metal tolerant, high salinity tolerant, and also produce amylase enzymes and can with stand heavy temperature. The microbe isolated is resistant to nickel, copper, manganese, ferrous, lithium and zinc. Heavy metals are very important and essential trace elements according to various studies. And microbes present in the nature have direct or indirect interactions with the heavy metals. Developments of heavy metal resistance also have some health implications because microbes which are metal resistant and are also antibiotics resistant. Heavy metal pollution is affecting land, air and water. And on inhalation or in consumption in excess amount they are toxic to the living beings. Heavy metal resistant microorganisms can be used for the bioremediation of pollution caused by heavy metals.[3] Multiple metal resistant microbes have advantages over the single metal resistant microbes. Heavy metals and biocides are two components which itself are not as such antibiotics but they have antibiotics properties and so help in selection of the microbes which are antibiotics resistant too. This is because genes responsible for the metal resistance lie near to the genes responsible for the antibiotics resistance.[3] Experiments are underway to develop and integrated bioremediation system using some of the resistant microbes.

REFERENCES

1. Cheng S. Heavy metal pollution in china: Origin, pattern and control. Environmental sciences and pollution research. 2003; 10:192-198.
2. Patterson JW. Waste water treatment technology. Ann Arbor Science publishers, Ann Arbor, MI, USA. 1977.
3. Singh V, Chauhan PK, Kanta R, Dhewa T, Kumar V. Isolation and characterization of pseudomonas Resistant to heavy metals contaminants. 2010; 2 (30):164-167.
4. Burhan A, Nisa, U, Gökhan C, Ömer C, Ashabil A, Osman G. Enzymatic properties of a novel thermostable, thermophilic, alkalineand chelator resistant amylase from an alkaliphilic Bacillus Sp. Isolate ANT-6. *Proc Biochem* 2003; 38: 1397-1403.
5. Van der Maarel MJEC, Van der Veen B,Utdehaag JCM, Leenhuis H, Dijkhuizen L. Properties and applications of starch converting enzymes of the alpha amylase family. *J Biotechnol. 2002;* 94: 137-155.
6. Gupta R, Gigras P, Mohapatra H, Goswami,VK and Chauhan B. Microbial alpha amylases: a biotechnological perspective. *Proc biochem.* 2003; 38 : 1599 – 1616.
7. Sindhu GS, Sharma P, Chakrabarti T, Gupt JK. Strain improvement for the production of a thermostable α-amylase. *Enzy Microbio Technol 1997;* 24:584-589.
8. Rao MB, Tanksale AM, Gathe MS, Deshpande VV. Molecular and Biotechnological aspects of microbial proteases. *Microbio Mol Biol Rev*. 1998; 62(3): 597-635.

9. Poonam N, Dalel S. Enzyme and microbial systems involved in starch processing. *Enzyme Micro Technol*. 1995; 17:770-778.

10. Crab W, Mitchinson C. Enzymes involved in the processing of starch to sugars. *Trends Biotechnology* 1997;15: 349-352.

11. Sarikayle E, Migossa T, Adachi M, Mikami B. Comparison of degradation abilities of alpha and beta amylases on raw starch granules. *Proc Biochem* 2000;35: 711-715.

12. Pandey A, Nigam P, Soccol CR, Soccol VY, Singh D, Mohan R. Advances in microbial amylases. *Biotechnol Appl Biochem . 2000 ;*31: 135–152.

13. Rehman A, Zahoor A, Munner BV, Hasnain S. Chromium tolerance and reduction potential of a bacillus sp.ev3 isolated from metal contaminates was water. *Bulletin of Environmental and Contamination toxicology*. 2008; 81:25-29.

14. Silver S, Phung LT. Bacterial heavy metal resistance: new surprises. *Annu. Rev. Microbiol.* 1956; 50:753-89.

15. Costa M, Klein CB,. Nickle carcinogenesis, mutation, epigenetics, or selection. Environmen Heal. Perspec. PartA. 1999; 107; 438-439.

16. Chung J, Nerenberg R, Rittmann BE. Bioreduction of solution chromate using a hydrogel based membrane biofilm reactor. *Water Research*. 2006; 40:1634-1642.

17. Thacker U, Parikh R, Shouche Y, Madamwar D. Hesavalent chromium reduction by provideria sp. *Process Biochemistry*. 2006; 41:112-1337.

18. McCall KA, Huang CC, Fierke CA. Function and mechanism of zinc metalloenzymes. *J of Nutri*. 2000; 130:1437s-1446s

19. Joselow MM, Tobias ED, Kohler R, Coleman S, Bogden J, Gause D (1978) Manganese pollution in the city environment and its relationship to traffic density. *Ann. J Pubic Health* 68:557-560.

20. Singh V, Chauhan PK, Kanta R, Dhewa J, Kumar V. Isolation and characterization of Pseudomans resistant to heavy metals contaminants. *Int. J. Of Pharma Sci. Review and Res*. 2010.3(2):164-167.

****