Isolation and Identification of Heavy Metal Resistant Bacteria Producing Enzymes from Industrial, Laboratory and Dumping Sites Wastes in Mombasa County

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Abstract

Industrial, laboratory and dumping sites wastes are sources of heavy metal introducing health risks to humans. However, bacterial strains living in such environments have capacity for wider industrial applications, including, productions of enzymes for detergent industry. The focus of the study was to screen and isolate enzymes produced by heavy metal-resistant bacteria. Wastewaters were collected from industrial discharge points at Tudor creek (TD) and laboratory effluent (TUL) at Technical University of Mombasa. Soil samples were collected from damping site in Kongowea 1 (KG1), Kongowea 2 (KG2), Tudor ground (TG) and Tudor district hospital (TH) in Mombasa County. Bacteria strains were isolated using nutrient agar media and further sub-cultured to obtain pure colonies. Pure strains were screened for production of protease and amylase using lytic activity. Heavy metal tolerance test was used to screen for copper and mercury resistance to isolated bacteria. Microscopic and biochemical characteristics were used to identify bacteria with ability to grow in heavy metals and actively produce viable enzymes. Positive growth was realized in all sample collected from the six sites in varying concentration with copper plates recording the highest counts. Activity of microbial protease and amylase ranged from 0.1080±0.0025 in KG1 to 0.3703±0.0014 in TUL and 0.200±0.0745 in TUL 0.3613±0.0014 in TH respectively at a concentration of 2.5 mg/L of copper. At a concentration of 2.5 mg/L of mercury, activity of microbial protease and amylase ranged from 0.0113±0.0014 in TD to 0.2047±0.0014 in TH. There was no significant difference ($p < 0.05$) in microbial community and enzyme activity from the six sampling points. In all the sampling points, S. aureus, N. Veillonella, B. licheniformis, B. azotoformans, Streptococcus, Peptostreptococcus, Enterococcus and Acinetobacter/Moraxella were identified. The sites studied contained bacteria adapted to heavy metal pollution that can be natured to produce enzymes for situations demanding resistant enzymes such as detergent industries.

Keyword: Heavy Metal Resistance, Enzymes; Industrial Wastes, Dumping Sites.

Background Information

In view of science, life involves catabolism and anabolism. Catabolism results in breakdown of complex molecules, a process that involves enzymes such as α-amylases, which are glycoside hydrolases acting on α-1, 4-glycosidic bonds (Alyer, 2005). These enzymes are produced by a wide range of living organisms, from bacteria to plants and humans. Enzymes have wide range of applications, from brewing to food industries, detergent industries and pharmaceutical industries, as well as textile industries. Enzymes producing microorganisms include *Bacillus subtilis, Bacillus licheniformis, Bacillus steriothermophilus, Bacillus megaterium, Lactobacillus, Escherichia, Proteus, Strepotmyces sp.,* and *Pseudomonas spp.,* among others (Parmar et al., 2012).

Use of microorganisms for production of industrial enzymes is due to cost effectiveness, consistency, less time and space required for production (Sindu et al., 1997). Further, microorganisms have ability to produce enzymes in bulk and they can easily be manipulated for production of desired products. Replacement of chemicals with microbial enzymes in industrial hydrolysis, pharmaceutical and fine chemical industries (Rao et al., 1998), has led to its increased use. This has placed greater stress on increasing indigenous enzyme production and search for more efficient processes for their production (Lonsanc, 1990). Owing to introduction of biotechnology, use of enzymes has widened in today's world to various fields.

Human activity has had a direct influence in the natural process of these microbial communities in their natural environment. This impact is felt in their natural metabolic process that involve enzyme production. For instance, pollution of soil and waste waters with heavy metal from industrial and laboratory processes is a common significant environmental problem (Cheng, 2003). It hampers production of enzymes for application in industrial products, both consumable and economically important. Release of wastewater from industries and sewage sludge has permanent toxic effects to humans and the environment (Rehman et al., 2008).

Thus byproducts of industrial and laboratory processes pose considerable health risks to human beings, as well as being a big challenge the microbial community in the soil and wastewaters. Existence of heavy metals in the microbial environment hinders use of microorganisms in production of industrial enzymes and its application in food, pharmaceutical, textile and detergent industries. There is therefore need to enforce measures to monitor presence and concentration of heavy metals in wastewaters before discharging them into the environment, as mandated by the various environmental protection agencies. This study sought to screen and isolate enzymes produced by bacteria species living in selected industrial waste discharge ports of Mombasa County.

Materials and Methodology

Target Site

The research was conducted in Mombasa County in the coastal region of Kenya within coordinates of 4.0500^0S, 39.6667^0E. The county has a population of approximately one million according to Kenya population census records of 2009. The major economic activity in this region is tourism. This county has had a challenge of dumping garbage for quite some time till recently when the county government devised improved mechanism managing the garbage.

Sampling Techniques

Waste water samples were collected from industrial discharge points to the ocean (Tudor beach) and at a laboratory at Technical University of Mombasa. Soil samples were collected from three garbage points (Kongowea 1, Kongowea 2 and Tudor garbage) and one from hospital damping site (Tudor district hospital). Using a sterile spatula and transferred to sterile disposable plastic tubes in aseptic conditions. Discharged waste water sample was collected in sterile plastic containers. Samples were transferred to the Department of Pure and Applied Sciences laboratories at Technical University of Mombasa for analysis and at the Kenya bureau of standards Laboratories in Mombasa.

1) *Isolation of Bacterial Strains from Waste Water and Soil Samples*

Bacteria strains from waste water and soil samples were isolated as described by Yavuz, (2003). Briefly, 1 ml of waste water and 1g of soil sample were used. Each sample was mixed with 9 ml of sterilized distilled water and serially diluted up to 10^{-5}. The serial dilutions was introduced into a sterile petri plates using the pour plate method into nutrient agar media fortified with 2% starch, lipid, and protein in different petri dishes. Plates were incubated at 37°C for 24 hrs and bacterial isolates further sub-cultured to obtain pure colonies. Pure colonies on respective agar slants were maintained at 4°C.

2) *Screening for Enzyme Producing Bacteria*

Isolated pure strains were screened for production of extracellular enzymes using amyl lytic activity. Microbial isolates were streaked on starch agar plate (amylase), agar plates containing skim milk as substrate (protease) and tributyrin agar (lipase), and incubated at 37°C for 48 hours. For amylase, 1% iodine solution was flooded with a dropper for 30 seconds on starch agar plate. The isolates that produced a clear zones of hydrolysis were considered as amylase producers (Gupta et al., 2003), were subjected to further analysis. For protease, micro-organisms that produced a zone of clearance on the culture media were subjected to further studies.

3) *Isolation and Assay for Amylase Activity*

A suitable volume of isolated culture broth incubated for 48 hrs was centrifuged at 5000 rpm for 20 min at 4°C and supernatant collected. Amylase activity was determined by spectrophotometric method. 1 ml crude enzyme and 1 ml 1% soluble starch in sodium phosphate buffer (pH 7) were mixed in test tubes covered and incubate at 35°C for 10 min. 2 ml DNS reagent was added in each tube to stop the reaction and incubated further for 10 minutes in boiling water bath. After cooling to room temperature, the final volume was made to 10 ml with distilled water and absorbance will be read at 540 nm by spectrophotometer (Kim et al., 1995). A control containing all the reagents with exception of the crude enzyme was run. Absorbance obtained from the control was subtracted from the ones read from respective sample tubes to obtain corrected absorbance which was tabulated as shown in tables 1 and 2 below.

4) *Isolation and Assay for Protease Assay*

Proteolytic activity was carried out according to Casein-Pholine method (Boethling, 1975). Culture media was centrifuged at 7200 rpm for 10 min. and supernatant used as enzyme source. However, 1% casein (in 0.1 M phosphate buffer pH 7.0) was used as substrate. 1 ml each of enzyme and substrate were incubated at 50°C for 60 min. The reaction was terminated by adding 3 ml of Trichloroacetic acid (TCA). Centrifuged at 5000 rpm for 15 minutes. From this, 0.5 ml of supernatant was taken, and 2.5 ml of 0.5M sodium carbonate was added, mixed and incubated for 20 minutes, added to 0.5 ml of folin phenol reagent and absorbance read at 660 nm using Spectrophotometer. A control was ran with all the reagent except the supernatant for enzyme source. Absorbance from control was used to calculate for corrected absorbance.

5) *Heavy Metal Resistance Screening*

Heavy metal tolerance test was used to screen for heavy metal resistance of isolated strains of bacteria. Cells of overnight grown cultures of bacteria that expressed ability to produce enzymes were inoculated on nutrient agar plates supplemented with different concentrations (0.5, 1.0, 1.5 and 2.5 mM) of different salts of heavy metals (Mercuric II chloride and Copper II sulphate). Cultures were incubated at 37°C for 24 hours and cell growth observed. Concentrations were dispensed from 0.5 to 2.5ml

6) *Identification and Characterization of the Soil and Waste Water Bacteria*

Colonies that expressed ability to grow in this condition and actively produce viable enzyme were considered for identification. Isolates were observed under a microscope to obtain their colony morphology (color, shape, size, nature of colony and pigmentation (Dipali et al., 2003). The following biochemical tests were used for oxidase and catalase activity tests: Voges-Proskauer (VP) test, methyl red-Voges-Proskauer (MR-VP) test, starch hydrolysis and gelatin hydrolysis, motility, indole production and citrate utilization, growth at 7%, 8%, 10% concentration of NaCl, Gram staining and growth at 55°C as described in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) and identified by means of taxonomic schemes and descriptions (Buchenan et al., 1974).

Result

Colonies on each plate were counted using a colony counter and the average count for each set calculated and results tabulated in the table 1 below. The growth proved positive for the entire plate sample and a cross the concentration.

Table 1: The Average Score of Viable Colonies Counted on the Three Sets of Each Sample Plates

Plate Count Results

Mercury Plates	Copper Plates

	0.5	1.0	1.5	2.0	2.5	0.5	1.0	1.5	2.0	2.5
TH	103	72	61	43	35	>200	107	109	74	63
KG1	32	37	31	26	21	73	61	71	57	45
KG2	67	54	56	47	32	81	34	51	21	19
TG	71	74	70	41	36	67	61	72	62	47
TD	61	60	43	31	21	67	66	64	59	29
TUL	41	42	33	27	23	107	94	61	57	47

Copper plates had the highest plate count, mercury the lowest plate count. There was a general growth decrease in all plates with high hits being recorded in samples obtained from Tudor hospital. This might be due to the plasmid that confers antibiotic resistance.

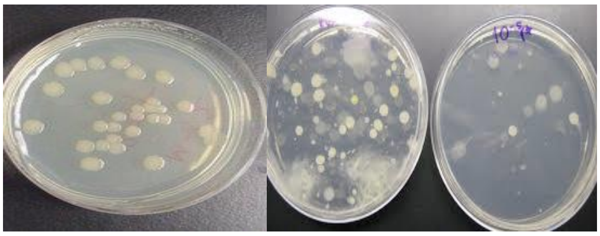

Figure 1: Microbes on Copper Plate Showing High Count and Low Count Plates in Mercury Plate

Table 2: Activity of Microbial Protease Produced Under Different Concentration of Copper (Mean±SEM)

Concentration of the Essayed Heavy Metals in the Test Samples

Crude enzymes were used to digest respective substrates and activity measured photo spectrometer. Along this a control was ran that contained all the reagents accept the crude enzyme. This was used to calculate for corrected absorbance. The obtained corrected absorbance were fed into excel and its Mean±SEM calculated for each set which was conducted in triplicate. Results of corrected absorbance are represented on tables 3 and 4 below.

Sample	**Concentration of Copper (mg/L)**				
	0.5	1.0	1.5	2.0	2.5
TH	0.4313±0.0014	0.3913±0.0014	0.3903±0.0014	0.2940±0.000	0.2047±0.0014
KG1	0.3187±0.0014	0.2967±0.0014	0.2713±0.0014	0.098±0.0014	0.0200±0.0025
KG2	0.2907±0.0087	0.1923±0.0014	0.1213±0.0014	0.1017±0.0029	0.0213±0.0025
TG	0.2073±0.0014	0.2073±0.0014	0.2057±0.0014	0.1760±0.000	0.0713±0.0014
TD	0.1923±0.0014	0.013±0.0014	0.1217±0.0029	0.0453±0.0014	0.0113±0.0014
TUL	0.3517±0.0029	0.2967±0.0014	0.2083±0.0014	0.1973±0.0014	0.0980±0.0025

Table 3: **Activity of Microbial Amylase Produced Under Different Concentration of Copper** (Mean±SEM)

SAMPLE	**Concentration of Copper (mg/l)**				
	0.5	1.0	1.5	2.0	2.5
TH	0.3947±0.0014	0.4013±0.0014	0.4190±0.000	0.3717±0.0014	0.3613±0.0014

Sample	**Concentration of copper (mg/L)**				
	0.5	1.0	1.5	2.0	2.5
TH	0.3907±0.00140	0.3927±0.0014	0.4033±0.0143	0.3673±0.1040	0.2010±0.000
KG1	0.3800±0.0025	0.3973±0.0014	0.391±0.000	0.2133±0.0143	0.1080±0.0025
KG2	0.394±0.000	0.3973±0.00014	0.3980±0.0025	0.3037±0.0137	0.2763±0.0014
TG	0.2963±0.0014	0.2970±0.0025	0.2987±0.0014	0.2917±0.0014	0.2817±0.0029
TD	0.03967±0.0014	0.3983±0.0014	0.3907±0.0014	0.3807±0.0014	0.3010±0.0025
TUL	0.3707±0.0014	0.3613±0.0052	0.3713±0.0029	0.3713±0.0014	0.3703±0.0014
KG1	0.3863±0.00014	0.3867±0.0014	0.3910±0.000	0.3183±0.0029	0.2803±0.0014
KG2	0.3673±0.0014	0.3553±0.0014	0.3680±0.000	0.3610±0.0025	0.3110±0.0025
TG	0.3753±0.0014	0.3780±0.0025	0.3770±0.00	0.2913±0.0014	002007±0.0014
TD	0.3907±0.0014	0.3980±0.0025	0.4013±0.0014	0.3203±0.0014	0.2933±0.0143
TUL	0.3907±0.0014	0.3914±0.0014	0.4027±0.0160	0.2810±0.0025	0.200±0.0745

Table 4: Activity of Microbial Protease Produced Under Different Concentration of Mercury (Mean±SEM)

Table 5: Activity of Microbial Amylase Produced Under Different Concentration of Mercury (Mean±SEM)

B. **Sample**	C. **Concentration of Copper (mg/L)**				
	D. **0.5**	**E. 1.0**	**F. 1.5**	**G. 2.0**	**H. 2.5**
I. TH	J. 0.4083±0.0180	K. 0.3917±0.0014	L. 0.3817±0.0038	M. 0.3013±0.0014	N. 0.2940±0.0151
O. KG1	P. 0.2907±0.0014	Q. 0.2907±0.0014	R. 0.2810±0.0050	S. 0.1023±0.0014	T. 0.0300±0.0025
U. KG2	V. 0.2940±0.000	W. 0.2003±0.0014	X. 0.2770±0.0043	Y. 0.0973±0.0014	Z. 0.0013±0.0014
AA. TG	BB. 0.2027±0.0038	CC. 0.2003±0.0029	DD. 0.2007±0.0014	EE. 0.0117±0.0014	FF. 0.0900±0.0248

GG. TD	HH. 0.3960±0.000	II. 0.2713±0.0029	JJ. 0.1633±0.0143	KK. 0.0407±0.0014	LL. 0.0214±0.0016
MM. TUL	NN. 0.3410±0.000	OO. 0.2917±0.0014	PP. 0.3010±0.0025	QQ. 0.1917±0.0014	RR. 0.0642±0.2058

Identification of the Microbes

Using the bar, the following microorganisms were able to be identified. The group (Streptococcus/Peptostreptococcus/Enterococcus) was the most dominant, *B. licheniformis* and *B. azotoformans* recoding the least dominant.

The following colonies were successfully characterizing

Table 6: Spread of Identified Microbes in the Respective Samples

	Sample					
Microbes	**Th**	**Kg1**	**Kg2**	**Tg**	**Td**	**Tul**
Staphylococcus aureus	+	+	+	-	-	+
Acinetobacter/Moraxella	-	-	+	+	+	+
Neisseria Veillonella	+	-	+	+	-	-
B. licheniformis	+	-	-	+	-	-
Streptococcus/Peptostreptococcus/ Enterococcus	+	+	+	+	+	+
B. azotoformans	-	-	+	+	-	-

Discussion, Conclusion and Recommendations

Discussion

Microorganisms exposed to heavy metal stress are vulnerable to toxic conditions caused by reactive oxygen species. The aim of this study was to investigate the tolerance of stressed bacteria and detect the ability of some resistant bacterial strains to produce antioxidant enzymes under heavy metal stress. The activities of antioxidant enzymes produced in the resistant bacterial strains were measured using a spectrophotometer. Results showed that of the two metals used, Hg was more toxic from plate count to enzymatic activity. Antioxidant enzymes can be used to biologically monitor heavy metal pollution. This is well explained from the results obtained from analysis of the samples for heavy metal. It shows that most of the samples obtained contain microorganisms adapted more to copper compared to mercury.

Analysis of results further revealed a gradual increase in enzymatic activity from concentration 0.5 to 1.0 or 1.5 then a drop as concentration rises through 2.0 to 2.5. This show that these microbes can tolerate certain concentration of heavy metal before the later turns to toxic concentration and hinder normal microbial physiological activity. These metals play an important role in the microbial activity probably by acting as co-factors.

An interesting outcome was experienced with the sample TH. This sample showed high degree of activity on both metals. Activity of enzyme isolated from microbes of this sample, had high tolerance to high concentration of metal. Considering the plate count results, there was relatively high growth in plates containing this sample in comparison to the other plates. This is might be linked to the point that bacterial resistance to heavy metals is determined by plasmids, which in many instances also encode resistance to antibiotics (Izaki, 1977). The plasmid-determined nature of resistance to heavy metal compounds was established by deter mining the ability for cotransduction (with other plasmid- encoded determinants) and high frequency of conjugal transfer of the Hg determinant (Summers et al. 1978). Isolation of covalently closed circular DNA from the Hg strains and its ability to transform Hgs recipients to the Hgr phenotype provided further evidence that the Hgr determinant is plasmid encoded, as did curing Hg strains with agents such as ethyl methanesulfonate (Nakahara et al., 1977). The relationship between resistance to mercury and other heavy metals and antibiotics in the hospital environment has been explored in numerous studies. There thus appears to be a strong correlation between antibiotic resistance and resistance to mercury and several other metals (Novick, et al., 1968). In most instances, the frequency of heavy-metal resistance is the same as or higher than that of antibiotic resistance (Kondo et al., 1974.).

From the anova analysis, the p values were less than 0.5. This implies that there were no statistically significant differences between group means as determined by one-way ANOVA. The activity of microbes obtained from the six sampling points is relatively the same. This serves two sets of information; thus there is equal distribution of industrial microorganism across the sampling points and there is relatively equal rate of pollution among the six sampling points.

Conclusion

Result of this study show that the immediate environment contains industrial microorganisms that are well adapted to functioning in environment polluted with heavy metal. These environs may be important source for microorganisms that can be natured to produce enzymes that function well in situations demanding heavy metal resistant enzymes such as detergent industries.

Recommendations

Feature studies should focus on shading light on the composition and stability of these microorganisms. As well studies focusing on purification and titre increment of the enzyme produced by these microorganisms should be conducted in order to facilitate full scale implementation of the project. On the other hand, regular studies into rate of pollution into the environment should be monitored to guarantee the public safety.

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