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Isolation, identification and characterization of microbes capable for bio remedial activities isolated from Jharia coalfield, Jharkhand

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Abstract
The present study focused on identifying heavy metal degrading bacteria in soil sample collected from jharia coalfield sites Jharkhand. For isolation of bacteria from the soil sample was serially diluted tenfold; the highest dilution (10^{-6}) sample was inoculated onto BBH agar plates and incubated at 37°C for 24 hrs. Colonies with diverse morphological features from the mixed cultures were sub cultured in order to obtain pure colonies. These colonies were then grown on nutrient agar containing diverse molarities of salts of heavy metals (Pb, Cu, Zn and Mn). The highest concentration of each heavy metal growth was observed were as follows: Zn-8mM, Cu-4mM, Pb-6mM and Mn-4mM. The bacteria having multiple tolerances were identified as *Bacillus spp.* on the basis of colony morphology, microscopic examination and biochemical tests. *Bacillus licheniformis* was identified through FAME GC. The tolerant isolate was further subjected to *in vitro* antibiotic cultural sensitivity assay and were found resistant to multiple antibiotics. The study is of significance in that such bacteria can be utilized for bioremediation of heavy metals in the environment particularly in the treatment of heavy metal contaminated soil.

Keywords: *Bacillus licheniformis*, bioremediation, antibiotic, heavy metal

Introduction

Industrial wastewater is a chief source of heavy metal pollution in environment. Heavy metals are economic importance in industrial utilize as well as the largest part of pollutants in the environment. Heavy metals have become a serious hazard to living organisms in environment (C. Su, 2014) [3]. Metal toxicity is large environmental distress because of their nonbiodegradability and bioaccumulation in nature (R. K. Gautam *et al*., 2014) [7]. A number of inorganic metals such as nickel (Ni), magnesium (Mg), chromium (Cr3+), copper (Cu), manganese (Mn), calcium (Ca), sodium (Na) and zinc (Zn) are essential elements required in tiny number for redox and metabolic functions. Heavy metals like lead (Pb), aluminium (Al), cadmium (Cd), mercury (Hg), gold(Au) as well as silver (Ag) do not have any toxic and biological role to living organisms (R. Turpeinen *et al*.,2002) [8].

Bioremediation is engaged in categorize to convert toxic heavy metals into a less harmful condition via microbes or its enzymes to clear out polluted environment (A. Akcil *et al*., 2015) [1]. Bioremediation is cost effective and environmentally friendly in the renewal of the environment (Y.Ma,M *et al*., 2016) [12]. Bioremediation of heavy metals has some limits. These are production of lethal metabolites by nonbiodegradability and microbes of heavy metals. The straight use of microorganisms with characteristic of catabolic potential and their products like bio surfactant and enzymes is a new approach to increase and enhance their remediation efficacy (T. T. Le *et al*., 2017) [9]. Dissimilar alternatives have also been probable to enlarge the microbiological techniques towards the remediation of heavy metals. Bio film mediated bioremediation could be useful for cleaning up of heavy metal infected environment.

Microbial-metal communications is mainly paying attention on metals elimination such as depollution and remediation. The current stimulation of the utilize solid-state electrodes as electron acceptors and donors for microbial growth has brought modern scenario, resulting to microbial-electrochemical technologies (METs) (J. C. Thrash and J. D. Coates, 2008) [4].

Microorganisms used as a green approach for the production of metallic nanoparticles (NPs) have been reported (T. Klaus-Joerger *et al*., 2001) [10]. Hereditarily modified microorganisms have also been using for remediation technique (V. Paliwal *et al*., 2012) [11]. Chemical modification and Genetic engineering could modify the mechanism of cells surface and could professionally progress the adsorption ability as well as selectivity to target-metal species. Numerous factors which limit and influences bioremediation efficiency consist of redox, temperature, nutritional status, pH, chemical composition and potential moisture of heavy metals (P. K. Shukla, *et al*., 2013) [5]. Microbes alone have exposed imperfect efficiency remaining to different factors together with excessive and poor competitiveness heavy metal concentrations. Effectiveness could be improved by numerous amendments with biosurfactants, inorganic nutrients, bulking agents, compost and biochar (A. Wiszniewska *et al*., 2016) [2].

Therefore, the main goal of this study was to get a strain Collection of heavy metal degrading bacteria able to apply wonderful degradation possible against high levels of heavy metal pollutants. Furthermore, our aim was to determine the heavy metal tolerance and antibiotic resistance of isolated and identified heavy metal-degrading bacterial strains.

Materials and Methodology

Soil sampling

Soil samples were collected from different areas of Jharia coalfield Jharkhand. Samples were collected during the month of June. The top 15 cm of soil was collected into sterile plastic bags for microbiological analysis. Samples were stored at 4^0C until further processing.

Purification and culturing of heavy metal degrading bacteria

Heavy metal degrading bacteria were isolated using enrichment containing: 40 ml BBH mineral broth medium supplemented with 0.1mM heavy metals and 4.0 g of contaminated soil sediment. And incubation at 32^0C for 2 weeks, the enriched cultures were serially diluted and inoculated onto BBH agar plates. Colonies with diverse morphologies were preferred as candidate heavy metal degrading strains and were maintained on standard Nutrient Agar (Hi Media).

Qualitative analysis (Dye method)

10 isolates were checked for the degradation of heavy metals. The setup was prepared in 250 ml sterilized conical flasks. Solid heavy metals (10 g/l) were dissolved in dichloromethane and filtered sterilized through 0.22 µm membrane. 2 ml of solution at a final concentration of 100 ppm were added in different conical flasks and left open in sterilized conditions for the solvent gets evaporated. Than 100 ml of BBH media with trace elements and 100 µl of resazurin was added in the flasks. 500 µl of bacterial culture was added (OD600=0.5) in BBH broth in the flasks and kept in shaking incubator at 120 rpm and 30^0C. Solvent, media and resazurin was used as control 1 and flask with inoculant and media was used as control 2.

Resazurin, which is also preferred to as Alamar blue. Alamar blue is a non-toxic dye condensed intracellular to resofurin through enzymes in the electron transport system. In the beginning a blue coloured resazurin is change into a pink colored resofurin through the loss one atom of oxygen. In second stage of reduction the pink colored resofurin is further more reduced to colorless hydroresofurin

Calculations

Formula used to calculate the residual concentration and % degradation are given below:

Residual concentration (in ppm)

$$= \frac{\text{Sample Area}}{\text{Standard Area}} X \frac{\text{Amount of std. injected (ng)}}{\text{Vol. of sample taken (ml)}} X \frac{\text{Sample make up volume}}{\text{Volume of sample injected}} X \text{ DF}$$

$$\%\text{Degradation} = \frac{\text{Initial Concentration} - \text{Residual concentration}}{\text{Initial Concentration}} X\ 100$$

Fame GC Analysis

The Sherlock TM Chromatographic Analysis System (CAS) automatically identifies fatty acid methyl esters (FAMEs) by gas chromatography. The FAME profile can be used for fatty acid compound identification (marine oil analysis, PLFA analysis), or the entire fatty acid profile can be compared by pattern recognition to one of Sherlock's microbial libraries (e.g. environmental aerobic bacteria).

Libraries & Methods

The primary Sherlock methods and libraries available include:

Fatty Acid Compound Naming and Quantitation

- Microbial Community Analysis (PLFA) - 150 soil phospholipid fatty acids
- Marine Oil Analysis (MARINE) - 147 marine fatty acids, includingOmega-3s, Omega-6s and Omega-9s

Sample Preparation

With inexpensive reagents, available from almost any chemical supply house, a technician averages 5 minutes per sample to prepare a batch of 30 samples. Each sample is prepared for analysis using a liquid-liquid extraction in a single test tube.

1. Harvesting a small quantity (~20mg cells) from the culture plate is the most labor-intensive step. It will typically take 1 hour or less to harvest cells from 30 plates into 30 test tubes.
2. The four-step liquid-liquid extraction process requires about 1½ hours or less for a batch of 30 samples. During the extraction process, there are approximately 35 minutes of "wait time" available for the technician to do paper work and other tasks.
3. The sample preparation is the same, regardless of microbial type. It is not necessary to do a Gram stain or other offline tests before preparing and analyzing a sample.

Gases

The Sherlock requires a specific type and quality of gases to function properly:

Carrier Gas

1. Hydrogen, 99.999%+, 150 cc/min+
2. Note: Helium cannot be used

Makeup Gas

1. Nitrogen, 99.999%+
2. Industrial Grade Air, < 1ppm THC

Testing heavy metal and antibiotic resistance of strains

Heavy metal resistance of strains was tested in nutrient broth tubes containing different concentrations (0.25, 0.5, 1.0, 2.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mm) of CuSO4.5H2O, Pb (NO3)2, ZnSO4.7H2O, MnSO4.H2O, C8H4K2O12Sb2.3H2O, SnCl2.2H2O (Hi Media). Stock solutions were prepared in deionized water and were also filter sterilized before inoculation. All tests were performed in triplicates and the results were evaluated visually for growth against heavy-metal free control cultures and blank tubes.

Antibiotics sensitivity of selected strains was performed by the Kirby Bauer's disc diffusion method using Muellere Hinton agar (Bauer *et al*., 1996; CLSI, 2013). Cephalosporins (cefuroxime

sodium-CXM30, cefotaxime-CTX30, cefoperazone-CFP75), penicillins (piperacillin-PRL100, penicillin G-10U, amoxyclav-30), tetracyclines (tigecycline-TGC15), carbapenems (imipenem-IPM10), quinolones (norfloxacin-NOR10). All the experiments were performed in triplicates.

Result

Purification and culturing of heavy metal degrading bacteria

5 diverse appearing colonies were recognized which were maintained in Agar slants after that enriching them from heavy metal contaminated soil samples.

Fig 1: Bacterial cultures isolated from soil sample.

Table 1: Morphological Characteristics of Isolates

Characteristics	JCJ-1	JCJ-2	JCJ-3	JCJ-4	JCJ-5
Colonial characteristics	Circular, white	Circular, white	Circular, white	Circular, white	Circular, white
Gram's reaction	+	+	-	-	+
Shape	Circular	Rod	Rod	Circular	Filamentous
Catalase	+	+	+	-	+
Oxidase	+	+	+	+	+
Methyl Red	-	-	-	-	-
Voges roskauer	+	+	+	-	-
Indole	-	-	-	-	-
Citrate	-	+	+	+	+

Qualitative degradation of the isolates (Dye method)

The heavy metal degrading of the 5 isolates by dye method is presented below tables. *Bacillus spp.* showed greatest microbial activity by dropping resazurin to pink colored resofurin. This one isolate was further applied for molecular identification, quantitative heavy metal degradation, antibiotic as well as heavy metal resistance.

Fig 2: Resazurin (purple) and resorfurin (pink) formation

Table 2: Qualitative degradation against Pb

SN.	Bacterial species	Sorption Efficiency (%)
1	Micrococcus	10.11
2	Bacillus	78.32
3	Pseudomonas	78.9
4	Staphylococcus	18.9
5	streptomyces	29.5

Table 3: Qualitative degradation against Cu

SN.	Bacterial species	Sorption Efficiency (%)
1	Micrococcus	35.78
2	Bacillus	67.33
3	Pseudomonas	67.33
4	Staphylococcus	76.8
5	streptomyces	29.5

Table 4: Qualitative degradation against Zn

SN.	Bacterial species	Sorption Efficiency (%)
1	Micrococcus	2.11
2	Bacillus	59.8
3	Pseudomonas	46.9
4	Staphylococcus	1.11
5	Streptomyces	11.12

Table 5: Qualitative degradation against Cr

SN.	Bacterial species	Sorption Efficiency (%)
1	Micrococcus	38.78
2	Bacillus	49.2
3	Pseudomonas	98.5
4	Staphylococcus	46.2
5	Streptomyces	73.1

Identification of the bacterial strain

The unknown bacterial culture was identified as *Bacillus-licheniformis* through FAME GC (Table 6).

Table 6: *Bacillus-licheniformis*

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.7599	1.59E+6	0.006	----	6.6052		----	< min rt	
0.7733	1.411E+9	0.021	----	6.6907	SOLVENT PEAK	----	< min rt	
1.0041	856	0.009	----	8.1775		----	< min rt	
1.1015	842	0.015	----	8.7922		----	< min rt	
1.2309	369	0.008	1.173	9.6099	10:0 iso	0.10	ECL deviates -0.006	Reference -0.014
1.4284	2065	0.010	----	10.6805		----		
1.5016	738	0.012	----	11.0392		----		
1.7369	7435	0.009	1.045	12.0139	12:0	1.84	ECL deviates 0.014	Reference 0.017
1.9185	4505	0.011	----	12.6744		----		
2.1970	4450	0.009	0.989	13.6286	14:0 iso	1.04	ECL deviates 0.001	Reference 0.006
2.3084	2537	0.009	0.979	13.9996	14:0	0.59	ECL deviates 0.000	Reference 0.006

2.5078	115394	0.009	0.964	14.6307	15:0 iso	26.32	ECL deviates -0.001	Reference 0.005
2.5372	161683	0.009	0.962	14.7238	15:0 anteiso	36.81	ECL deviates -0.001	Reference 0.005
2.6230	1194	0.012	----	14.9952	15:0	----	ECL deviates -0.005	
2.7576	3433	0.010	0.950	15.4111	16:1 w7c alcohol	0.77	ECL deviates -0.003	
2.8282	19649	0.009	0.946	15.6292	16:0 iso	4.40	ECL deviates -0.004	Reference 0.001
2.8765	4104	0.011	0.944	15.7785	16:1 w11c	0.92	ECL deviates -0.003	
2.9465	14037	0.009	0.941	15.9950	16:0	3.13	ECL deviates -0.005	Reference -0.001
3.0285	565	0.012	0.938	16.2490	15:0 2OH	0.13	ECL deviates -0.006	
3.0647	627	0.010	----	16.3612		----		
3.0815	4097	0.009	0.936	16.4133	17:1 iso w10c	0.91	ECL deviates -0.001	
3.1124	5188	0.010	0.935	16.5090	Sum In Feature 4	1.15	ECL deviates -0.003	17:1 anteiso B/iso I
3.1264	2870	0.009	0.935	16.5524	17:1 anteiso w9c	0.64	ECL deviates 0.000	
3.1532	30805	0.009	0.934	16.6354	17:0 iso	6.81	ECL deviates -0.002	Reference 0.001
3.1846	58557	0.009	0.933	16.7327	17:0 anteiso	12.93	ECL deviates 0.000	Reference 0.002
3.4742	411	0.009	0.927	17.6350	18:0 iso	0.09	ECL deviates -0.001	Reference -0.001
3.5258	2702	0.014	0.926	17.7960	18:1 w9c	0.59	ECL deviates 0.002	
3.5920	1324	0.011	0.926	18.0026	18:0	0.29	ECL deviates 0.003	Reference 0.001
3.6528	884	0.012	0.925	18.1969	17:0 iso 3OH	0.19	ECL deviates 0.004	
3.6876	1915	0.014	----	18.3082		----		
3.7695	597	0.013	0.924	18.5702	17:0 3OH	0.13	ECL deviates 0.005	
3.7996	2232	0.012	----	18.6663		----		
3.8231	1049	0.015	0.924	18.7415	19:0 anteiso	0.23	ECL deviates 0.004	Reference -0.002
4.0129	985	0.024	----	19.3574		----		
----	5188	---	----	----	Summed Feature 4	1.15	17:1 iso I/anteiso B	17:1 anteiso B/iso I

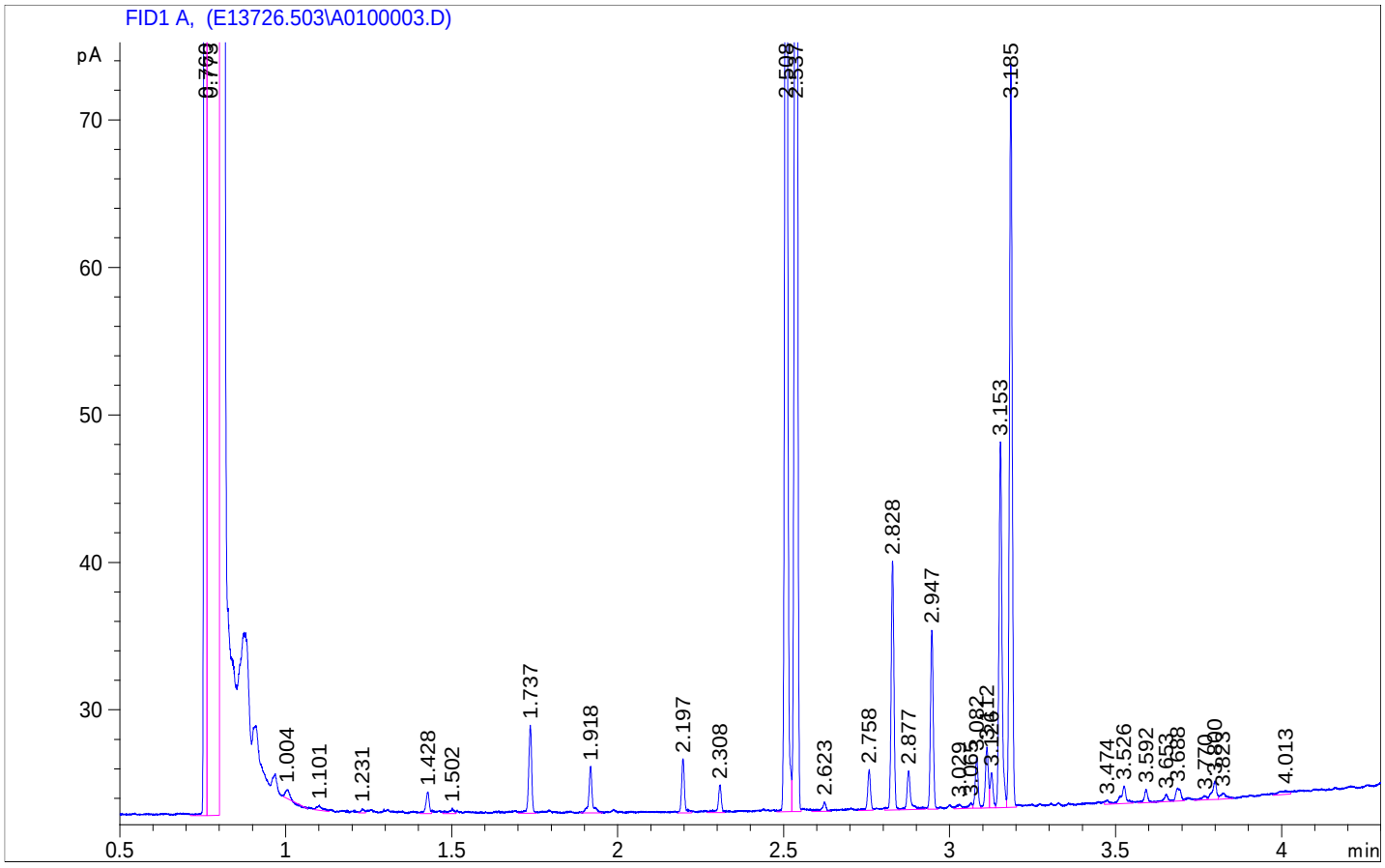

Fig 3: Chromatogram of *Bacillus-licheniformis*

Heavy metal resistance of identified bacteria

Maximum tolerance was shown by *Bacillus licheniformis* against (15 mM) and least tolerance was shown by four bacterial species except *B. licheniformis* MTC values of 0 mM for Stannous Chloride. *Staphylococcus spp.* showed least tolerance against Cu+ (0.25 mM) but was able to tolerate 10 mM of Mn2+.

Table7: Maximum tolerated concentration (MTC) values of tested heavy metals

Strains	MTC values of heavy metals (mm)					
	Lead Nitrate (PbNO3)	Copper Sulphate (CuSO4.5H2O)	Stannous Chloride (SnCl2.2H2O)	Zinc Sulphate (ZnSO4.7H20)	Manganese Sulphate (MnSO4H2O)	Potassium Antimony Tartarate (C8H4K2O12Sb2.3H2O)
Micrococcus	5	3	3	5	9	6
Bacillus	7	4	4	8	11	16
Pseudomonas	6	4	4	5	3	5
Staphylococcus	6	4	4	6	8	6
Streptomyces	5	5	4	5	6	7

Antibiotic resistance of strains

All identified bacteria showed resistance against penicillin G and cefuroxime, impenem, amoxyclav. *B. licheniformis* showed resistance against 4 out of 6 antibiotics and the other four (Micrococcus, Pseudomonas, Staphylococcus, Streptomyces) were resistant only to 2 out of 6 antibiotics used (Table 8).

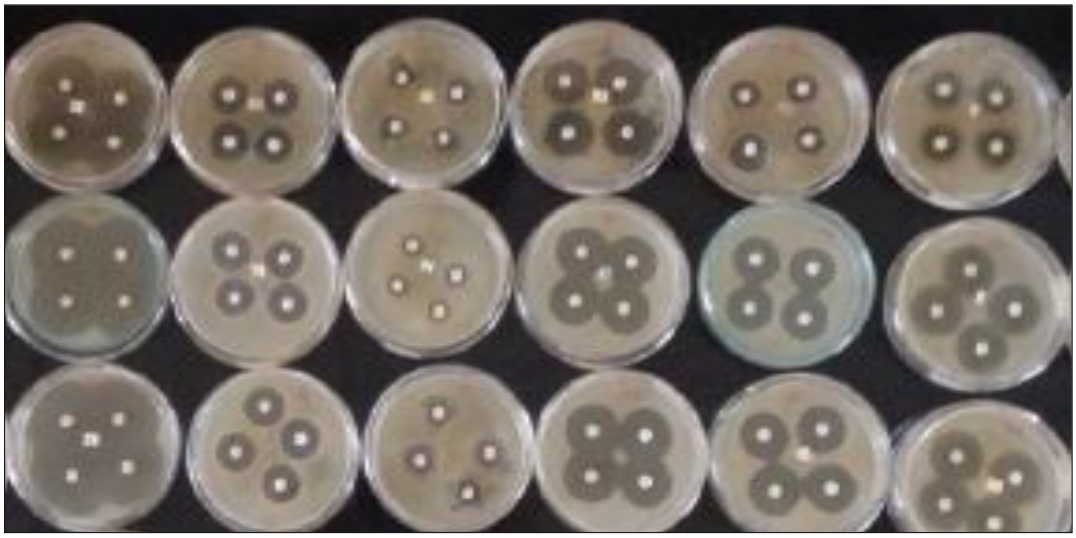

Fig 4: Antibiotic sensitivity pattern of the *Bacillus licheniformis.*

Table 8: Susceptibility of identified strains against antibiotics

Antibiotic & concentration →	Penicillin G	Piperacillin	Norfloxacin	Imipenem	Cefotaxime	Amoxyclav
Strains ↓						
Micrococcus	9.3 ± 0.57	10.3 ± 0.56	7.3 ± 0.55	13 ± 0	09.6 ± 0.58	15.3 ± 0.58
Bacillus	25 ± 0.1	25.4 ± 0.5	28.6 ± 0.58	40.3 ±0.58	26 ± 1	30.6 ± 0.58
Pseudomonas	13.6 ± 0.58	14.3 ± 0.58	16.6 ± 0.58	40.3 ±1.15	22.3 ±0.58	29 ± 1
Staphylococcus	2.6 ± 0.58	7.6 ± 0.58	2.6 ± 0.58	4.6 ±0.58	15 ± 0	15 ± 0
Streptomyces	2.3 ± 1.1	2 ± 0	3.3 ± 0.58	4 ± 0	2± 1	3 ± 0

Conclusion

The present study was of the bacterial bioremediation characters and their heavy metal and antibiotic tolerance from the heavy metal contaminated soil located in Jharia Coalfield Jharkhand, India. Mainly strong strains characterized were predominantly from the *Bacillus licheniformis* which showed mixed resistance against antibiotics and heavy metals. Highest degradation was observed by *B. licheniformis. Bacillus licheniformis* strain showed resistance against most of heavy metals and the most active bacteria to degrade the selected heavy metals. All the isolates except *Bacillus licheniformis* were resistance against 4 antibiotics out of 6 antibiotics and other bacterial *spp.* showed less sensitive to antibiotic resistance.

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