

Characterization and Potential Applications of Heterotrophic Bacteria Inhabit Nickel Rich Soils in Çanakkale, Turkey*

Furkan Öztürk¹ , Nurcihan Hacıoğlu Doğru² 



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¹Çanakkale Onsekiz Mart University, Faculty of Arts and Sciences, Department of Biology, Çanakkale, Turkey

ORCID: F.Ö. 0000-0002-9752-0584;
N.H.D. 0000-0002-5812-9398

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Correspondence: Nurcihan Hacıoğlu Doğru
nurcihan.n@gmail.com

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Introduction

Heavy metals which are the most important pollutant sources in terms of soil pollution. They mixed and accumulated in soils led to many environmental and human health problems ranging from microbial activity, soil fertility, biodiversity and yield losses of products, food chain through to poisoning (Özay & Mammadov, 2013). Nickel (Ni) metal is one of these chemicals; it is classified by the Environmental Protection Agency (EPA), as one of 129 important pollutants and 14

Abstract

Microorganisms inhabit extreme environments such as high nickel rich soils are novel in terms of diversity and also valuable source of extracellular hydrolytic enzymes. The present study focused on isolation and characterization of heterotrophic bacteria from Nickel rich soils in Çanakkale, using culture dependent method and assessment of their heavy metal, antibiotic resistance and potential for production of some industrially important enzymes. Total 35 bacterial isolates were characterized morphologically, biochemically and these analysis of strains revealed that these strains were able to grow between 4-50 °C. These isolates also showed high heavy metal and antibiotic resistance and ability to produce one or more extracellular enzymes like amylase, protease, lipase and DNase. Thus, the isolates from Ezine, Çanakkale could be potential candidates for industrial applications.

Keywords: Soil bacteria, Nickel, Heavy metal, Antimicrobial sensivity, Enzymatic activity

important toxic heavy metals (EPA, 2002). In addition, nickel is considered to be one of the 25 important compounds which are poisonous to human health.

Ni-containing plants are called high-level metal accumulators (hyper accumulators). This term represents a concentration 100 times higher than the maximum expected for non-accumulating species grown in serpentines (Brooks, 2000). More than half *Alyssum* taxa of the Flora of Turkey, has high levels of heavy metal accumulation properties. Some of the regions in the Ezine district of Çanakkale are very important areas for the development of

the *Alyssum pinifolium* (Nyar, T.R. Dudley) plant. As a result of the soil analyzes performed by Esen (2016), the structure of the soil of the Ezine Road is fertile, the salinity 0.33 dS/m (without salt), the pH 7.52 (slightly alkaline), the lime 0.81 % (low), the organic matter 0.85 %. (low), phosphorus 3.99 kg/da (low), potassium 13.95 kg/da (low), calcium 810 ppm (low), magnesium 619 ppm (medium), sodium 399 ppm (high), iron 7.62 ppm (very high), copper 0.22 ppm (low), manganese 5.44 ppm (medium), zinc 0.17 ppm (low), and nickel 1702 mg/g (very high) was determined. In addition, the amount of nickel collected in the plant samples obtained from the *A. pinifolium* distribution areas was analyzed: 1781 mg/g in the Ezine road population (Esen, 2016).

It is possible to develop new technologies from these adapted organisms by reducing the amount of nickel in soil and water, especially by understanding the metabolic activities of nickel-related microorganisms. Therefore, it is important to know the microbial diversity in these areas. In this study, it was aimed to determine the bacterial diversity in nickel-containing *A. pinifolium* soils and to determine some biochemical properties, environmental requirements and antibiotic-heavy metal resistance and enzymatic activities of isolates that could be used in possible Ni bioremediation.

Material and Methods

Site description and sample collection

The region which covers about 3208 m² and found species of *A. pinifolium* in slope with serpentine soil (39° 52'N and 26° 19'E) by the highway where locate 6 km North away from Ezine district of Çanakkale province (Table 1). Appointed soil samples were collected from 10 cm depth of soil surface with the help of sterile spatula from different five locations that *A. pinifolium* exists and transferred into sterilized polythene bags and transported aseptically to laboratory.

Isolation and purification of bacterial isolates

10 g of collected soil samples were suspended in 90 g distilled water 0.90% and the suspension were made 10⁻⁵ fold serial dilution. 100 µL of each fold was transferred to appropriate growth medium. Nutrient Agar and Tryptic Soya Agar were used for bacterial isolation. The plates were incubated at 35 ± 2°C for 24 h (Tamer et al., 1989). The colonies grown on the plates were purified by successive streaking on nutrient agar plates. Isolated bacterial strains were then stored at 4°C in refrigerator for further study.

Biochemical characterization

The pure cultures were obtained. The appearance of colony such as shape, color was observed by magnifying lens (10X) with the simple strain technique after the growth of isolated strains. Gram staining process was performed according to the method determined by Bozkurt (2016). A series of basic biochemical tests were performed with oxidase, catalase, indole, citrate, voges proskauer (VP), methyl red (MR) test and Kilger's iron agar (KIA) test (Tamer et al., 1989).

Determination of physiological growth characteristics of isolates

Isolates transferred to calibrated medium for determining the appropriate growth requirement such as pH, temperature, salt, using carbon resource by using culture dependent method as described previously by Bozkurt (2016). The effect of pH on growth of isolates was tested with the pH range from 5.0 to 11.0. The temperature range for optimum growth was determined by incubating the isolates from 4 to 50°C (Bozkurt, 2016). The effect of the salt on growth of isolates was tested with the salt range from 2.0 to 23.0% (Karaboz & Ozcan, 2005).

Table 1. Location, altitude and coordinates of soil samples.

Samples	Location	Altitude	Coordinates
I	Ezine/Çanakkale (Near of Araplar strait)	111 m	39.873416 N 26.323919 E
II	Sarımsaklı bridge, Kendirlik site	55 m	39.840276 N 26.320107 E
III	Araplar strait, Ahlatlı site	50 m	39.853860 N 26.318425 E
IV	Menderes Mountain	273 m	39.960355 N 26.374708 E
V	Ovacık, Küçük Uludağ	423 m	39.982499 N 26.416317 E

Antibiotic sensitivity testing

Susceptibility testing was performed by an agar diffusion method (Bauer et al., 1966), using Mueller–Hinton Agar (Oxoid) and 15 antibiotic discs: Sulfamethoxazole (SMZ100), Oxytetracycline (T30), Cephotaxime (CE30), Cefoxitin (CN30), Trimethaprim (TR10), Cephalothin (CH30), Chloramphenicol (C30), Kanamycin (K30), Furazolidone (FR50), Cefmetazole (CMZ30), Tobramycin (TB10), Erythromycin (E15), Ampicillin (A10), Gentamicin (G120), Amoxicillin (AX25). The isolates were determined to be sensitive to antibiotics according to the information supplied by the manufacturer (NCCLS, 2007). Reference strain of *Escherichia coli* ATCC 11230, were used as control organisms for verification of the antibacterial effect of the discs.

Determination of the MIC of heavy metals

The minimal inhibitory concentration (MIC) for each bacterial isolate for seven heavy metals was determined by using Mueller–Hinton Agar which is containing Cd^{2+} , Cr^{3+} , Cu^{2+} , Ni^{2+} , Mn^{2+} , Pb^{2+} , and Zn^{2+} at concentrations ranging from 100 to 12800 $\mu\text{g}/\text{mL}$. The metals were added as $\text{CdCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{K}_2\text{Cr}_2\text{O}_7$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{Ni}(\text{CO})_4$, $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{Pb}(\text{NO}_3)_2$ and ZnCl_2 . The isolates were considered resistant if the MIC values exceeded that of the *E. coli* K-12 strain which was used as the control (Matyar et al., 2008).

Screening of isolates for extracellular hydrolytic activities

All isolates were examined for their enzyme activity like DNase, lipase, protease and amylase by using standard methods (Sokol et al., 1979; Collins et al., 1989; Collins et al., 2003).

Results

A total of 35 bacteria were isolated from five stations soil samples. According to Gram staining reaction, 31 Gram positive and 4 Gram negative, rod shaped isolates were determined. 34 bacteria have endospore structure; 5 bacteria were found to be oxidase positive and 14 bacteria to be catalase positive. All to bacteria were found give results for indole test (+/-), 15 bacteria citrate tests were positive, 24 bacteria were tested for methyl red positive and 29 bacteria were tested for VP test positive (Table 2).

All isolates showed optimum growth in the temperature range of 20-37°C. 20 isolates at 4 °C; 9 isolates did not grow at 50 °C (Fig. 1). The optimum pH range for growth

of all strains was observed 5.0 to 11.0, except 1T1 and 3T13 (at pH 9) and 4T30 (at pH 11) (Fig. 1). Isolates growth rate at different salt concentrations was examined; these rates were 100%, 80%, 25.71%, 31.42% at the salt concentration of 2%, 6%, 12%, 18%, respectively. There was no growth at the salt concentration of 23% (Fig. 2).

It was found that most effective antibiotic was G120 and about 94.28, 85.71, 82.85 and 80% of the isolates were resistant to CN30, A10, T30 and CMZ30, respectively (Fig. 3).

Results of heavy metal resistance of isolates were shown in Figure 4. The highest concentration of Cd^{2+} , Cr^{3+} , Cu^{2+} , Ni^{2+} , Mn^{2+} , Pb^{2+} , Zn^{2+} metals which observe the bacteria growth are 40%, 17.14%, 17.14%, 17.14%, 31.42%, 8.57%, 20%, respectively.

The isolates were tested for their ability to produce four industrially important hydrolytic enzymes (Table 3). Interestingly, it was noted that except 5N12 and 3N2, all other strains showed multi hydrolytic enzyme production ability. 28 isolates showed the ability to produce also 4 enzymes. 5 bacteria showed the ability to produce 3 enzymes and 2 bacteria just showed the ability to produce 1 enzyme (Table 3).

Discussion

Nowadays, the increasing industrialization rate in the world is threatening for the quality of abiotic resource such as soil, water and atmosphere. Contaminating of ecosystem and the amount of toxic and dangerous substance has been increased by using of industrial activities and the application of various chemicals (Eghomwanre et al., 2016). Toxic metals, including extremely basic metals, disrupt their biological structures and systems into reversible or irreversible compatibility, leading to the impaired organ function or ultimate death. The environment contains less amount of Ni which known heavy metal. Environmental pollution increase day by day because of the using of vast industrial of nickel containing material production includes recycling and disposal of them. By Ni mining or by various industrial processes, power plants or incinerators, rubber and plastic industries, nickel-cadmium battery industries and electroplating industries are caused of charging of Ni into the atmosphere. The widespread using or occupational exposure of Ni in various industries is definitely a matter of serious impact on human health.

There are many studies where new isolates with different isolates and conditions are adapted to different soils, such as

Table 2. Morphological features and biochemical characteristic of isolates (+: positive; -: negative; Y = yellow (acid), P = pink (alkaline), Black = H₂S)

Station	Isolate	Characteristics											
		Gram staining	Shape	Endospore	Oxidase	Catalase	Indole	Citrate	KIA			MR	VP
									Slant	Butt	Gas		
I	1N4	+	Rod	+	-	-	+/-	-	alkaline	acid	-	+	-
	1N5	+	Rod	+	-	+	+/-	-	alkaline	alkaline	-	+	+
	1N7	-	Rod	+	-	+	+/-	+	alkaline	acid	-	+	+
	1N18	+	Rod	-	+	+	+/-	+	alkaline	acid	-	+	+
	1T1	+	Rod	+	-	+	+/-	-	alkaline	acid	-	+	+
	1T2	+	Rod	+	+	-	+/-	-	alkaline	alkaline	-	+	+
	1T3	+	Rod	+	-	-	+/-	-	alkaline	acid	-	+	+
II	2N15	-	Rod	+	-	+	+/-	+	alkaline	acid	-	+	+
	2N19	+	Rod	+	-	-	+/-	-	alkaline	alkaline	-	+	+
	2T4	+	Rod	+	-	+	+/-	+	alkaline	acid	-	-	-
	2T6	+	Rod	+	-	+	+/-	-	alkaline	alkaline	-	+	+
	2T24	+	Rod	+	-	-	+/-	+	alkaline	acid	-	-	+
III	3N1	+	Rod	+	-	+	+/-	-	alkaline	acid	-	+	+
	3N2	-	Rod	+	-	+	+/-	+	alkaline	acid	-	+	+
	3N3	+	Rod	+	+	-	+/-	-	alkaline	alkaline	-	-	+
	3N17	+	Rod	+	+	-	+/-	-	alkaline	acid	-	-	-
	3T13	-	Rod	+	-	-	+/-	+	alkaline	alkaline	-	+	-
	3T14	+	Rod	+	-	-	+/-	+	alkaline	alkaline	-	+	+
IV	4N9	+	Rod	+	-	-	+/-	+	acid	alkaline	-	+	+
	4T9	+	Rod	+	-	+	+/-	-	acid	acid	-	+	-
	4T10	+	Rod	+	-	-	+/-	-	alkaline	acid	-	-	+
	4T12	+	Rod	+	-	+	+/-	-	alkaline	acid	-	+	-
	4T26	+	Rod	+	-	-	+/-	+	alkaline	acid	-	+	+
	4T27	+	Rod	+	-	+	+/-	-	alkaline	alkaline	-	-	-
	4T30	+	Rod	+	-	-	+/-	+	alkaline	acid	-	+	+
	4T31	+	Rod	+	+	-	+/-	+	alkaline	alkaline	-	-	-
	4T32	+	Rod	+	-	+	+/-	-	alkaline	alkaline	-	-	-
V	5N12	+	Rod	+	-	+	+/-	-	alkaline	acid	-	-	-
	5N13	-	Rod	+	-	-	+/-	-	alkaline	acid	-	-	-
	5N14	+	Rod	+	-	-	+/-	-	alkaline	acid	-	+	+
	5T15	+	Rod	+	-	-	+/-	+	alkaline	acid	-	-	-
	5T17	+	Rod	+	-	-	+/-	-	alkaline	alkaline	-	+	+
	5T19	+	Rod	+	-	-	+/-	-	alkaline	acid	-	+	-
	5T20	+	Rod	+	-	-	+/-	+	alkaline	alkaline	-	+	-
	5T28	+	Rod	+	-	-	+/-	+	alkaline	acid	-	+	-

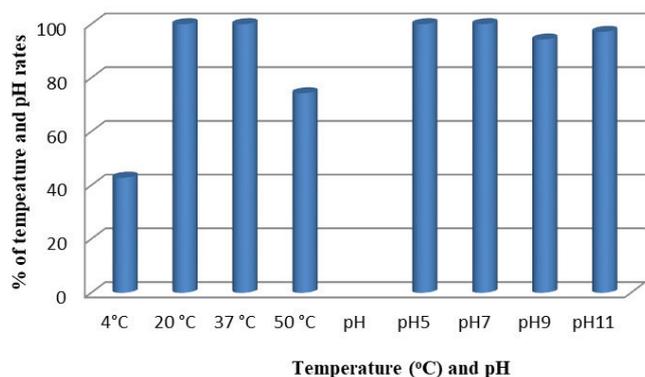


Figure 1. Percent of temperature and pH growth range of isolated soil bacteria.

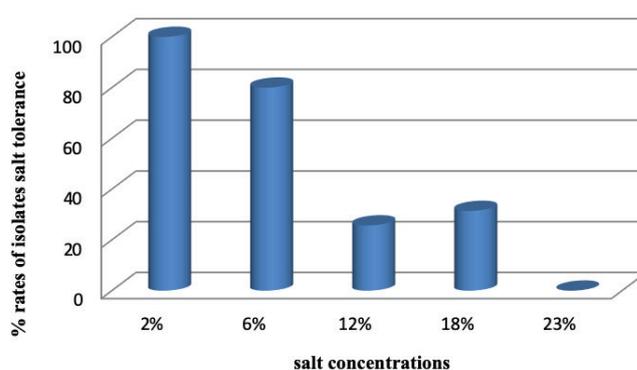


Figure 2. Percent of salt tolerance of isolated soil bacteria.

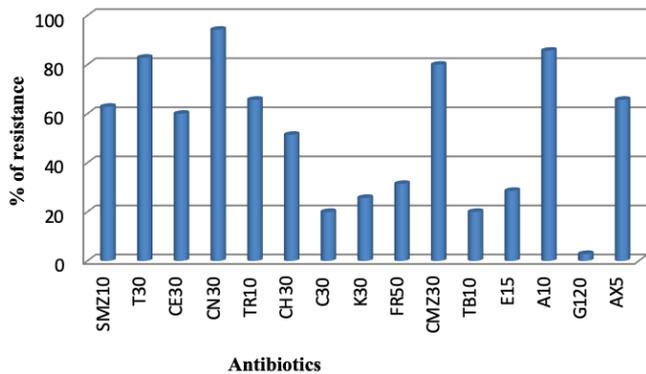


Figure 3. Percent of antibiotic resistance against isolated soil bacteria.

heavy metal chemicals or extreme physical conditions (Gülcan, 2006; Sevgi, 2007; Saraç et al., 2008; Dülger, 2012; Eghomwanre et al., 2016; Neelam et al., 2018).

Neha et al. (2015) isolated and identified heavy metal resistant bacteria from petroleum soil of Loni and found that the strains showed diverse metabolic pattern of carbon sources and other growth factors. They also showed tolerance to other heavy metals, such as copper, lead and nickel.

Eghomwanre et al. (2016) researched some selected bacteria that are from contaminated soils and sediments around Warri area of Delta State, for the tolerances of antibiotic resistance patterns and heavy metals such as Pb, Zn, Cd and Fe. The most resistant isolates were

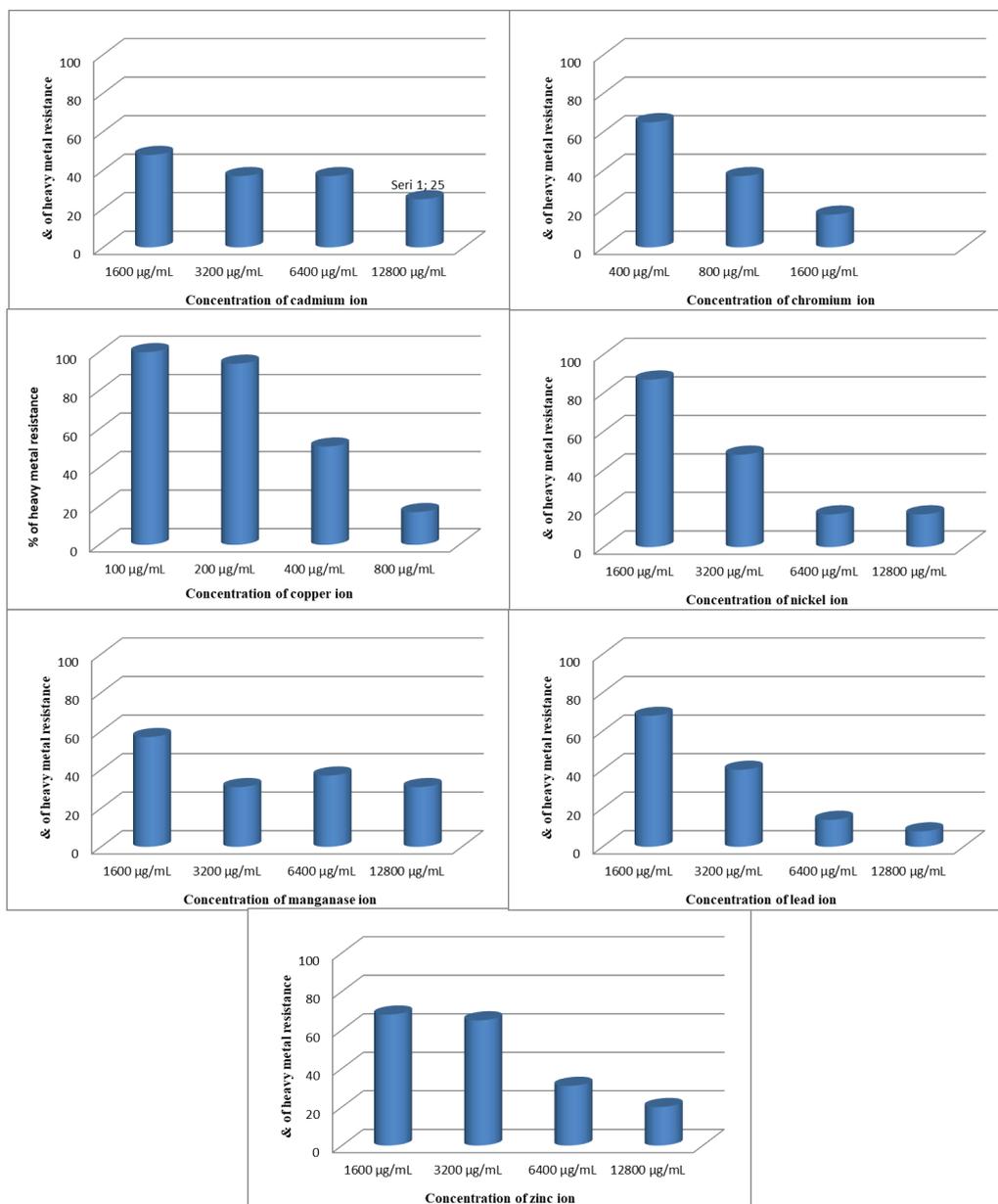


Figure 4. Percent of heavy metal resistance of isolated soil bacteria.

Table 3. Screening of industrially putative enzymes from isolated soil bacteria (+: positive; -: negative)

Isolates no	DNase	Lipase	Protease	Amylase
1N4	+	+	+	+
1N5	+	+	-	+
1N7	+	+	+	+
1N18	+	+	+	+
1T1	-	+	+	+
1T2	+	+	+	+
1T3	+	+	+	+
2N15	+	+	+	+
2N19	+	+	+	+
2T4	+	+	+	+
2T6	+	+	+	+
2T24	-	+	+	+
3N1	+	+	+	+
3N2	-	-	-	+
3N3	+	+	+	+
3N17	+	+	+	+
3T13	+	+	+	+
3T14	+	+	+	+
4N9	+	+	+	+
4T9	+	+	+	+
4T10	+	+	+	+
4T12	+	+	+	+
4T26	+	+	+	+
4T27	+	+	+	+
4T30	-	+	+	+
4T31	+	+	+	+
4T32	+	+	+	+
5N12	-	-	-	+
5N13	-	+	+	+
5N14	+	+	+	+
5T15	+	+	+	+
5T17	+	+	+	+
5T19	+	+	+	+
5T20	+	+	+	+
5T28	+	+	+	+

Staphylococcus aureus, *Escherichia coli* and *Pseudomonas aeruginosa* from the experiment of multiple drug resistance on the bacterial isolates; while *Klebsiella mobilis* exhibited the least resistance. At different concentrations of Pb and Cd, all the bacterial isolates exhibited various degree of sensitivity; meanwhile the organisms showed abundant and moderate growth in the Fe and Zn even at higher concentrations. Our results show similarities with these studies. In the present study high degree of heavy metals resistance associated with multiple heavy metals was detected in Ni rich soil bacteria. These metal resistant bacteria can be utilized in bioremediation of metal contaminated environments. In addition to heavy metal resistance, our isolates have high antibiotic resistance and enzymatic activity. Inimitableness and characteristics of them could be used as agents of potential bioremediation for taking out the heavy metals

from the environment and source of industrial enzymes. More studies are required to assess the heavy metal extraction ability of those isolates.

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