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# Screening of plant growth promoting traits in Cr(VI) reducing *Rhizobium* strains isolated from root nodules of *Phaseolus vulgaris*

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**ABSTRACT**

A total of five *Rhizobium* strains were screened from root nodule of *Phaseolus vulgaris* grown in leather industrial effluent contaminated soil and their heavy metal tolerances were studied. The bacterial strains NA2, NB2, NE2 and NG1 were able to reduce Cr(VI) by 100% at 20µg/ml ofCr(VI) completely after 120, 80, 100 and 100 h of incubation, respectively. Moreover, selected *Rhizobium* strains produced a considerable amount of indole acetic acid (IAA) in the presence of L-tryptophan. A maximum amount of IAA production was observed in strain NB2, it produced 8.71 and 23.84 µg/ml of IAA with 50 and 100 µg/ml of L-tryptophan concentrations, respectively. The strain NA2 was found superior in ammonia production; it produced 8.58 µg/ml of ammonia after 96 h incubation. Further, all the tested *Rhizobium* strains showed positive results for exopolysaccharide (EPS), catalase, amylase and protease production. The present observations suggested that the heavy metal tolerance and plant growth promoting activities of *Rhizobium* strains could be exploited for bio-remediation of leather industrial effluent contaminated soil and to enhance the productivity of the legume crops.

**Keywords**: *Rhizobium* strains, Heavy metals, Cr(VI) reduction, IAA, plant growth promotion.

## 1. INTRODUCTION

Chromium (Cr) is one of the prominent heavy metal discharged into the environment due to industrial process such as tannery, textile, electroplating, galvanizing, dyes and pigment, metallurgical, paint industries and other metal processing industries. More than 170,000 tonnes of Cr wastes are discharged into the environment annually as a consequence of industrial and manufacturing activities [1]. Cr exists in nine valence states from -2 to +6. Among all, Cr(VI) is having highly environmental significance because of its stability in the natural environment. The chromate anions are highly soluble and have rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids. It has been reported that Cr(VI) causes lung cancer, chromate ulcer, perforation of the nasal septum and kidney damage in human and it is also toxic to other organisms as well [2].

Conventionally Cr(VI) reduction methods used to relieve the toxicity of chromium include chemical reduction followed by precipitation, ion exchange and adsorption on activated coal, ash or alum. However, most of these methods involve high energy or large quantities of chemical reagents and are not cost effective. In contrast, the detoxification of chromium by biological method is considerably cheaper and considered an alternative remediation for chromium pollution[3].

Soil heavily contaminated with toxic metals, including chromium, which at elevated concentrations adversely affect not only the beneficial rhizospheric microbes but also the growth and development of plants. Therefore, vegetation establishment on Cr(VI) polluted sites is still a problem during the process of phytoremediation.Recent studies to evaluate the role of PGPR in improving phytoremediation efficiency proved that PGPR's could play major role in plant growth promotion even under metal stress [5]. Hence, metal resistant PGPR's could protect the plants from acute toxicity by its bio transformation and plant growth promoting ability. Thus, the present study was designed to 1) isolate and characterize heavy metal tolerant

*Rhizobium* strains, 2) evaluate their Cr(VI) reducing ability and 3) determine the plant growth promoting ability of the *Rhizobium* strains.

## 2. MATERIALS AND METHODS

### 2.1. Soil sample collection and bacterial isolation

Cr(VI) tolerant *Rhizobium* strains were isolated from the root nodules of 60 days old *P. vulgaris*plant grown in leather industry effluent contaminated soil. The nodules of *P. vulgaris* were surface sterilized in 2.5% of sodium hypochlorite for 2 min followed by rinse with 95% ethanol (v/v). Finally, surface sterilized root nodules were washed and squashed with 0.5 ml of sterile distilled water. Root nodule suspensions were diluted and plated on YEMA medium (g/1: mannitol 10; $K_2HPO_4$ 0.5; $MgSO_4$. $7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 2; agar 20; pH 6.8 ± 0.2) supplemented with 100 µg/ml of Cr (VI). Inoculated plates were incubated at 28±2° C for three to five days. The pure culture was characterized based on their colony morphology and biochemical characterization.

### 2.2. Evaluation of multi metal resistance levels

Multi metal tolerance of the *Rhizobium* strains were tested by the agar dilution method [6]. The freshly prepared LB agar medium was amended with various heavy metal salts such as $K_2Cr_2O_7$, $Pb(NO_3)_2$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $MnCl_2.4H_2O$. Further, overnight grown culture was inoculated onto the heavy metal amended plates for observing the growth after 3-5 days of incubation.

### 2.3 Chromium (VI) reduction analysis

For Cr(VI) reduction study, bacterial strains were inoculated in YEM broth supplemented with 20, 30 and 50 µg/ml concentrations of Cr(VI). All the inoculated culture tubes were incubated at 28±2°C for 120 h with shaking at 200 rpm. The reduction was estimated by 1, 5-diphenyl carbazide method [7] at regular time intervals every 20 h only.

### 2.4. Bioassay of plant growth promoting substances under Cr(VI) stress

#### 2.4.1. Indole-3-acetic acid production

The plant growth promoting activities such as, IAA, ammonia, EPS, amylase, protease and catalase production of the *Rhizobium* strains were performed. The IAA production of *Rhizobium* strains was quantitatively analyzed by the method of Libbert*et al.,*[8] using YEM broth supplemented with 50 and 100 µg/ml of L-tryptophan. The culturewas incubated for 30 h at 28±2° C with 200rpm shaking. After 30 h incubation, *Rhizobium* culture was centrifuged at 10,000 rpm for 10 min and 2ml of Salkowski's reagent (2% 0.5M $FeCl_3$ in 35% Perchloric acid) was added to 2ml of the supernatant. The reaction mixture was incubated in darkness at room temperature for 30 min. The absorbance of the developed pink color was read at 530 nm. The IAA concentration was determined using a calibration curve of pure IAA as a standard (HiMedia, India).

#### 2.4.2. Ammonia and EPS production

The ammonia producing ability of the *Rhizobium* strains was quantitatively analyzedin peptone water brothby Cappuccino and Sherman [9] method. After incubation, 1 ml Nessler's reagent was added to 1 ml of culture free supernatant and volume of this mixture was made up to 10 ml by addition of ammonia free sterile distilled water. The development of yellow color, indicated the production of ammonia and its optical density was measured by spectrophotometer at 450 nm. The concentration of ammonia was estimated based on a standard curve of ammonium sulfate (HiMedia, India). The EPS production was analyzed by qualitative by the protocol of Sayyed*et al.,*[10]. The inoculated plates were incubated for four days at 28±2°C. Emission of gummy substances on the edge of the bacterial colonies indicated the production of EPS.

#### 2.4.3. Catalase and hydrolytic enzyme production

Catalase producing ability of the *Rhizobium* strains were studied qualitatively using the method described by Cappuccino and Sherman [9]. Overnight grown bacterial culture was mixed with an appropriate amount of 3% hydrogen peroxide on a glass slide to observe the evolution of oxygen gas formation. In case of amylase production, bacterial strains were streaked on starch agar medium and incubated at 28±2 ° C for two days. The plates were flooded with 1% iodine solution. A colorless zone formation around colonies indicated the production of amylase enzyme. For protease production, the *Rhizobium* strains were streaked on casein hydrolyzed medium and the plates were incubated at 28±2 °C for 24 h. A clear zone around the colonies indicated the proteolytic activity of the strain.

### 2.5. Statistical analysis

Each experiment was carried out in the triplicates (n=3) and standard error (SE) was calculated. All the data were analyzed using Analysis of Variance (ANOVA) with the statistical software Prism 5.0 version. The analyses were done with 95% confidence.

## 3. RESULTS AND DISCUSSION

### 3.1. Isolation of Cr(VI) tolerant *Rhizobium* strains

In the present study, a total of five bacterial strains were isolated from the root nodules of the *P. vulgaris* grown chromium contaminated leather industrial soil. The bacterial isolates NA2, NB2, NE2, NF3 and NG1 showed promising tolerance against Cr(VI). Therefore the strains were selected for further studies. The selected strains were found to be Gram negative, rod shaped and produced circular, raised, non pigmented and translucent gummy colonies on YEMA plates and no fluorescence emission was observed under the Ultra Violet light (UV). All the tested bacterial isolates were shown positive results for oxidase, catalase and glucose peptone broth test. However,bacterial isolatesdisplayed negative reaction for indole, methyl red, Voges-Proskauer, triple sugar ion, citrate, Hofer's alkaline broth and keto lactose tests. On the other hand, isolates NB2, NE2 and NG1 showed negative results for urease. However, isolate NF3 showed negative result for hydrogen sulfide test. The morphological and biochemical characterizations of the selected strainswere discussed in table 1.

### 3.2. Multi metal resistance ability of *Rhizobium* strains

The selected *Rhizobium* strains were tested for their ability to tolerate various concentrations of heavy metals like Cr(VI), Pb(II), Zn(II), Cu(II) and Mn(II). The strains showed a varied level of tolerance to the tested heavy metals. Among the five tested *Rhizobium* strains, the strain NA2 showed highest tolerance to most of the tested heavy metals. Strain NA2 tolerated Cr(VI), Pb(II), Zn(II), Cu(II) and Mn(II) at a concentration of 350, 250, 350, 50 and 500µg/ml respectively. Followed by NA2 strain, NB2, NE2, NF3 and NG1 strains aslso showed considerable tolerance to tested heavy metals (Table 2). From the heavy metal tolerance results we conclude that, Cu is a toxic metal to *Rhizobium* strains as compared to other tested heavy metals. The results indicated that the strains displayed resistance to different heavy metals at different concentrations. This might be due to the development of resistance mechanism of microbes to a variety of toxic heavy metals for their survival in the heavy metal contaminated environment [11]. Different heavy metal tolerance pattern of the selected *Rhizobium* strains probably due to their unique genetic makeup and biochemical nature of the bacterial strains. In addition, media composition and growth condition of bacterial cells play a key role in multimodal tolerance.

### 3.3. Cr(VI) reducing ability of the *Rhizobium* strains

Chromium reduction was monitored at different initial Cr(VI) concentrations ranging from 20, 30 and 50µg/ml in aerobic conditions at pH 6.8 ± 0.2 and 28±2°C. Among the five *Rhizobium* strains, four strains NA2, NB2, NE2 and NG1 showed complete reduction in 20µg/ml concentration of Cr(VI) after 120, 80, 100 and 100h of incubation respectively. However, in case of 30µg/ml concentration of Cr(VI) only one *Rhizobium* strain NB2 showed a complete reduction after 120h incubation. On the other hand, at the increased concentrations of Cr(VI) (50µg/ml) tested bacterial strains were unable to reduce the Cr(VI) completely even after 120h incubation (Figure 1). It seems that the increased concentrations of Cr(VI) may inhibit the reducing ability and enzymatic process of the cell. Similar evidence on the effect of different Cr(VI) concentrations on chromium reduction by naturally occurring microbes is reported. For instance, *Mesorhizobium* strains RC1 and RC4 completely reduce 50µg/ml of Cr(VI) after 120h incubation [12], whereas *Pseudomonas* strain CRB5 completely reduce 20 µg/ml of Cr(VI) after 120 h incubation [13]. Microorganisms eliminate heavy metals from the surrounding environment either through enzymatic or non-enzymatic pathway. Moreover, microorganisms protect themselves from toxic heavy metals by masking the entry of the metal ions into cells or by reducing the free ions in the cytosol. The bacteria capable of reducing fewer amount of Cr(VI) in liquid medium and showing a higher level of resistance to Cr(VI) on agar medium. These results clearly denoted that Cr(VI) reducing the ability of the rhizobacterial strain was not related to their Cr(VI) tolerance on agar medium. Similar to our report, Rajkumar et al[14] reported in *Pseudomonas sp.*

### 3.4. Plant growth promoting ability *Rhizobium* strains

#### 3.4.1. IAA production

The IAA synthesized by PGPR's can act as a signaling molecule during the plant development and it enhances several physiological behavior of plant such as, root initiation, cell division, cell elongation, seed germination, root elongation and plant growth rate, etc[15]. In our study, we observed that, all the Cr(VI) reducing *Rhizobium* strains released a substantial amount of IAA in YEM broth after 30 h of incubation. A maximum amount of IAA observed in NB2 strain. It produced 8.71 and 23.84µg/ml of IAA with 50 and 100µg/ml of L-tryptophan concentrations respectively. Followed by this, strains NE2, NG1, NA2 and NF3 produced 19.90, 18.08, 17.18 and 14.67 µg/ml of IAA at 100µg/ml of L-tryptophan concentration respectively. While comparing the

**Table - 1: Morphological and Biochemical characterization of Cr (VI) tolerant *Rhizobium* strains**

| Characteristic features | *Rhizobium*strains | | | | |
|---|---|---|---|---|---|
| | **NA2** | **NB2** | **NE2** | **NF3** | **NG1** |
| **Morphological characteristic** | | | | | |
| Grams reaction | -Ve | -Ve | -Ve | -Ve | -Ve |
| Cell shape | Short rods | Short rods | Short rods | Short rods | Short rods |
| Colony morphology | Transparent, raised circular and gummy | Transparent, raised circular and gummy | Transparent, raised circular and gummy | Transparent, raised circular and gummy | Transparent, raised circular and gummy |
| Pigments | - | - | - | - | - |
| Fluorescent under the UV light | - | - | - | - | - |
| **Biochemical reaction** | | | | | |
| GPB | No growth | No growth | No growth | No growth | No growth |
| Keto-lactose test | No yellow ring | No yellow ring | No yellow ring | No yellow ring | No yellow ring |
| Hofer's Alkaline broth | No growth | No growth | No growth | No growth | No growth |
| Indole | - | - | - | - | - |
| MR | - | - | - | - | - |
| VP | - | - | - | - | - |
| Citrate | - | - | - | - | - |
| TSI | - | - | - | - | - |
| Oxidase | + | + | + | + | + |
| $H_2S$ | + | + | + | - | + |
| Urease | + | - | - | + | - |

**Note:** '+' Positive reaction, '-' negative reaction, GPB- Glucose Peptone Broth, TSI- Triple sugar ion, MR-Methyl red, VP-Vogesproskauer, $H_2S$- Hydrogen sulfide.

effect of various concentrations of L-tryptophan on IAA production, 100µg tryptophan/ml showed a significant ($p<0.05$) increase in IAA production compared with 50µg tryptophan/ml concentration (Figure 2). This result demonstrated that the tryptophan is a key precursor compound for IAA production. Plant growth promoting bacteria's are known to produce several plant growth promoting substances and phytohormones such as auxins, cytokinins, gibberellines etc. In which IAA is an efficient molecule known to enhance plant growth by stimulating apical dominance and root growth [16]. Patten and Glick [17] suggested that the secretion of microbial IAA into the rhizospheric ecosystem can modify the plant endogenous auxin level to an optimum level or even above it, resulting in optimized plant growth. Similar trends in IAA production from chromium tolerant bacteria were reported by Rajkumar*et al.,*[14] and Oveset al[18] in *Pseudomonas sp*.

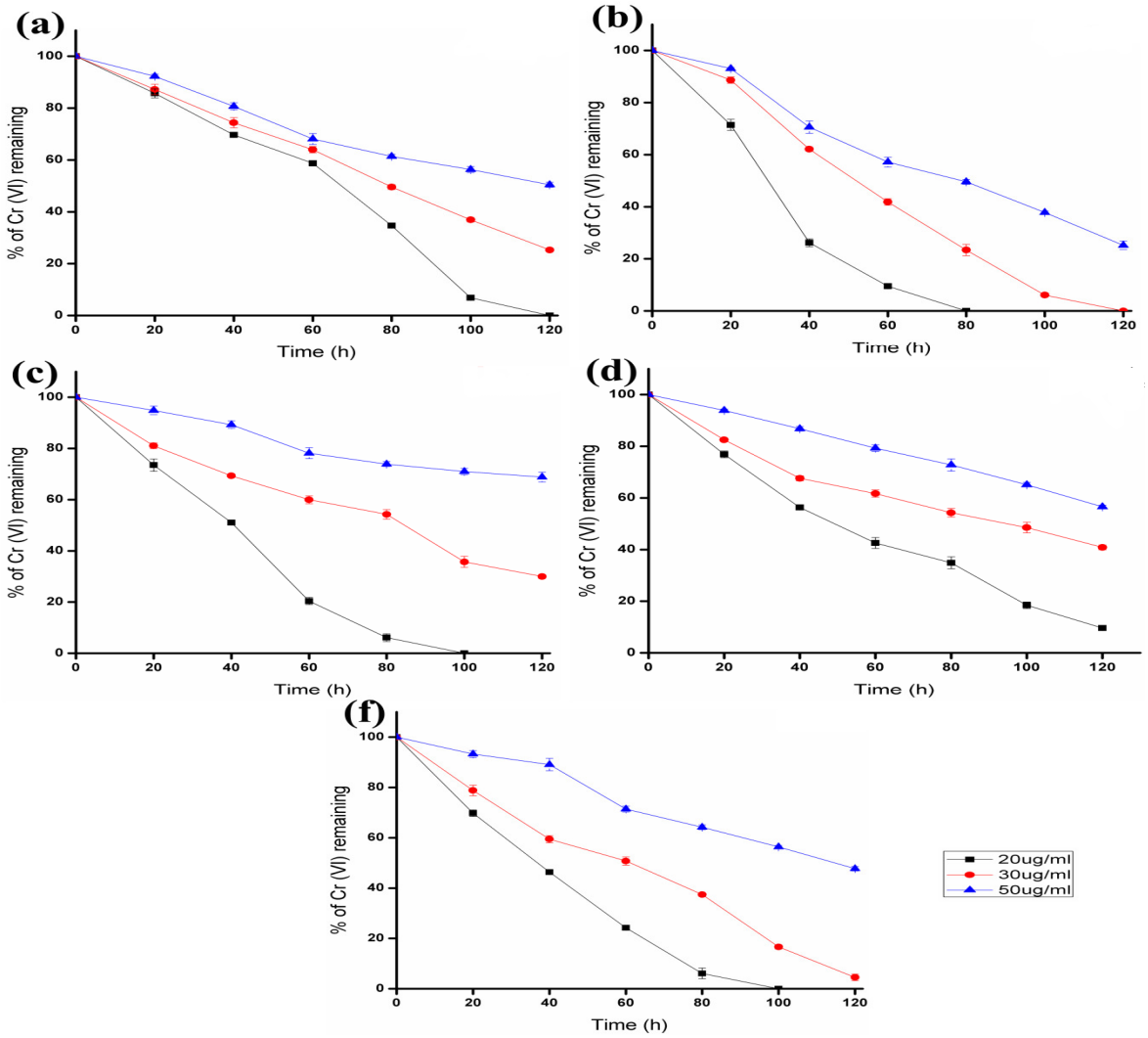


**Figure - 1: Effect of different concentrations of Cr(VI) on Cr(VI) reduction ability of the selected *Rhizobium* strains a) NA2, b) NB2, c) NE2, d) NF3 and f) NG1 in YEM broth after 120 h of incubation. Each value represents the mean of three independent triplicates.**

**Table - 2: Multi Metal resistance ability of *Rhizobium* strains**

| ***Rhizobium*strains** | **Heavy metals (µg/ml)** | | | | |
|---|---|---|---|---|---|
| | **Cr (VI)** | **Zn (II)** | **Pb (II)** | **Cu (II)** | **Mn (II)** |
| NA2 | 350 | 250 | 350 | 50 | 500 |
| NB2 | 350 | 150 | 250 | - | 500 |
| NE2 | 150 | 100 | 150 | - | 300 |
| NF3 | 150 | 50 | 50 | - | 100 |
| NG1 | 350 | 250 | 300 | 50 | 400 |

Note: - no growth

**Table - 3: Plant growth promoting abilities of the selected *Rhizobium* strains**

| ***Rhizobium*strains** | **EPS** | **Protease** | **Amylase** | **Catalase** |
|---|---|---|---|---|
| NA2 | + | + | - | + |
| NB2 | + | ++ | + | ++ |
| NE2 | ++ | + | + | + |
| NF3 | + | + | - | + |
| NG1 | ++ | + | - | ++ |

**Note:** '+' Slight activity, '++' moderate activity and '+++' intense activities

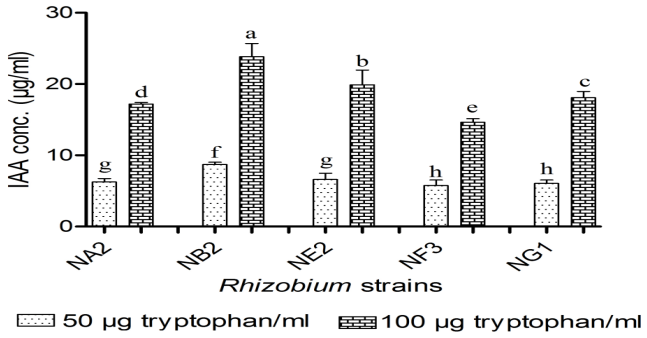


**Figure - 2: IAA production by *Rhizobium* strains. Error bars represent standard error of the individual. An asterisk (*) denotes a value significantly greater than the corresponding 50µg /ml tryptophantreatment.**

### 3.4.2. Ammonia and EPS production

The ammonia production is another important attribute of PGPR, which exhibits plant growth promotion ability[19]. In case of ammonia production, all the five *Rhizobium* strains produced a considerable amount of ammonia. Among the five *Rhizobium* strains, the strain NA2 produced 8.58 µg/ml of ammonia. Followed by this, strains NG1, NE2 and NF3 produced 7.15, 5.35, 4.66 and 4.10 µg/ml of ammonia respectively (Figure 3). Hence, we can conclude that the strains can promote the plant growth without being affected by the high chromium concentrations prevailing at the contaminated sites. A similar trend of ammonia production was reported by Karuppiah and Rajaram[20] in Cr(VI) reducing plant growth promoting *Bacillus* sp. Previously, Marques et al[21] reported that, the ammonia produced from bacteria supply nitrogen source to their host plant and promote root and shoot elongation, consequently increasing plant biomass. Moreover, it is worthwhile mentioning that excess production of ammonia can act as a triggering factor for virulence of opportunistic plant pathogens [22].

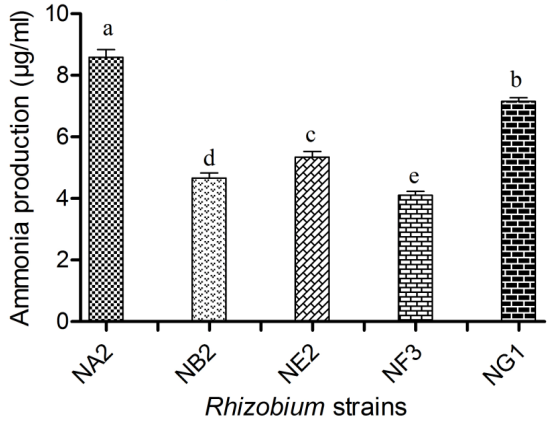


**Figure - 3: Ammonia production by*Rhizobium* strains. Error bars represent standard error of the individual.**

The EPS production is an important plant growth promoting trait of bacteria, because it provides protection of cells against pathogens, desiccation, phagocytosis and metal stress [23]. In case of EPS production, all the tested *Rhizobium* strains showed positive results. In which *Rhizobium* strains NE1 and NG2 produced moderate level of EPS as compared to other tested *Rhizobium* strains (Table 3). The excess production of EPS by rhizobia [24] denotes the innate protection mechanism by masking the effect of metals while growing in stressed environments. The present result suggests that the Cr(VI) tolerant ability of the *Rhizobium* strains could probably due to entrapment of Cr(VI) within their cell wall.

### 3.4.3. Hydrolytic enzyme and Catalase production

All the tested *Rhizobium* strains showed positive result for catalase production. The catalase production shows the resistance ability of the strains against oxidative, environmental, mechanical and chemical stress [25]. Therefore, this result concluded that the *Rhizobium* strain with catalase activity indirectly involved in the promotion of plant growth. In addition, the *Rhizobium* strains NB2 and NE2 showed positive results for amylase production. But in case of protease production, all the tested five *Rhizobium* strains exhibited positive results for protease activity (Table 3). The *Rhizobium* strain NB2 showed moderate levels of protease enzyme production compared to other tested strains. The production of hydrolytic enzymes by the *Rhizobium* strain denoted the antimicrobial property of the strain by cell lysis in the close vicinity of the rhizospheric plant pathogens[26].In nature, the cell wall of fungal pathogens contains fibrillar materials bound together by sugar, proteins, lipid and a variety of polysaccharides like β-1,3-glucan and chitin which compounds are essential for fungal growth and pathogenicity [27]. Lysis of the cell wall by hydrolytic enzymes producing microbes leads to cause serious effects

on pathogens and inhibit their population in rhizospheric environment. Previously, Malleswari and Bagyanarayana[28] reported that, production of hydrolytic enzymes from plant growth promoting bacteria would be the most prominent traits of antagonism.

**4. CONCLUSION**

The present research demonstrated that the heavy metal tolerance and Cr(VI) reducing ability of the *Rhizobium* strains at laboratory scale. With these interesting characteristics, the isolated *Rhizobium* strains produce plant growth promoting substances like IAA, EPS, ammonia, catalase,amylase and protease enzyme.. Phytoremediation in combination with PGPR is a cost effective and eco-friendly solution for the remediation of heavy metal contaminated sites. Therefore, *Rhizobium* strains with multiple properties might be a good nominee for multi component bio-remediation system in chromium contaminated leather industry soils as well as useful biofertilizer to improve the growth improvements of various economically important agricultural crops.

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