In vitro cyanide degradation by Serretia marcescens RL2b
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ABSTRACT
Detoxification of cyanide compounds using biological systems is gaining much attention due to various advantages over the traditional physical and chemical methods. In present study, a cyanide degrading bacterial strain RL2b was isolated from forest soil of Himachal Pradesh. Based on the morphology, physiological, biochemical tests and its 16S rDNA sequence, the bacterial isolate RL2b was identified as Serretia marcescens. In vitro degradation of cyanide by this organism was investigated by varying several cultural conditions viz. medium, carbon and nitrogen sources, pH and temperature. Serretia marcescens RL2b exhibited maximum cyanide degradation in medium M1 containing glycerol and tryptone as carbon and nitrogen source respectively. Cyanide degradation was maximum at pH 6.0 and 35°C temperature. This bacterial isolate exhibited cyanide tolerance up to 16 mM and highest cyanide degradation at 12 mM in 40 h. The present study revealed that the strain Serretia marcescens RL2b has high cyanide tolerance and degradation potential at wide pH and temperature range and thus has very good potential for efficient cyanide removal from environment.

Keywords: Serratia marcescens, cyanide degradation, cyanide tolerance, 16S rDNA sequence

1. Introduction
Cyanide is used in the extraction of gold from its ore, electroplating, steel manufacturing, polymer synthesis and dye making. Due to its extensive applications in industries, it is inevitable to use cyanide (Luque-Almargo et al., 2005). Several problems are associated with the discharge of large amounts of cyanide compounds in the environment (Das and Santra, 2011). When cyanide is released in the soil, it may leach through the soil and affects its physical and biological components. Its entry into soil or water system imposes a serious threat to sustainability of the ecosystem (Seepulveda et al., 2010). A number of industrial effluents contain cyanide at a concentration exceeding 100mg/L (Watanabe et al., 1998: Gurbuz et al., 2009). The acceptable limit of cyanide in industrial effluents and polluted environment is 0.2mg/L. Cyanide is toxic to a wide spectrum of organisms because it has the ability to form complexes with metals (Fe^{2+}, Mn^{2+} and Cu^{2+}) which act as cofactor of many enzymes (Raybuck, 1992: Dumestre et al., 1997). Some microorganisms and plants are capable of metabolizing cyanide due to presence of alternative pathways to utilize or degrade cyanide (Daniel et al., 1994). Microorganisms are able to convert cyanide into other less toxic products like ammonia, formic acid and formamide depending upon the enzyme system they possess (Huertas et al., 2010: Luque-Almargo et al. 2011). The presence of such pathways in biological systems encourages researchers to develop novel processes involving biomaterials for the removal of cyanide from the environment (Kao et al., 2003: Dhillon and Shivaraman, 1999).
Although, there are various treatment processes reported for cyanide remediation including chemical and physical methods, yet their applications are limited due to environmental variations, high cost and operational hazards. Thus biological method of cyanide removal or precisely cyanide bioremediation is more attractive. It is cost effective, environment friendly, and easily operable (Dash et al., 2009). Beside this, the maximum level of cyanide that can be degraded by the microbes reported hitherto is up to 10 mM to 12 mM. Therefore, it will be worthwhile to isolate cyanide degrading microbes from less explored habitats with higher levels of cyanide tolerance and degradation potential and such microorganisms will have immense scope for bioremediation of cyanide contaminated soil/water. Keeping in view the importance of biological remediation of cyanide, present study is focused on isolation and characterization of a high cyanide tolerant and degrading bacterial isolate and in vitro degradation of cyanide.

2. Materials and methods

2.1 Materials

Potassium cyanide was obtained from SD Fine Chemicals Ltd. India. Other medium components were obtained from commercial sources and were of analytical grade.

2.2 Medium

The selective enrichment medium contained glucose (10 g), \(\text{Na}_2\text{HPO}_4\) (2.5 g), \(\text{KH}_2\text{PO}_4\) (2 g), \(\text{MgSO}_4\) (0.5 g), \(\text{FeSO}_4\) (0.03 g), \(\text{CaCl}_2\) (0.06 g) and 12 mM KCN (0.78 g) in 1 L distilled water. The pH of the medium was adjusted to 7.0 and then autoclaved at 121°C for 20 min. The strain was incubated for 72 h at 30°C and 150 rpm. Cyanide levels were measured at different intervals of time.

2.3 Analytical methods

Samples were withdrawn in small aliquots directly from flask at different intervals of time, centrifuged and cyanide level in the supernatant was assayed using picric acid assay method (Fischer and Brown, 1952). Absorbance was measured at 540 nm and concentration of cyanide was calculated from standard curve of cyanide estimation (prepared by assaying KCN ranging from 1.5µM-13.5µM).

2.4 Isolation of cyanide degrading isolates

Soil and water samples were collected from different locations of Himachal Pradesh, India. One ml water sample or 1 g soil sample was added to 50 ml enrichment medium containing glucose and potassium cyanide as sole carbon and nitrogen source and incubated at 30°C for four days. The culture was then transferred to new medium and process was repeated three times under similar conditions. The culture was then plated on to enrichment medium containing 2% agar. Microbial colonies appeared on to these plates were streaked individually on to nutrient agar plates. Isolate RL2b which exhibited maximum cyanide degradation was selected for further studies.

2.5 Identification and characterization

2.5.1 Phenotypic and biochemical characterization
The physiological and biochemical features were identified by the methods described in Bergey’s Manual of Systematic Bacteriology (Holt et al., 1994). Carbon source utilization profile of the isolate was studied with a carbohydrate test kit from Himedia Chemicals, India.

2.5.2 16S rDNA sequencing

Chromosomal DNA of the isolate was extracted manually by Marmur’s procedure (Sambrook and Russel, 2006). The 16S rDNA genes were amplified using polymerase chain reaction with the universal primers 16S rRNA For (5’-AGAGTTTGATCCTGGCTCAG-3’) and 16S rRNA Rev (5’-ACGGCTACCTTGTTACGACTT-3’) (Integrated DNA Technologies) and were sequenced at Xcelris Genomics. Related matching sequences were downloaded from the GenBank database (NCBI, USA) using blast search program (Altschul et al., 1990). The sequences were aligned using multiple sequence alignment software, Clustal W version 2.0. A phylogenetic tree was constructed with MEGA align software version 5.1 (Tamura et al., 2011) based on the partial 16S rDNA sequences of 21 bacterial species similar to isolate RL2b selected in the present.

2.6 In vitro cyanide degradation

The isolate RL2b was grown in different media, carbon sources, nitrogen sources, pH, temperature and cyanide concentrations in order to optimize conditions for in vitro degradation of cyanide. Cyanide was assayed in medium at different time intervals. Five different media were used (Table 2). This was grown in selected medium containing different carbon sources (viz. glucose, fructose, sucrose, glycerol, and sodium succinate) and nitrogen sources (viz. ammonium sulfate, beef extract, peptone, tryptone, malt extract and yeast extract). The isolate was grown at pH 4.0-10.0 with a difference of 1.0 pH unit and temperature ranging from 25°C-50°C. Cyanide tolerance was also monitored. Cyanide levels in the culture were measured by taking small aliquots at an interval of 24h, 48h and 72 h.

3. Results and discussion

3.1 Screening of microorganisms

Fifteen soil samples and five water samples were collected. Twenty seven isolates were obtained by enrichment using 10 mM cyanide as sole source of nitrogen. Pure colonies were obtained and then each one was cultured for cyanide degradation. Among these, eight isolates exhibited potential for cyanide degradation and isolate RL2b was found to possess highest cyanide degrading ability with 99% cyanide removal of 10 mM cyanide in 72 h. The degradation of cyanide by biological systems has increasingly attracted the focus of researchers in the past several years (Ebbs, 2004). In the present investigation, bacteria were isolated from rhizosphere soil of various cyanogenic plants or industrial effluents containing cyanide compounds.

3.2 Identification of isolate RL2b

3.2.1 Morphological and physiological characteristics

The colonies of RL2b isolate on agar plate were creamish white, concave, smooth and wet. It was found that cells were large and rod like. The details of physiological and biochemical tests carried out for this isolate are given in Table 2. The isolate was Gram negative, oxidase negative, indole negative. The nitrate reduction, catalase and citrate tests were positive. H₂S
production was negative. On the basis of these data, the isolate was tentatively identified as *Serratia* sp.

### Table 1: Phenotypic characteristics of isolate RL2b

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Properties</th>
<th>Characteristics</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>_</td>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>_</td>
<td>Adonitol</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>Cyanide</td>
<td>+</td>
</tr>
<tr>
<td>H₂S production</td>
<td>_</td>
<td>D-Fucose</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>D-Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>+</td>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>D-Maltose</td>
<td>+</td>
<td>Indole Production</td>
<td>_</td>
</tr>
<tr>
<td>Lysine Decarboxylase</td>
<td>_</td>
<td>DNase</td>
<td>+</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>+</td>
<td>Trehloose</td>
<td>+</td>
</tr>
</tbody>
</table>

### 3.2.2 16S rDNA sequencing and phylogenetic analysis

The partial 16S rDNA sequence of RL2b was determined and a phylogenetic tree was constructed based on 16S rDNA sequence (Figure 1). Comparison of ribosomal DNA gene sequence analysis revealed a strong relationship between isolate RL2b and members of genus *Serratia*. The isolate RL2b had 100% sequence similarity with *Serratia marcescens* strain XJU PA-6. The sequence was deposited in GenBank database with accession number JX549409. On the basis of physiological and biochemical characteristics and results of 16S rDNA gene sequence comparison, isolate was identified as *Serratia marcescens* RL2b.

![Phylogenetic dendrogram of isolate RL2b 16s rDNA sequence based on neighbor joining method](image)

**Figure 1:** Phylogenetic dendrogram of isolate RL2b 16s rDNA sequence based on neighbor joining method. Number in parentheses is accession number of published sequences. Bootstrap values were based on 1000 replicates.
3.3 Optimization of conditions for *in vitro* degradation

3.3.1 Effect of medium

Optimum degradation of cyanide was observed in M1 medium (Table 2) with 99% of cyanide degradation in 72 h. The isolate RL2b showed rapid growth and maximum degradation in M1 medium. Bushneel-Hass medium was used to degrade cyanide using different bacterial isolates (Parmar et al., 2012). LB medium (Zlosnik and Williams, 2004) and modified Vogel’s medium (Ozel et al., 2010) were also reported for degradation of cyanide. Optimization of medium did have a significant effect on the cyanide degradation potential of the strain because components of medium play important role in switching on the enzymes systems responsible for degradation.

Table 2: Percent degradation of cyanide in different media

<table>
<thead>
<tr>
<th>Medium code</th>
<th>Medium constituents (per litre)</th>
<th>% Cyanide Degradation (72 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>2.5 g Na₂HPO₄, 2.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 30 mg FeSO₄·7H₂O, 60 mg CaCl₂, 1% glucose (pH=7.0) (Bhalla <em>et al.</em>, 1992)</td>
<td>99</td>
</tr>
<tr>
<td>M2</td>
<td>2.5 g Na₂HPO₄, 2.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 30 mg FeSO₄·7H₂O, 60 mg CaCl₂, 1% glucose and yeast extract100 mg (pH=7.0) (Bhalla <em>et al.</em>, 1992)</td>
<td>97</td>
</tr>
<tr>
<td>M3</td>
<td>Luria broth (Sambrook <em>et al.</em>, 1989)</td>
<td>42</td>
</tr>
<tr>
<td>M4</td>
<td>7.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 0.045 g FeCl₃, 0.0020 g CaCl₂, 0.25 g NaCl, 0.01g MnSO₄, 0.01g ZnSO₄, 0.02g CuSO₄, 0.003 g COCl₂, 0.003 g NiCl₂, 0.5% glucose (pH=7.0) (Ingoversen <em>et al.</em>, 1991)</td>
<td>94</td>
</tr>
<tr>
<td>M5</td>
<td>12.8 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.1 g MgSO₄·7H₂O, 0.2 g CaCl₂, 0.5 g NaCl, 0.01g MnCl₂, 0.1 g ZnSO₄, 0.2 g COCl₂, 0.03 g H₃BO₃ 20 mM glucose (pH=7.0) (Sabatini <em>et al.</em>, 2011)</td>
<td>96</td>
</tr>
</tbody>
</table>

3.3.2 Effect of carbon source

In current study, it was seen that glycerol as carbon source in medium M1 favored higher cyanide degradation as compared to other carbon sources used (Figure 2). Glycerol (0.25%) in combination with acetate, glucose and sucrose had been used earlier for cyanide degradation using *Alcaligenes xylosoxidans* subsp. *denitrificans* (Ingoversen *et al.*, 1991). Glucose and KCN had been reported as carbon source for *Pseudomonas flouorescens* NCIMB 11764 (Kunz *et al.*, 1994) and *Psedomonas* sp. (White *et al.*, 1988).
3.3.3 Effect of nitrogen source

The purpose of optimizing nitrogen source was to reduce stress from isolate RL2b so that it could grow well and combat toxic effects of cyanide. Among the several nitrogen sources tested in the present investigation, tryptone turned out to be the best nitrogen source with a decrease in time course of cyanide degradation from 72h to 60h (Figure 3). However KCN had been preferred as sole nitrogen source for degradation of cyanide (Harris and Knowles, 1983: Parmar et al., 2012).

3.3.4 Effect of pH

The acidity and alkalinity of growth medium affect physiological and biochemical
features of microorganisms. The isolate RL2b showed a wide range of pH stability. Usually it exhibited maximum degradation in acidic pH. Optimum pH observed was 6.0 (Figure 4) and there was a significant decrease in time required by the isolate to degrade cyanide from 60 h to 48 h. *Rhodococcus* sp. UKMP-5M has been reported to actively degrade cyanide at neutral pH and only 50% of the added 12 mM cyanide could be degraded by the organism (Maniyam et al., 2011).

![Figure 4: Percent cyanide degradation at different pH](image)

### 3.3.5 Effect of temperature

The isolate RL2b showed maximum degradation of cyanide at 35°C (Figure 5). Most of the biological systems reported for cyanide degradation worked under 25°C to 40°C (Maniyam et al., 2011; Motaung et al., 2011). There was a further decrease in degradation time from 48 h to 40 h.

![Figure 5: Percent cyanide degradation at different temperatures](image)
3.3.6 Cyanide tolerance

Most of the biological systems reported can tolerate cyanide up to 10 mM. *Rhodococcus* sp. UKMP-5M showed 50% degradation of 12 mM cyanide (Maniyam et al., 2011). In present study, isolate RL2b exhibited a maximum tolerance of 16 mM and 99% degradation potential of cyanide up to 12 mM (Figure 6).

![Graph: Percent cyanide degradation and dry cell weight at different cyanide concentration](image)

**Figure 6:** Percent cyanide degradation and dry cell weight at different cyanide concentration

4. Conclusion

This investigation resulted in isolation and characterization of a novel cyanide degrading bacterial isolate *Serretia marcescens* RL2b which tolerate 16 mM of cyanide and degrade 99% of 12 mM of cyanide *in vitro*. It has broad pH and temperature range for cyanide degradation. The bacterial strain can be used for bioremediation purposes as an alternative to chemical and physical remediation because it has highest tolerance and degradation potential for cyanide to the best of our knowledge.

Acknowledgement

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