



## A study over pattern of Zinc Tolerance among Rhizobial isolates of *Trifolium alexandrinum*

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### Abstract

All over the world Heavy metal pollution of soil is a severe environmental problem and has drastically increased due to beginning of industrillization. This pollution affects the growth ,morphology and metabolic activities of soil microorganisms through functional disturbance of enzyme activities and protein denaturation. Organisms which can resist such conditions provides an economical and cheap alternative technology to clean up heavy metal contaminated soils. Characterisation, CFU/ml counts , biomass yield., enzymes (cellulase and amylase) and proteins of zinc tolerant and sensitive Rhizobium sp. In the present study bacteria were isolated from the Barseem (*Trifolium alexandrinum*) root nodules. On the basis of colonial, morphological, biochemical and nodulation ability it was found that all the isolates belongs to Rhizobium species. On the basis of CFU/ml counts and biomass yield zinc sensitive isolates were selected Based on the above parameters, it was concluded that the strain R1 is most sensitive and the strain R2 is tolerant to zinc. The tolerant strain produces an increased amount of the enzymes (cellulase and amylase) and proteins than the sensitive strain. The similarity coefficient calculated revealed that the tolerant and the commercial (MTCC 905) strains are 72.3% similar. The R2 strain had the highest level of enzymes (cellulase and amylase) and proteins.

**Keywords:** Rhizobium ,*Trifolium alexandrinum*, Zinc tolerant

### Introduction

Heavy metals associated with pollution and toxicity are those having densities greater than 5g cm<sup>-3</sup>, but some of these elements (essential metals) are toxic to microorganisms at low concentrations<sup>1</sup>. For example, zinc (Zn) found in many enzymes (dehydrogenases, proteinases, peptidases) and is also involved in the metabolism of carbohydrates, proteins, phosphate, auxins, in RNA and ribosome formation in plants. Rapid industrialization, urbanization Smelters, mines power stations, metal – rich pesticides, fertilizers, waste waters are the different activities through which soils and water can contaminated . Over the past ten years, there has been a growing concern over increased heavy metal pollution of soils and waters and the resulting toxicity to plants, animals and microorganisms, as well as their irreversible binding to various soil components. Secondly, the other problem that arises from heavy metals in soils is that unlike organic pollutants, they cannot be biodegraded and therefore reside in the environment for long periods of time.

Heavy metals has negative impact on soil microorganisms, by affecting their growth, morphology and activities, therefore severe ecosystem disturbance occurred. Soil heavy metal contamination influences the microbial proliferation that changes in enzyme activity<sup>2</sup> and influence the biochemical process also. There is growing evidence that soil biological parameters may have a potential as early and sensitive indicators of soil ecological stress and restoration<sup>3</sup>. Soil enzyme activities are the driving force behind all the biochemical

transformations occurring in the soil since they catalyze all biochemical reactions and are an integral part of nutrient cycling and soil fertility<sup>4</sup>. With the growing importance of enzymes in industries, there is an increasing worldwide interest in the screening of new microorganisms producing enzymes suitable for new industrial application.

Metals can also exert a selective pressure to the organisms, resulting in microbial populations with higher tolerance, which can survive and adapt due to their genetic characteristics. The bacterial resistance properties can be used for different purposes, like in the case of mercury pollution, the insertion of the microbial mercury reductase in a transgenic plant improved significantly the phytoextraction process<sup>5</sup>.

Importance of legumes in animals and human consumption and their use in maintaining soil fertility, there is a need to explore the effects that heavy metals exert on *Rhizobium* isolates. Taking into account Zn, which was identified by Chaudri *et al.*<sup>6</sup>, as having a strong effect on the number of Rhizobia in sludge treated soil<sup>7</sup>. Zn is an essential trace element and plays a key role as a structural constituent or regulatory co-factor of a wide range of different enzymes. It is important in forming complexes (such as zinc fingers in DNA) and as a component in cellular enzymes<sup>8</sup>.

### Material and Methods

**Collection of Bacterial strains:** Bacteria were isolated from the root nodules of Barseem (*Trifolium alexandrinum*) collected

from different region of Dehradun. For phenotypic traits comparison to the field isolates, a commercial strain *Rhizobium trifolii* (MTCC 905) was obtained from Institute of Microbial Technology (IMTECH) Chandigarh.

**Isolation of Rhizobium species:** Effective root nodules of Barseem were collected from the plants grown in a field. The collected nodules were first surface-sterilized with 75% ethanol, then followed by 0.1% mercuric chloride and finally washed with distilled water. *Rhizobium* strain was obtained by streaking the crushed root nodules on YEMA plates and incubated at  $28\pm 2^{\circ}\text{C}$  for 48 hours. Further streaking, spreading and colony morphology helped in isolation of pure cultures of *Rhizobium*. Pure isolates were used for further study<sup>9</sup>.

Biochemical characterization of recovered isolates was performed according to Bergey's Manual of Determinative Bacteriology<sup>10</sup>.

**Symbiotic properties: Surface sterilization and germination of seeds:** The *Trifolium alexandrinum* seeds were rinsed in 95% ethanol, then immersed in 0.2% Mercuric chloride followed by acidification with 0.5% HCl for two minutes. The seeds were then washed thoroughly with at least 5 charges of sterile distilled water and directly spread on 1% agar petri plates. The plates were incubated at  $28\pm 2^{\circ}\text{C}$  in inverted position to provide the seedlings with uniform straight growth<sup>11</sup>.

**Testing of nodulation ability of the isolates:** Two seedlings which were two days old were transferred to tubes containing nitrogen free plant growth medium. It was inoculated with 2 days old culture of *Rhizobium* isolates and incubated at  $28^{\circ}\text{C}$ . The roots were protected from direct exposure to light with the help of black paper. After 4 weeks, data on individual plant were recorded for nodule appearance.

**Evaluation of the response of Rhizobium isolates to the presence of zinc in the growth medium: Determining the CFU/ml counts:** Cell suspensions were prepared by inoculating 50 ml of YEM broth with approximately  $10^6$  Rhizobial cells and then incubated in a shaker (80 rpm/minute) for 48 hours. The cultures were centrifuged at 3000g for ten minutes. The supernatant was discarded and the pellet re-suspended in 30 ml of sterile distilled water and this washing was repeated twice. The washed cells were finally suspended in sterile deionised water. Stock solution of Zinc was made using Zinc chloride from which solutions of different concentration i.e. 0, 0, 25, 50, 75 and 100 mg/ml zinc chloride were prepared and pH of each solution was adjusted to 6.5-7.0. The solutions were then filtered using filter with  $0.2\mu\text{m}$  pore size. Test solution were prepared by pipetting 10 ml of above solutions in a 50ml flask and inoculated with  $30\mu\text{l}$  of washed cells. The flasks were then placed in the shaker incubator at 80 rpm/minute at  $30^{\circ}\text{C}$  for a period of 72 hours. Cell viability was assessed after 24, 48 and 72 hours using the technique in which 0.1 ml of each test solution and their successive dilutions were spread onto a YEM

plate. After overnight incubation at  $30^{\circ}\text{C}$ , the plates were observed for number of colonies (cfu/ml).

**Measurement of the growth/biomass yield:** Washed rhizobial cell were inoculated in 50 ml of YEM broth containing different Zinc concentrations (0, 5, 20, 50 & 100 mg/ml) and incubated at  $30^{\circ}\text{C}$  for 48 hours in shaker at 80 rpm/minute. After incubation, the broth was centrifuged at 6500 rpm for 10 minutes. The supernatant was discarded and the pellet left at the bottom of the tube was re-suspended in saline water and centrifuged in order to remove the residual spent medium. The pellet was finally re-suspended in 2ml of saline water and O.D. of the suspensions was measured at 540 nm using saline water as blank.

**Screening of the isolates:** On the basis of the Cfu/ml counts obtained after 72 hours, the isolate giving maximum number of colonies in the highest Zinc concentration (100 mg/ml) was regarded as the tolerant strain and the one giving minimum number of colonies as the sensitive strain.

**Enzymatic assay (Cellulase and Amylase): Production of crude enzyme:** *Rhizobium* strains (tolerant, sensitive and standard MTCC 905) were inoculated into 100 ml of YEM broth and incubated at  $30^{\circ}\text{C}$  for 48 hours in a shaker at 80 rpm/minute. After incubation the cultures were centrifuged at 6000 rpm for 10 minutes. The supernatant was transferred into another tube and used as crude enzyme for measurement of enzymatic activity.

**Qualitative estimation of Amylase and Cellulase activity:** For the estimation of cellulase and amylase enzymes, the isolates were spot inoculated on the media containing carboxy methyl cellulase and starch respectively. After an incubation period of 48 hours the plates were observed for the presence of zone of hydrolysis around the colony.

**Quantitative estimation of Amylase and Cellulase activity:** The procedure used to estimate the enzymatic activity essentially consisted of estimating reducing sugars formed by the action of the enzyme on suitable substrate. Dinitrosalicylic acid (DNSA) method was used to estimate amylase activity<sup>12</sup>. Maltose solution (1%) was used as enzyme substrate. In a tube, 0.4 ml of enzyme extract, 1.8 ml of the substrate and 2 ml of DNS were added and incubated at  $37^{\circ}\text{C}$  for 10 min. To stop the reaction, 1 ml of 40% solution of sodium potassium tartarate was added. Change in color was observed and OD was taken at 575 nm. OD is proportional to the concentration of the enzyme present. The more the enzyme activity, more will be the color and thus, the higher the OD. Cellulase activity was also estimated by dinitrosalicylic acid (DNSA) method. All the reagents used were the same as that used for the amylase activity assay instead of substrate, wherein 1% Carboxy methyl cellulose (CMC) solution was used as substrate for the enzyme.

**Protein profiling of the sensitive and tolerant isolates: Production of crude protein extract:** YEM broth (100 ml) was

inoculated with the tolerant, sensitive and standard MTCC 905 *Rhizobium* strains, separately and incubated at  $28 \pm 2^\circ\text{C}$  for 48 hours at 80 rpm/minute. After 48 hours the cultures were centrifuged at 6000 rpm for 10 minutes. The supernatant thus obtained was used as crude protein extract.

**Quantitative estimation of proteins:** Protein content was estimated as described by Lowry *et al.*<sup>13</sup>

## Results and Discussion

**Isolation of *Rhizobium* from root nodules:** Present study resulted in the isolation of root nodulating bacterial isolates from *Trifolium alexandrinum* that were collected from different regions of Dehradun. Eighteen *Rhizobium* strains were recovered from root nodule of *Trifolium alexandrinum*.

**Cultural and Biochemical characteristics:** The colonies obtained on YEMA medium after incubation at  $28 \pm 2^\circ\text{C}$  (2 days) having sticky appearance showing production of mucous. Colony morphology indicated round colonies which was white colored till 3-4 days of growth and turned yellowish after 4 days. Colonies diameter was around 5-7 mm Microscopic observation showed that the isolates were gram negative rods<sup>9</sup>. Biochemical characterization revealed that the selected isolates were oxidase, catalase and urease positive. Isolates were able to utilize citrate, which indicated the finding of Lupwayi and Hague<sup>14</sup>. None of the isolate showed growth on medium containing dyes i.e. methylene blue and gentian violet at 1% concentration, which correlates with the earlier studies by Wei *et al.*<sup>15</sup>. All the isolates were able to grow on lactose peptone agar and growth was absent on glucose peptone agar. The isolates were not producing gelatinase enzyme and it is shown by Hunter<sup>16</sup> that negative gelatinase activity is a characteristic of *Rhizobium*. Positive results were obtained when the isolates were subjected to medium containing starch which complements the report of De Oliveria *et al.*<sup>17</sup>. All the isolates showed negative fluorescence assay. Triple iron sugar test indicates positive results for selected isolates<sup>18</sup>. Characteristics similar to commercial strain (MTCC-905) were shown by all the isolates (table -1). From the above isolates only four showed maximum nodulation with *Trifolium alexandrinum*.

**Enumeration of (*Rhizobium*):** In the present study growth of bacteria were measured using two different parameters: cell biomass and cell number.

Optimum growth of different isolates occurred at 50 mg/ml of Zn concentration after 24 (2.56 cfu/ml), 48 (1.47 cfu/ml) and 72 (1.0 cfu/ml) hours of incubation. Isolate R2 showed highest cfu count at all Zn concentrations i.e. 0 mg/ml (3 cfu/ml); 25 mg/ml (3 cfu/ml); 50 mg/ml (2.56 cfu/ml); 75 mg/ml (2.48 cfu/ml) and 100 mg/ml (0.99 cfu/ml), while least count was observed in case of R1 i.e. 0 mg/ml (2.36 cfu/ml); 25 mg/ml (2.20 cfu/ml); 50 mg/ml (1.80 cfu/ml); 75 mg/ml (1.40 cfu/ml) and 100 mg/ml (0.55 cfu/ml), after 24 hours of incubation (figure-1). Similar

observation were recorded after 48 (figure-2) and 72 hours (figure-3) of incubation. The cfu count of all the isolates increased up to 48 hours of incubation, thereafter it decreased. At 72 hours the count was even less than the count observed at 24 hours on incubation. This is in accordance to the work carried out by Bogic *et al.*<sup>19</sup>. The isolated strains of *Rhizobium* showed retardation of growth when the concentration of Zn was further increased beyond 50 mg/ml and it is clearly evident from the results of cfu and biomass. This is due to microbiostatic effect of higher concentration, as evident from the work done by Gauri *et al.*<sup>20</sup>. Maximum growth was recorded by R2 isolate, while it was least in case of R1 isolate, when the isolates were subjected to different Zn concentrations i.e. 0, 25, 50 75 and 100 mg/ml.

**Biomass yield:** Maximum biomass was recorded in case of R2 isolate at all the levels of Zn concentration and minimum in R1 isolate (figure-4). This is in accordance to the increase in cfu count of different isolates at different Zn concentrations similar trend was reported by Angel and Chaney<sup>21</sup> in which lowest *R. meliloti* population density was found in highly contaminated zinc soil and large population were in soils which were moderately contaminated.

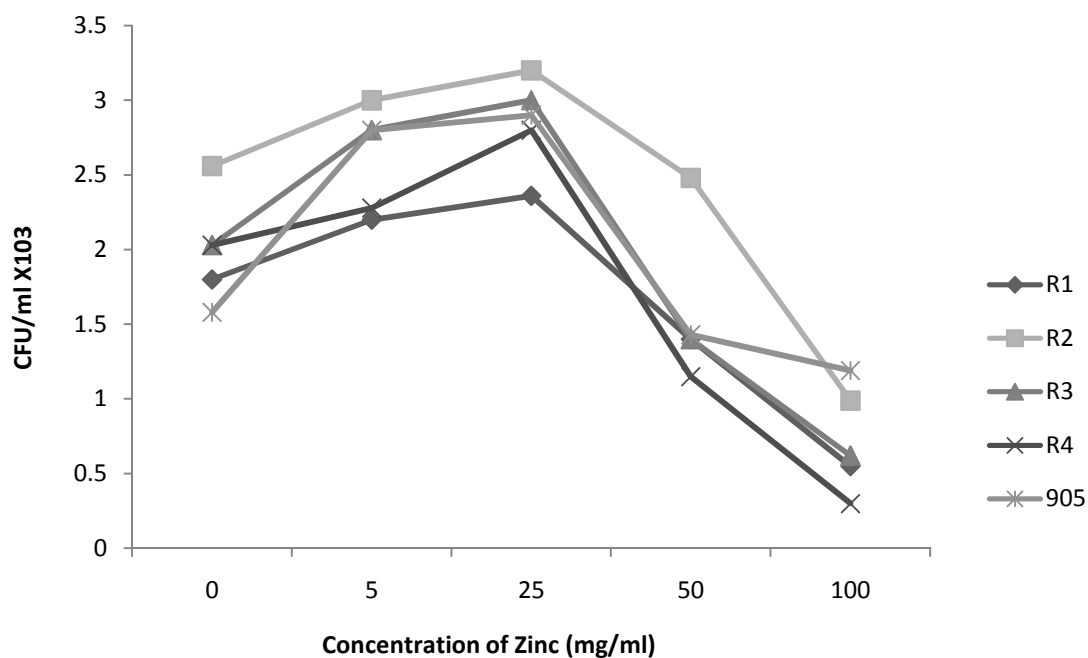
**Estimation of Cellulase and Amylase:** Qualitative analysis as indicated by zone of hydrolysis showed that tolerant strain (R2) produce more amount of cellulase as compared to standard and sensitive strain (R1). Amount of cellulase and amylase produced by tolerant strain is highest followed by standard and sensitive strain (table-2) Result of enzyme estimation revealed that R2 strain produce more amount of cellulase and amylase as compared to standard strain. Least production of enzyme was shown by R1 strain at 50 mg/ml of Zn concentration. In a similar manner Arlem *et al.*<sup>22</sup> also observed effect of Zn on the production of amylase.

**Quantitative estimation of Protein:** Quantitative estimation of protein revealed maximum production by R2 (0.22mg/ml) followed by standard strain (0.20 mg/ml). Protein production was maximum production by R2 (0.22mg/ml) followed by standard strain (0.20 mg/ml). Sofia Isabel *et al.*<sup>23</sup> observed decreases in polypeptide expression in metal tolerant rhizobial isolates of *Rhizobium leguminosarum* biovar *viciae*.

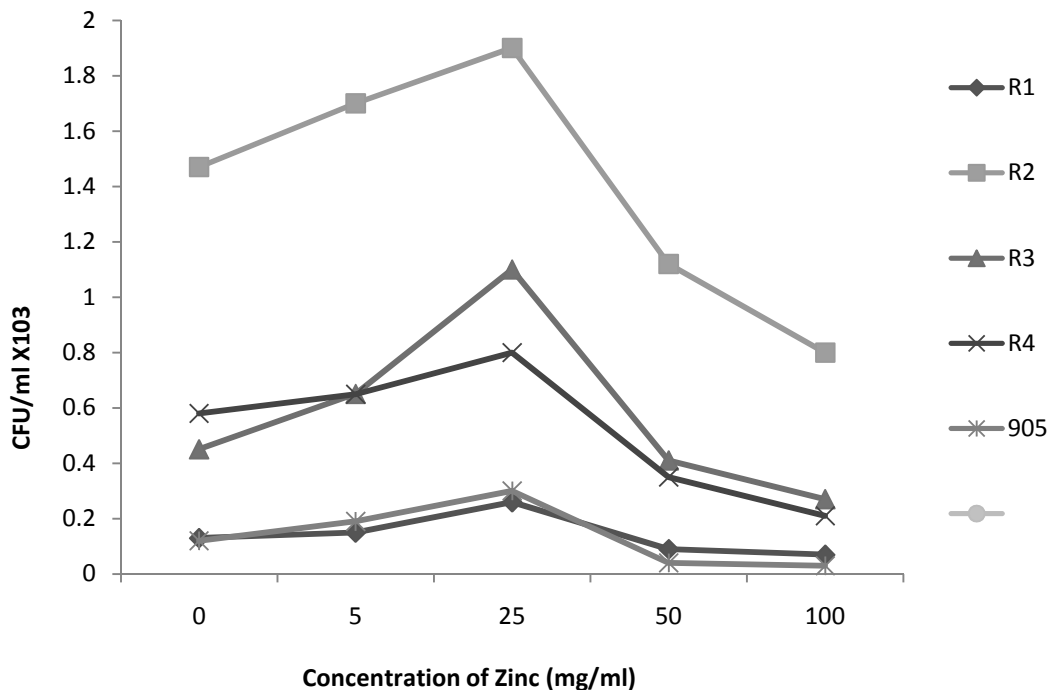
**Protein Profiling:** The protein profiling of the tolerance strain revealed it to be 72.3% similarity with commercial strain (MTCC 905).

## Conclusion

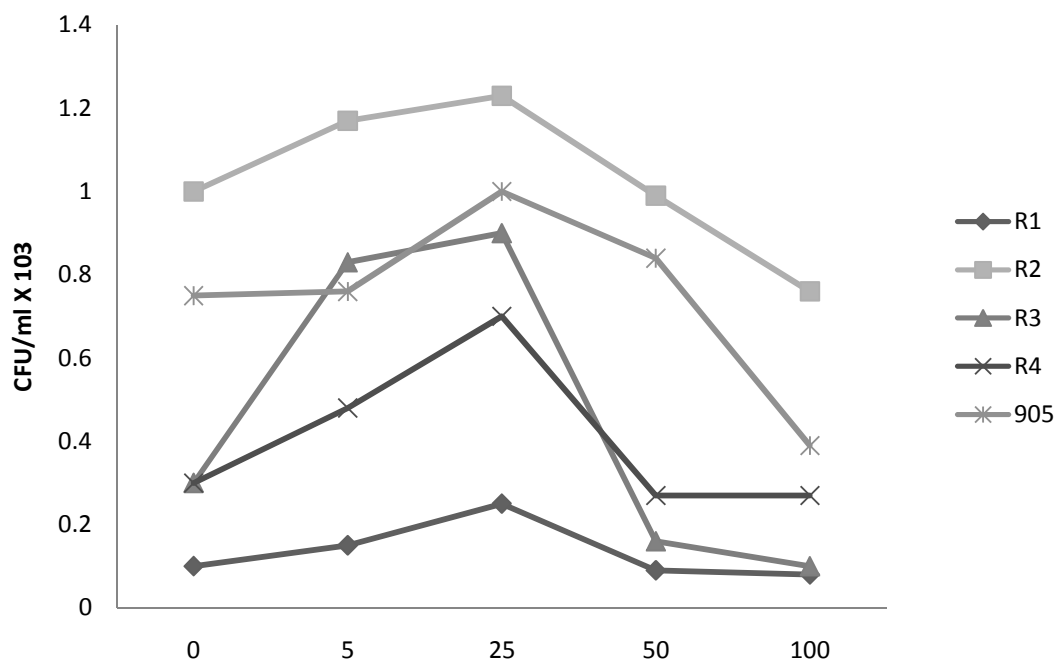
It was clearly evident from the study that although zinc at higher concentration is very toxic to *Rhizobium*, but some strains resist the presence of zinc, by increasing the enzymes production and proteins estimation as shown by the R2 strain.



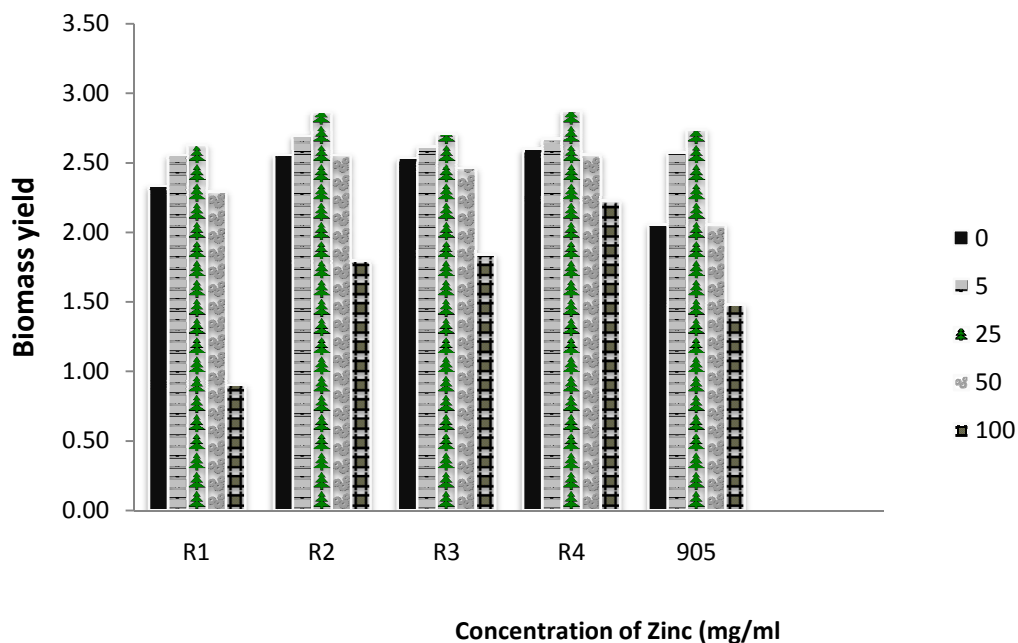
**Figure-1**  
CFU/ml count observed after 24 hour incubation period at different Zn concentrations



**Figure-2**  
CFU/ml count observed after 48 hour incubation period at different Zn concentrations



**Figure-3**  
CFU/ml count observed after 72 hour incubation period at different Zn concentrations



**Figure-4**  
Biomass yield at different Zn concentrations

**Table-1**  
**Biochemical characteristics of Rhizobium isolates recovered from *Trifolium alexandrinum***

Isolate	Characteristics															
	Gram Reaction	Catalase	Oxidase	Acid From Glucose	Growth on GPA	Growth On 0.1 % Methylene Blue	Growth on 0.10% Gentian Violet	Starch Hydrolysis	Urea Hydrolysis	Growth On HAB	Gelatin Hydrolysis	Citrate Utilization	8% KNO <sub>3</sub> Tolerance	2% NaCl Tolerance	Ppt. in Calcium Glycerophosphate	TSI test
R 1	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 2	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 3	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 4	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 5	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 6	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 7	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 8	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 9	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 10	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 11	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 12	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 13	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 14	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 15	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 16	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 17	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
R 18	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K
905	-	+	+	+	-	-	-	+	+	+	-	-	+	+	+	A/K

**Table -2**  
**Qualitative and Quantitative estimation of Cellulase, Amylase and protein**

Isolates	Qualitative (Zone of hydrolysis)		Quantitative (mg/ml)		
	Cellulase	Amylase	Cellulase	Amylase	Protein
Tolerant (R2)	+++	+++	1.03	2.68	0.22
Sensitive (R1)	+	+	0.73	2.63	0.18
Standard (MTCC 905)	++	++	0.96	2.65	0.20

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