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Characterization of zinc solubilization and organic acid detection in *Pseudomonas* sp. RZ1 from rice phyllosphere

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Abstract
A number of sixty four bacterial isolates from rice phyllosphere region were screened for dissolution of zinc under *in vitro* studies. Among them the isolate RZ1 showed positive results for solubilization of zinc components was further characterized and identified into *Pseduomonas* sp. The isolate showed sharp decline in the pH of the growth medium after 12 hrs of incubation and the titratable acidity was measured as 1.44 mg/ml after 48 hrs of incubation. The culture filtrate of RZ1 isolate subjected to HPLC, GC-MS and LC-MS indicating the presence of acetic acid and gluconic acid in the extracellular supernatant.

Keywords: *Pseudomonas*, gluconic acid, HPLC, MS, zinc

Introduction
Zinc is considered as a trace element and generally found in the range between 10-300 ppm in wide range of soils. In the soil it is predominantly adsorbed to solid surfaces results in crop deficiency to zinc nutrition (Lindsay, 1972) [7]. The influence of zinc in agriculture and its impact in livestock production is enormous. The zinc nutrition is not only important for green plants but also for livestocks in poultry, swine and cattle (Nielsen, 2012) [10]. Zinc play a vital role in plant metabolism involving photosynthesis, protein synthesis, phytohormone activity, disease resistance, carbohydrate metabolism, gene expression, enzyme activation, fertility and seed production. The availability of zinc is considerable low in half of the arable soil results in poor yield and hampered crop growth (Sadeghzadeh and Rengel, 2011) [11].
Soils with improved microbial activity favouring better uptake of zinc nutrient by the crop plants. The microbial activity improves the dissolution of insoluble zinc components into plant uptake form by production of complex organic acids. A number of literatures reported the solubilization of insoluble zinc components including zinc oxide, zinc carbonate and zinc phosphate by plant growth promoting rhizobacteria (Saravanan *et al.*, 2003; Sarathambal *et al.*, 2010; Goteti *et al.*, 2013; Krithika and Balachandar, 2016) [15, 13, 4, 5]. Based on the experiments Whiting *et al.* (2001) [18] reported that the microbial activity in the rhizosphere region enhances the uptake of zinc by *Thlaspi caerulescens* into 4-fold compared to axenic control.
Shaikh and Saraf (2017) [16] evaluated the zinc solubilization potential of three bacterial and four fungal isolates with enrichment of growth medium. Apart from zinc solubilization the isolates MSSZB4 and MSS-ZF3 also produce higher quantity of growth promoting substances in the presence of 0.1% ZnO, dextrose as carbon source and ammonium sulphate as nitrogen source in the growth medium. Wang *et al.* (2013) [17] reported that the well-known phosphorus solubilizing bacteria (PSB) *P. fluorescens* and *P. poae*, isolated from dripping water in Heshang cave, Central China are observed to solubilize $Zn_3(PO_4)_2$ with an efficiency of 16.7% and 17.6%, respectively.
In this present study several bacterial strains were isolated from rice phyllosphere region and evaluated for zinc dissolution under *in vitro* studies. The RZ1 isolate showing zinc solubilization potential was further characterized for production of organic acids by chromatographic techniques. The results of the experiments are presented in this paper.

Materials and methods
Microbial culture
The bacterial strain used in this present study were isolated from rice phyllosphere region and grown in Nutrient agar slants for five days at 28ºC and maintained under refrigerated conditions.

Chemicals

The chemicals used in the present study were of analytical reagent grade. It was purchased from Himedia, Sigma, Qualigens, and SD Fine Chem., India.

Screening of zinc solubilization by rice phyllosphere bacterial isolates

Totally sixty four bacterial strains isolated from rice phyllosphere region were screened for dissolution of zinc by the procedure described by Fasim *et al.* (2002) [3] under *in vitro* conditions. Three different components of zinc respectively zinc oxide, zinc carbonate and zinc phosphate were used separately for testing the dissolution. The each zinc components were added to the Bunt & Rivera medium separately at 0.1 percent level and evaluated for zinc dissolution.

The sterilized Bunt and Rivera medium was poured into the petriplates and allowed to solidify. Then each petriplate was divided into four equal quarters. Using a sterile cork borer, wells of 6 mm diameter were made in the plate containing the media. For each organism, 10 µl of the test sample was loaded in each well. Three replications were maintained for each treatment. For each test sample the negative control (three replications each) were also loaded in a separate well. The plates were incubated for a week and the observations were taken periodically. The observations were made by measuring halo like area which indicates the dissolution of zinc around the well.

Identification of bacteria by 16S rRNA sequencing

The RZ1 isolate were initially categorized by preliminary microscopic examination after gram staining and employing various biochemical tests. Thereafter the RZ1 isolate identified by molecular technique using 16S rRNA sequencing studies. The total genomic DNA from the RZ1 strain was isolated using the method given by Marmur (1961) [8] with slight modifications. The genomic DNA subjected to PCR using the forward primer 5'-AGAGTTTGATCCTGGCTCAG -3' and reverse primer 5'-ACGGCTACCTTGTTACGACTT-3' for the amplification of 16S rRNA gene.

PCR products were sequenced through single pass analysis from forward and reverse direction (Sanger *et al.*, 1977) [12]. Sequence data was compared with available data by BLAST analysis in NCBI sequence data bank. Relevant sequences were collected and data was plotted with Mega 7.0 software and phylogenetic tree was constructed (Kumar *et al.*, 2016) [6].

Detection of organic acids by chromatographic techniques

The promising zinc solubilising isolate RZ1 were further characterized for their ability to produce organic acids by several techniques. The drop in pH of the broth, titratable acidity and chromatographic detection of organic acid were evaluated to found out the zinc dissolution ability by the selected isolate. The RZ1 isolate were inoculated into nutrient broth and the above said parameters were evaluated periodically. The titratable acidity was calculated by following the procedure of AOAC (Association of Official Analytical Chemists) method (AOAC, 1990) [1].

The analysis of organic acids was carried out using HPLC and GC-MS techniques. The extracellular fraction obtained from zinc solubilising isolate RZ1 were subjected to HPLC separation using the mobile phase Acetonitrile: water (80:20), with a flow rate of 1 ml per minute and the detection wavelength set at 210 nm. The organic acid standards like acetic acid and gluconic acid were separately analyzed for HPLC detection for comparison. The samples were derived from growth medium after 72 hours of incubation and tested in HPLC after filtration using 20 µm membrane filters.

The Gas chromatography mass spectrometry (GC-MS) was performed for the culture filtrate of RZ1 isolate with helium as carrier gas (Model, Agilent Technologies GC 7890B, mass spectrometer 5977A). The samples were analysed in GC-MS with a source temperature of 200ºC and ionizing voltage of 70 eV and operated in a scan mode (50–700 m/z) using a temperature gradient of 70-260ºC (Saravanan *et al.*, 2007) [14].

The liquid chromatography mass spectrometry (LC-MS) for the culture filtrate of RZ1 isolate was carried out in Shimadzu LC-8040 system with injection volume of 10µl. The mobile phase Acetonitrile: water (80:20) used for the separation in a C_{18} reverse phase column. The separated fractions were analyzed for their mass using LC8040 mass spectrometer *via* Electrospray Ionization Interface (ESI) in both positive and negative ion modes.

Results and discussion

Zinc is an important component of enzymes and phytohomones associated with photosynthesis and growth of the plants. Deficiency of plants to zinc nutrient is wide spread in calcareous, water logged and phosphorus enriched soil. In those soils the zinc made complex with clay minerals, phosphates and become unavailable for plant uptake (Nielsen, 2012) [10]. Microbial activity in the rhizosphere region enhances zinc solubilization by a number of mechanisms including organic acid production, proton extrusion and excretion of chelating agents (Goteti *et al.*, 2013) [4].

In this present study sixty four isolates of bacteria from rice phyllosphere screened for zinc solubilization under *in vitro* condition. The isolate RZ1 showed positive results for zinc dissolution of all the three tested zinc components *viz.*, zinc oxide, zinc carbonate and zinc phosphate (Fig. 1) compared to all other isolates. The RZ1 isolate utilized the insoluble zinc component in the order of preference of zinc oxide, zinc carbonate and zinc phosphate respectively. The dissolution of zinc observed as clear halo zone around the colony after the incubation period of 3 days. The elite RZ1 isolate further taken for culture identification and characterization studies.

The RZ1 isolate observed as short rods under phase contrast microscope and showed gram negative reaction in staining. Further the isolate RZ1 identified into *Pseudomonas* sp. based on 16S rRNA sequencing studies. The obtained gene sequence was analyzed using BLAST software in GenBank website and the phylogenetic tree were constructed using Mega 7n software (Fig. 2).

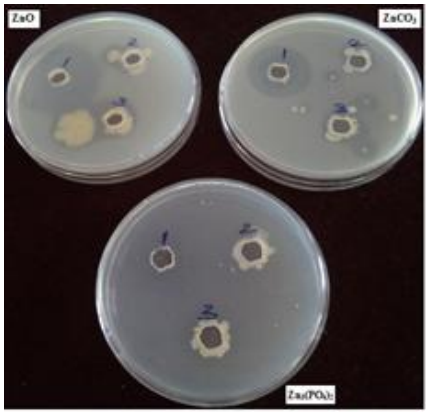


Fig 1: Zinc dissolution by RZ1 isolate

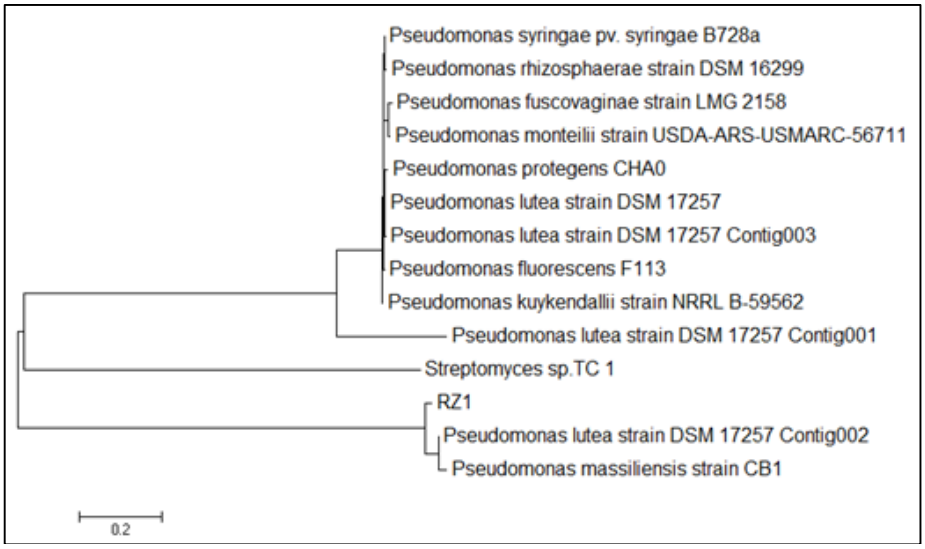


Fig 2: Phylogenetic tree of RZ1 isolate

Characterization of RZ1 isolate

The plausible mechanism of zinc dissolution by RZ1 isolate evaluated by a number of means. The RZ1 isolate inoculated broth periodically observed for pH and titratable acidity. The initial broth pH of 6.5 reduced to 3.0 after 48 hours of incubation. The titratable acidity was measured at 1.44 mg/ml after 48 hours of incubation (Table 1). The steep drop in pH and increase in titratable acidity observed after 12 hrs. of incubation. Hence the organic acids produced by the RZ1 isolate might be reason behind pH drop and also for zinc dissolution. To this contrast the solubilization of zinc achieved by a bacterium of forest soil *Pseudomonas fluorescens* 3a by increase in the H^+ concentration of the medium. In the presence of zinc phosphate the secondary production of gluconic acid by *Pseudomonas fluorescens* 3a were observed in higher quantities. The gluconic acid production and ammonia assimilation probably the main reasons behind decline in pH of the growth medium favouring zinc solubilization (Di Simine *et al.* 1998) [2].

Mishra *et al.* (2017) [9] isolated ten numbers of zinc solubilizing bacterial isolates from rice rhizosphere region. Phylogenetic analysis revealed that four of the elite bacterial isolates belong to phyla proteobacteria and identified into *Pseudomonas aeruginosa*, *Ralstonia picketti*, *Burkholderia cepacia* and *Klebsiella pneumonia* respectively. These isolates able to solubilize both ZnO and $ZnCO_3$ and produce a clear halo zone in the screening experiments. They also observed a positive correlation between zinc solubilization and pH drop in the medium. According to Wang *et al.* (2013) [17] there is a positive correlation observed between aqueous Zn(II) concentration with H^+ activity which confirms the presence of acidification mechanisms widely exploited by PSB. Hence the RZ1 isolate further screened for the production of organic acids by HPLC and GC-MS techniques.

Table 1: Characterization of zinc solubilising isolate

Time interval (in hrs)	pH of the broth	Titratable acidity (mg/ml)
0	6.5	0.00
3	6.0	0.18
6	6.0	0.18
12	5.0	0.54
24	4.0	1.08
30	3.5	1.26
48	3.0	1.44

Detection of organic acids

The culture filtrate of RZ1 isolate were subjected to HPLC separation using the mobile phase Acetonitrile: water (80:20). A number of distinct peaks were observed for the sample and were compared with standard organic acids. The retention time of 1.62 and 2.25 obtained for the culture filtrate were corresponding to the gluconic acid and acetic acid respectively (Fig. 3). Similar kinds of retention time were observed for standard gluconic acid and acetic acid in the HPLC chromatogram (Fig. 4).

The GC-MS analysis of the culture filtrate produce distinct peaks in chromatogram and produce respective mass spectrum corresponding to gluconic acid and acetic acid. The fragmentation ions produced at 177, 147, 133, 119, 105, 73, 55, 44 in mass spectrum indicated the presence of gluconic acid in the culture filtrate. Whereas the fragmentation ion produced at 60 and 43 indicated the presence of acetic acid (Fig. 5). In the GC-MS analysis the gluconic acid observed in gluconolactone form as the sample undergoes complete dehydration during sample injection. Saravanan *et al.* (2007) [14] reported the zinc solubilization potential of *Gluconoacetobacter diazotrophicus* and GC-MS analysis of the culture filtrate produce distinct fragmentation ions of 147, 133, 119, 73 corresponding to 5-ketogluconic acid. The similar fragmentation pattern observed for the RZ1 isolate confirms the presence of gluconic acid in the culture filtrate.

The gluconic acid production by the RZ1 isolate thus confirmed by HPLC and GC-MS analysis. Further it was analyzed using LC-MS for confirmation. The culture filtrate produces mass spectrum with m/z 195 corresponds to gluconic acid in LC-MS chromatogram. The retention time of 1.65 were also observed in the total ion chromatogram (TIC) for the m/z 195 corresponding to gluconic acid (Fig. 6). The gluconic acid undergoes M^- ionization by ESI probe and the mass spectrum observed under negative ionization mode. Hence, all the chromatographic techniques confirm the production of gluconic acid by *Pseudomonas* sp. RZ1.

The above experiments and its result suggest the possible nature of zinc solubilization by *Pseudomonas* sp. RZ1 isolate. Hence, this isolate can be efficiently used for solubilization of insoluble zinc and exploited for field application.

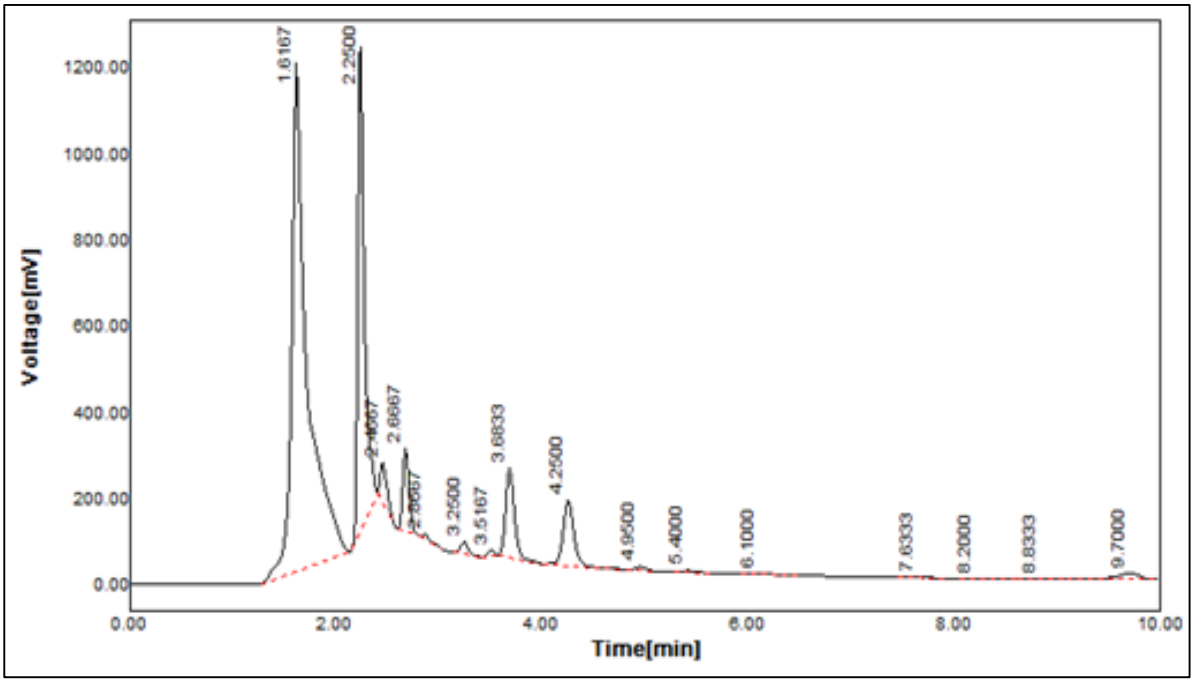
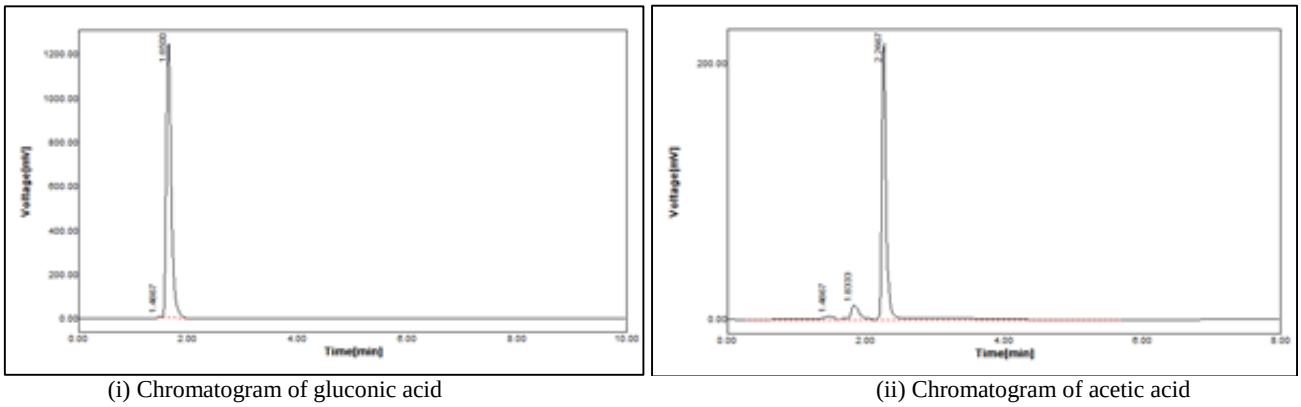
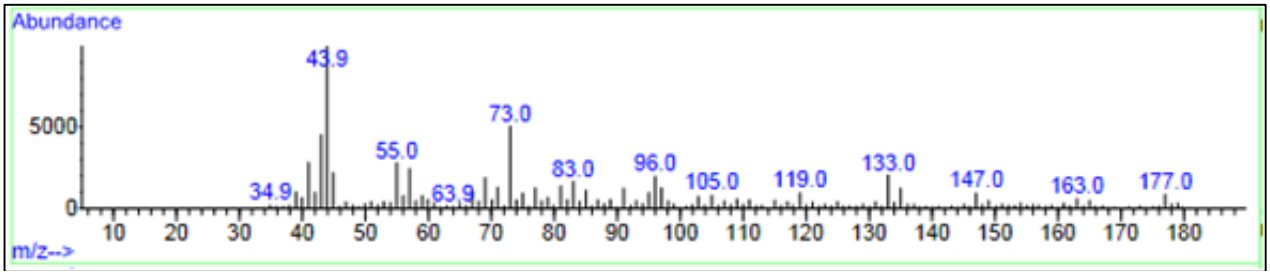


Fig 3: HPLC chromatogram of culture filtrate of RZ1 isolate

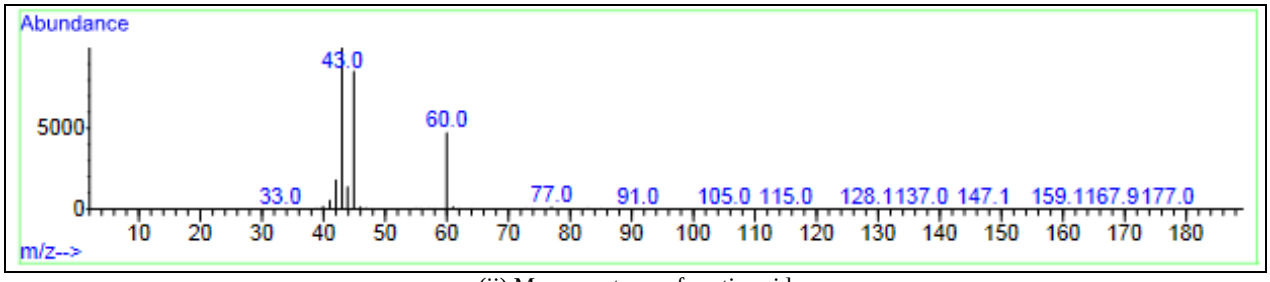


(i) Chromatogram of gluconic acid (ii) Chromatogram of acetic acid

Fig 4: HPLC chromatogram of standard organic acids

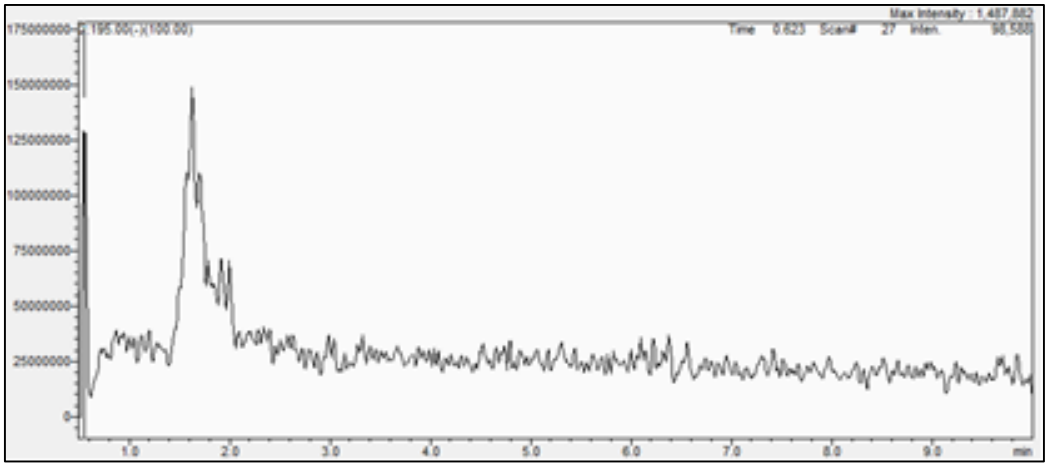


(i) Mass spectrum of gluconic acid

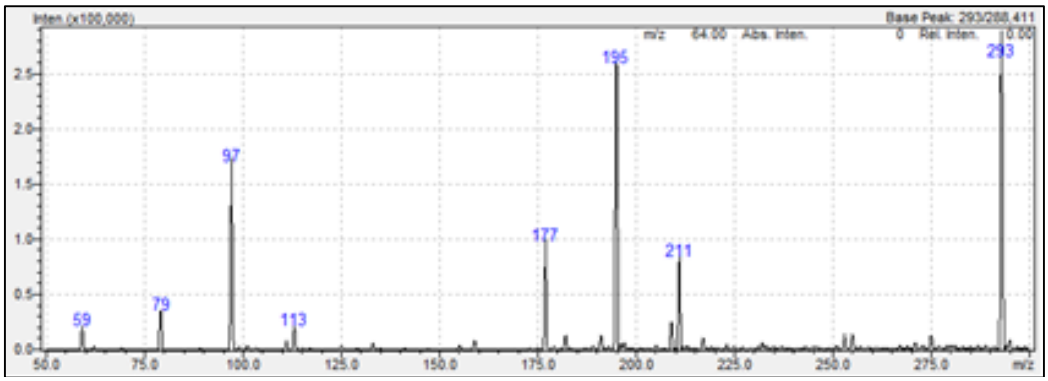


(ii) Mass spectrum of acetic acid

Fig 5: GC-MS chromatogram of culture filtrate of RZ1 isolate



(i) Total Ion Chromatogram of gluconic acid



(ii) Mass spectrum of gluconic acid

Fig 6: LC-MS chromatogram of culture filtrate of RZ1 isolate

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