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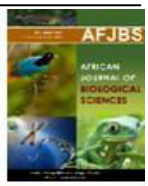

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# Identification And Structure Prediction Of Potentially Active Antimicrobial Molecule Isolated From Soil Of Dibrugarh, Assam.

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**ABSTRACT:**
The present work is carried out to isolate antibiotic producing soil bacteria from Dibrugarh district and elucidate the structure of the metabolite. 100 samples were collected from 10 different zones of the district and serial dilution upto $10^{-9}$was done. Samples were cultured in different culture media. Total 160 isolates were isolated of which 147 are bacteria and 13 are fungi. Among all these two potentially viable isolates namely NAA and EMBA were selected for further studies. The molecular identification by sequencing of the 16S rDNA gene confirms that the isolates NAA and EMBA were strains of *Enterobacter cloacae* with 95% and *Klebsiella pneumonia*e with 92% similarity . The 16S rDNA sequence of the isolate NAA and EMBA were compared with that of other bacterial sequence by the way of BLAST and phylogeny was constructed. Our findings are in agreement with the earlier identification of *Enterobacter* and *Klebsiella* strains( Zhou et al.,2018). On the basis of sequence alignment it was found that *Enterobacter cloaca*e culture clone CLM107 and *Enterobacter cloacae* strain VITADJ are 99% similar with our sequence. The selectively amplified 16S rDNA gene sequences from total genomic DNA by PCR with the help of universal primer helps in construction of phylogeny. The obtained 16S rDNA sequence were deposited in NCBI GenBank and accession numbers for NAA was KX859176 and for EMBA was KY111020. The extract of isolate NAA was found to be more active than the isolate EMBA and further LC-MS,FTIR and NMR study of the extract NAA was done. The molecular weight of the extract is 582.6. The molecule contains a **furan ring** which is responsible for its antimicrobial activity. In the subtropical regions of the world, around 20,000 deaths per year were due to parasitic bacterial infections(Rani et al.,2015).In spite of different available antibiotics in the market , there is an ample requirement for novel antibacterial agents in view of rapid developing of antibiotic resistant bacteria(Sekhar,2010).Keeping this in mind our finding of new molecule can serve as a new potent source of antibiotic in the field of antibiotic study.

**Keywords**: alignment, strains, *Enterobacter cloacae, Klebsiella pneumonia*e, GenBank, furan ring.

## Introduction:

Soil, the surface layer of earth on which the human civilization depends for its existence. Biodiversity of soil means the global environment encompassing distinct communities of organisms (lithosphere). In case of soil organisms basically there are two divisions—first, those organisms which can be studied satisfactorily only with a microscope and under this comes bacteria, cyanobacteria, micro fungi, microalgae and protozoans. Bacteria constitute most dominant group of microorganisms in soil and probably equal to one half of the microbial biomass

of the soil. The greatest amount of them is found generally in the top layer of soil upto a depth of 5-15 cm. The most commonly occurring soil bacteria belonging to the genera *Pseudomonas*, *Arthobacter*, *Clostridium*, *Baccilus*, *Micrococcus* (Waites et al, 2008). Antibiotics (gk:anti-against,bios-life)constitute the major part of chemotherapeutic agents of modern times that are used for treatment of diseases. They are defined by S.A.Waksman (1945) as "chemical substances produced by microorganisms which can kill or inhibit growth of other organisms at a very low concentration".Antibiotics are the most important bioactive and chemotherapeutic compounds made by microbiological synthesis Among the microbial secondary product, antibiotics are the most important organic compound which plays an important role in biological control. Antibiotic producing process, which is known as antibiosis is where the metabolic products of one organism directly inhibit the growth of other pathogenic organisms (Thomashow and Weller, 1995). Antibiotics no matter how they are released may cause extensive antibiotic resistance building up in soil. Numerous antibiotic resistant bacteria (ARB) have been detected and isolated from soil samples. Many investigators have shown that compounds, that are produced by soil microbes are selective in their action; others, such as penicillin, inhibit the growth of a wide variety of microorganisms. Antibiotic resistant genes (ARGs) have also been widely detected in soil in past few decades. (Jechalke *et al*.,2014).Antibiotic resistant bacteria and genes in soils have the potential to pose risk to human health(Richard *et al*.1998) as susceptible pathogenic bacteria can become resistant by acquiring resistance genes in the environmental media or other microorganisms that are already resistant. At elevated concentrations of ARB and ARGs, therapeutic potential of antibiotic against pathogens may be compromised as the likelihood of pathogens to acquire resistance increases.

The NE region of India comprises the states namely Arunachal Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura and Sikkim can be physiologically categorized Eastern Himalayas, Northeast Hills and Brahmaputra and Barak Valley plains. At the confluence of the Indian-Malayan, Indo-Chinese and Indian bio geographical realms, the NE region is unique in endemism. The NE region has been under the focus for its high biodiversity content. The WWF has identified the Entire Eastern Himalayas as a priority Global 200 Eco region; the Conservation International up scaled the Eastern Himalaya 'hotspot' to Indo Burma Hotspot which now includes all the eight states of North East India. The Eastern Himalaya Biodiversity 'hotspot' which has been currently scaled up as Indo Burma hotspot is the second largest in the world.

This region is characterized by the presence of pristine natural ecosystems which are yet to be exploited by anthropogenic activities. The pristine area along with some extreme habitats are bound to provide scope for discovery of novel organisms yet to be known to the scientific community and waiting to be beneficial for human race. The undulating contour with great variation in altitude, soil types, vegetation are the main reason behind the richness in diverse groups of flora and fauna provided by a plethora of habitats harbouring a genetic treasure house of plant, animal and microbial resources. Although there have been substantial amount of scientific literature generated on flora and fauna of the region, the microbial documentation and exploration still is very scarce and calls for a concerted attention of microbiologists. The microbial diversity from the diverse ecological niches of the Indo-Burma biodiversity hotspot holds promise for isolation of biotechnologically significant strains as well as novel species, and provide vistas for studies on the micro flora of the biodiversity rich region of the lower belt of eastern Himalayas(Joshi et al.,2015).Regarding Assam, except few exceptions the major soil are acidic. The Brahmaputra valley soil can be classified as new alluvial and old alluvial soil types. The new alluvial soil again can be classified as loamy soil, sandy soil and clayey soil (Taher,2016).This varied types of soil under the influence of hot and humid climatic condition is harbouring large array of micro fauna.

The emerging problem of antibiotic resistance (AMR) is a serious threat to global public health (Hogberg,2010) and the multi-resistant bacterial strains worldwide is placing a significant burden on healthcare systems and society.(Hawkey,2008).According to World Health Organisation (WHO) AMR as "so serious that it threatens the achievements of modern medicine". The need for this study includes these vital points(According to Health Technology Assessment, HTA: Schaffer, 2017)

## MATERIALS AND METHOD

**Study area**:

The study area i.e. Dibrugarh district was devided to 10 different quadrents (A,B,C,D,E,F,G,H,I,J) with their co-ordinates depicted in the table.Collection of soil sample is done following judgement sampling sampling method which comes under non probability sampling method.

**Soil samples collection:**

A total of 100 soil samples were collected randomly from different habitats of various ecological niches of Dibrugarh district. The location of the sample collection sites are shown in figure. The soil samples were collected after removal of 8-10 cm of surface soil and brought to the laboratory in sterile safety bags.

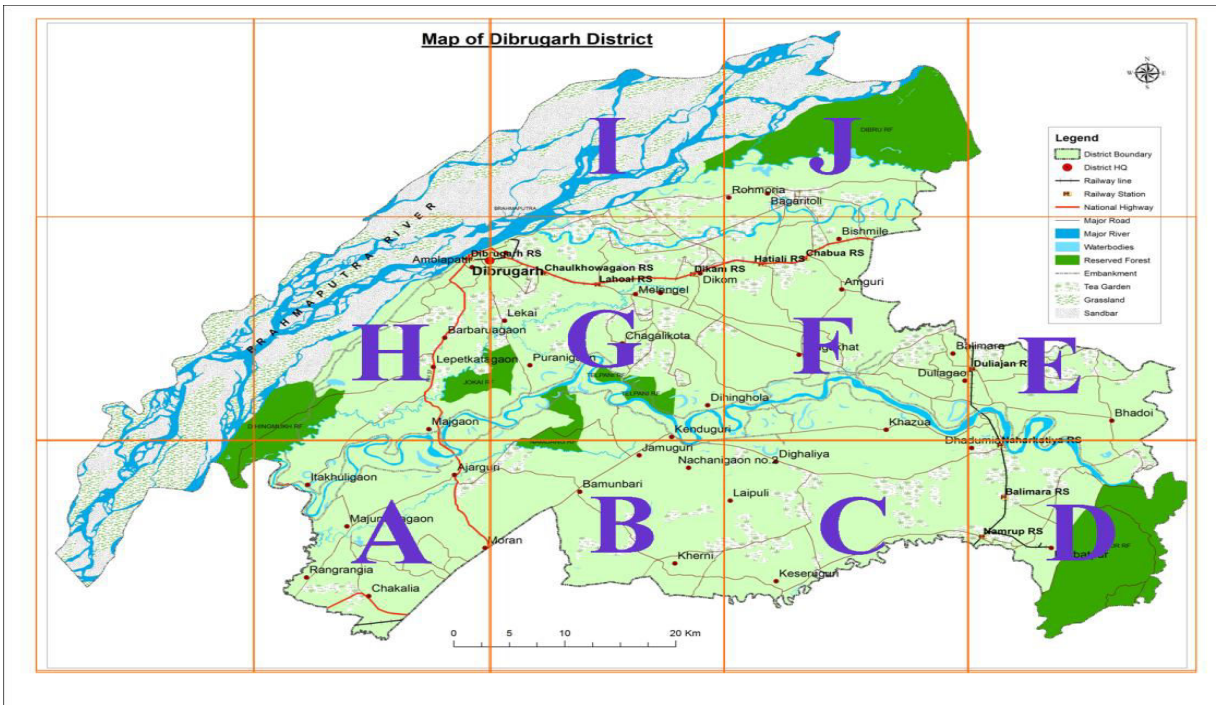



Fig1:Map of Dibrugarh District

Bacterial strains were isolated from collected samples by the standard serial dilution spread plate technique method mentioned in the manual of Microbiological Methods (Buchanan and Gibbon,1974) using different media composition. Ten gram of soil sample from each quadrants of the study area were powdered and mixed well.

Antimicrobial activity of the isolates were determined by disk diffusion method (N.G.Heatly,1944) against following test organisms Characters of the 4 test organisms

| NAMES | MTCC | GRAM REACTION |
|---|---|---|
| *Pseudomonas aeuroginosa* | 4673 | *Gram negative* |
| *Escherichia coli* | 40 | *Gram negative* |
| *Bacillus subtilis* | 121 | *Gram positive* |
| *Saccharomyces cerevisiae* | 3090 | *Both positive and negative* |

**Fig2: Antimicrobial activity of different fractions of 6 active isolates against 10 test organisms**

| Name of the test organism | | *S. cerevisiae* | *E.coli* | *B.subtilis* | *P.aeruginosa* | *C. acetobutylicum* | *S. ficaria* | *E. aerogens* | *A. aceti* | *C.albicans* | *S. mutans* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NAA | CB | 28.00± 1.00 | 31.00± 2.00 | 28.00± 3.51 | 29.33± 0.66 | 27.66±1 .25 | 19.66± 1.00 | 19.66± 0.57 | 16.33± 1.00 | 15.33± 0.58 | 23.66± 1.00 |
| | CFCS | 23.66± 1.00 | 27.66± 0.57 | 27.00± 0.36 | 27.00± 0.57 | 25.00±1 .00 | 20.33± 0.57 | 15.00± 1.00 | 15.00± 0.57 | 16.00± 1.00 | 21.00± 1.25 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cell | 21.3±0.57 | 25.00±1.00 | 25.00±1.52 | 23.66±1.25 | 16.33±0.57 | 15.00±1.00 | 16.33±1.25 | 19.66±1.25 | 18.66±1.00 | 20.33±0.36 |
| | Control | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| NAA1 | CB | 19.66±2.51 | 20.66±2.08 | 21.00±1.52 | 20.60±2.51 | | 15.00±1.00 | | -- | -- | 12.33±1.28 |
| | CFCS | 18.00±1.00 | 17.00±1.00 | 17.00±1.00 | 14.00±0.57 | -- | 13.66±0.33 | -- | -- | -- | 14.66±0.57 |
| | Cell | 15.66±1.00 | 14.00±1.75 | 15.00±1.00 | 13.66±1.57 | -- | 12.00±1.00 | -- | -- | -- | 13.00±1.00 |
| | Control | 10.00 | | 10.00 | | | 10.00 | | -- | -- | 10.00 |
| NAA8 | CB | 17.00±1.00 | 19.66±1.52 | 16.00±1.53 | 23.33±1.52 | | | 16.00±1.00 | | 15.33±1.00 | 13.33±1.00 |
| | CFCS | 17.00±0.57 | 12.33±1.25 | 13.00±0.57 | 16.00±1.00 | | | 13.00±1.25 | -- | 16.00±0.57 | 12.66±0.36 |
| | Cell | 18.33±1.28 | 11.66±1.00 | 12.00±1.00 | 13.66±1.00 | | | 11.00±0.57 | -- | 14.66±1.52 | 11.00±1.00 |
| | Control | 10.00 | 10.00 | 10.00 | 10.00 | -- | -- | 10.00 | -- | 10.00 | 10.00 |
| MNS1 | CB | 20.00±1.00 | 16.00±1.00 | 22.33±1.82 | 17.00±1.00 | -- | -- | -- | | -- | |
| | CFCS | 18.33±0.66 | 16.33±1.52 | 22.33±1.82 | 18.00±0.57 | -- | -- | -- | | -- | |
| | Cell | 15.00±0.57 | 11.00±0.66 | 17.66±1.00 | 14.00±0.57 | -- | -- | -- | -- | -- | -- |
| | Control | 10.00 | 10.00 | 10.00 | 10.00 | | -- | -- | -- | -- | -- |
| MNS15 | CB | 20.66±1.00 | 20.00±1.00 | 16.00±1.00 | 16.00±1.00 | -- | -- | -- | 15.33±1.00 | -- | -- |
| | CFCS | 16.00±0.57 | 11.00±0.33 | 11.00±1.00 | 15.00±1.52 | -- | | -- | 16.00±0.57 | -- | -- |
| | Cell | 16.33±0.36 | 13.00±1.00 | 15.00±1.00 | 13.00±1.25 | -- | | | 14.66±1.52 | | |
| | Control | 10.00 | 10.00 | 10.00 | 10.00 | -- | | | 10.00 | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EMBA | CB | 20.00± 1.28 | 25.33± 1.52 | 23.33± 1.00 | 28.00± 1.00 | 21.66±1 .00 | 16.00± 1.00 | 19.33± 1.00 | 18.00± 1.00 | 22.00± 1.00 | 13.00± 1.00 |
| | CFCS | 26.00± 1.00 | 21.00± 1.00 | 27.00± 1.00 | 27.00± 1.00 | 16.00±1 .00 | 12.33± 1.00 | 16.66± 1.00 | 17.00± 1.25 | 19.00± 0.36 | 15.33± 1.00 |
| | Cell | 23.66± 1.28 | 22.00± 1.00 | 28.66 ±1.52 | 28.33± 1 | 17.33±. 1.25 | 14.66±. 36 | 17±1 | 13.33± 1.00 | 17.00± 1.52 | 16±.36 |
| | Control | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |

**Fig:3 Morphological characterisation of most potential isolates NAA and EMBA**

| Tests | NAA | EMBA |
|---|---|---|
| Gram staining | Negative | Negative |
| Spore | Non spore forming | Non spore forming |
| Motility | Motile | Non-Motile |
| Shape | Rod | Rod |

**Fig:4: Growth rating of the isolates NAA and EMBA at different concentration of NaCl**

| Conc. Of NaCl (in %) | Growth rating | |
|---|---|---|
| | NAA | EMBA |
| 0.5 | +++ | ++ |
| 1.0 | +++ | +++ |
| 1.5 | ++ | +++ |
| 2.0 | ++ | ++ |
| 2.5 | ++ | ++ |
| 3.0 | ++ | ++ |
| 3.5 | ++ | ++ |
| 4.0 | + | ++ |
| 4.5 | + | + |
| 5.0 | + | + |
| 5.5 | + | + |
| 6.0 | + | + |
| 6.5 | - | - |
| 7.0 | - | - |
| 7.5 | - | - |

**Fig5:Growth rating of the isolates NAA and EMBA at different temperatures (thermal death point (TDP).**

| Temperature | Growth rating | |
|---|---|---|
| | NAA | EMBA |
| 20 | + | + |
| 25 | +++ | ++ |
| 30 | +++ | ++ |
| 35 | +++ | +++ |
| 40 | ++ | +++ |
| 45 | ++ | +++ |
| 50 | ++ | ++ |
| 55 | ++ | ++ |
| 60 | + | ++ |
| 65 | + | + |
| 70 | + | + |
| 75 | + | + |
| 80 | - | + |
| 85 | - | - |
| 90 | - | - |
| 95 | - | - |

| | | |
|---|---|---|
| 100 | - | - |

**Fig6: Growth rating of isolates NAA and EMBA at different pH level:**

| pH | Growth rating | |
|---|---|---|
| | NAA | EMBA |
| 3 | ++ | + |
| 4 | +++ | + |
| 5 | +++ | ++ |
| 6 | ++ | +++ |
| 7 | ++ | +++ |
| 8 | + | ++ |
| 9 | - | + |

**Fig7: Comparison of Morpho biochemical characters of isolate NAA with *Enterobacter cloaca* and EMBA with *Klebsiella pneumonia***

| Biochemical tests | *Enterobacter cloaca* | NAA | *Klebsiella pneu.* | EMBA |
|---|---|---|---|---|
| Catalase | + | + | + | + |
| Oxidase | - | - | - | - |
| β-galactosidase | + | + | + | + |
| Gas from glucose at 37°C | + | + | + | + |
| Nitrate reduced | + | + | + | + |
| Carbohydrates | | | | |
| Arabinose | + | + | + | + |
| Esculin | - | - | - | - |
| Lactose | + | + | + | + |
| Maltose | + | + | + | - |
| Mannitol | + | + | + | + |
| Sorbitol | + | - | + | - |
| Sucrose | + | + | + | + |
| Trehalose | + | + | + | + |
| Xylose | + | + | + | + |
| Related carbon source | | | | |
| Citrate | + | + | + | + |
| Malonate | + | + | + | + |
| M.R. | - | - | - | - |
| VP | - | - | - | - |
| Protein reaction: | | | | |
| Gelatine hydrolysis | + | + | - | – |
| $H_2S$ from TSI | - | - | - | - |
| Indole | - | - | - | - |
| Ornithine | + | - | - | - |
| Urea hydrolysed | - | + | - | - |
| Cellobiose | + | + | - | - |
| glycerol | + | + | + | + |
| Sorbose | + | + | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NAA | | 0.0027 | 0.0027 | 0.0027 | 0.0029 | 0.0029 | 0.0029 | 0.0029 | 0.0029 | 0.0029 | 0.0029 |
| JF489149.1 | 0.0095 | | 0.0000 | 0.0000 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 |
| HQ686272.1 | 0.0095 | 0.0000 | | 0.0000 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 |
| HQ686278.1 | 0.0095 | 0.0000 | 0.0000 | | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 | 0.0008 |
| KJ543849.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | | 0.0000 | 0.0000 | 0.0011 | 0.0011 | 0.0000 | 0.0000 |
| KJ543843.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0000 | | 0.0000 | 0.0011 | 0.0011 | 0.0000 | 0.0000 |
| KJ543834.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0000 | 0.0000 | | 0.0011 | 0.0011 | 0.0000 | 0.0000 |
| KP305912.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0016 | 0.0016 | 0.0016 | | 0.0000 | 0.0011 | 0.0011 |
| KP305911.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0016 | 0.0016 | 0.0016 | 0.0000 | | 0.0011 | 0.0011 |
| KF828866.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0000 | 0.0000 | 0.0000 | 0.0016 | 0.0016 | | 0.0000 |
| KF434609.1 | 0.0103 | 0.0008 | 0.0008 | 0.0008 | 0.0000 | 0.0000 | 0.0000 | 0.0016 | 0.0016 | 0.0000 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EMBA | | 0.0038 | 0.0038 | 0.0038 | 0.0039 | 0.0039 | 0.0038 | 0.0039 | 0.0038 | 0.0038 | 0.0040 |
| KJ803910.1 | 0.0195 | | 0.0000 | 0.0007 | 0.0012 | 0.0010 | 0.0010 | 0.0010 | 0.0007 | 0.0007 | 0.0012 |
| KJ803908.1 | 0.0195 | 0.0000 | | 0.0007 | 0.0012 | 0.0010 | 0.0010 | 0.0010 | 0.0007 | 0.0007 | 0.0012 |
| KU199000.1 | 0.0202 | 0.0007 | 0.0007 | | 0.0010 | 0.0012 | 0.0007 | 0.0012 | 0.0010 | 0.0010 | 0.0010 |
| KT001207.1 | 0.0209 | 0.0021 | 0.0021 | 0.0014 | | 0.0016 | 0.0012 | 0.0016 | 0.0014 | 0.0014 | 0.0014 |
| KJ803941.1 | 0.0209 | 0.0014 | 0.0014 | 0.0021 | 0.0036 | | 0.0014 | 0.0010 | 0.0012 | 0.0012 | 0.0016 |
| KJ803925.1 | 0.0202 | 0.0014 | 0.0014 | 0.0007 | 0.0021 | 0.0029 | | 0.0014 | 0.0012 | 0.0012 | 0.0012 |
| KJ803920.1 | 0.0209 | 0.0014 | 0.0014 | 0.0021 | 0.0036 | 0.0014 | 0.0029 | | 0.0012 | 0.0012 | 0.0016 |
| KJ803904.1 | 0.0202 | 0.0007 | 0.0007 | 0.0014 | 0.0029 | 0.0021 | 0.0021 | 0.0021 | | 0.0010 | 0.0014 |
| KJ803871.1 | 0.0202 | 0.0007 | 0.0007 | 0.0014 | 0.0029 | 0.0021 | 0.0021 | 0.0021 | 0.0014 | | 0.0014 |
| KJ806501.1 | 0.0216 | 0.0021 | 0.0021 | 0.0014 | 0.0029 | 0.0036 | 0.0021 | 0.0036 | 0.0029 | 0.0029 | |

**Fig:8 6S rDNA sequence of the isolate NAA showed maximum evolutionary relatedness (95%)with *Enterobacter cloaca* NR_028912.1The other isolate EMBA showed maximum closeness (92%) with *Klebsiella pneumonia* (KU 199000.1)**

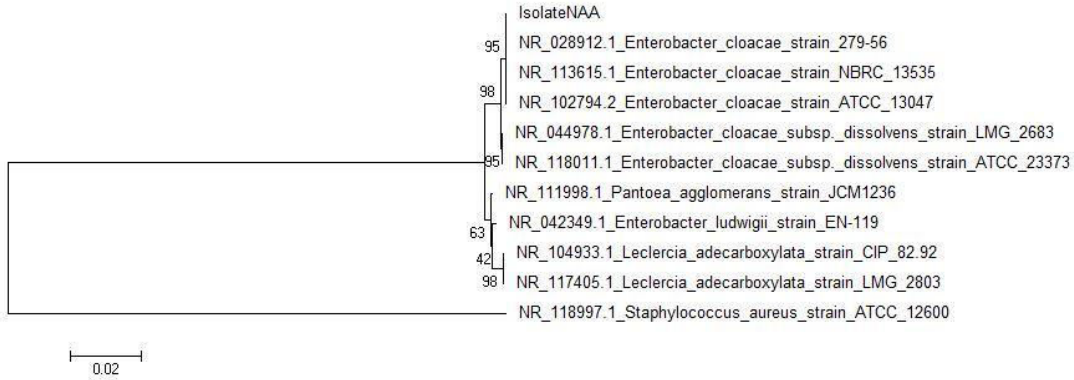



**Fig 9:Phylogenetic relationship of NAA with other most closely related species of *Enterobacter* species based on 16SrDNA sequencing .**

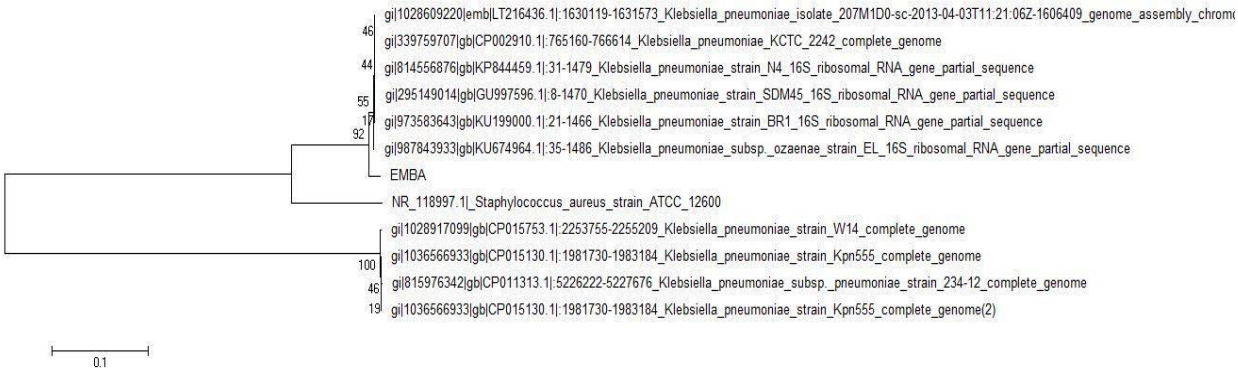



**Fig 10:Phylogenetic relationship of EMBA with other most closely related *Klebsiella* species based on 16SrDNA sequencing**

**Fig:11 Effect of different media on the growth of the isolates NAA and EMBA**

| Media | Zone of inhibition in mm | | | |
|---|---|---|---|---|
| | EMBA | | NAA | |
| | Mean±SD | OD(nm) | Mean±SD | OD(nm) |
| Nutrient Broth | 12.00±3.05 | 0.105 | 32.66±1.25 | 0.297 |
| Luria Broth | 14.70±1.52 | 0.634 | 21.00±0.66 | 0.201 |
| Mullear Hinton | 21.90±1.00 | 0.724 | 18.66±2.08 | 0.162 |
| Starch Casein | 14.50±4.92 | 0.219 | 16.66±0.57 | 0.150 |

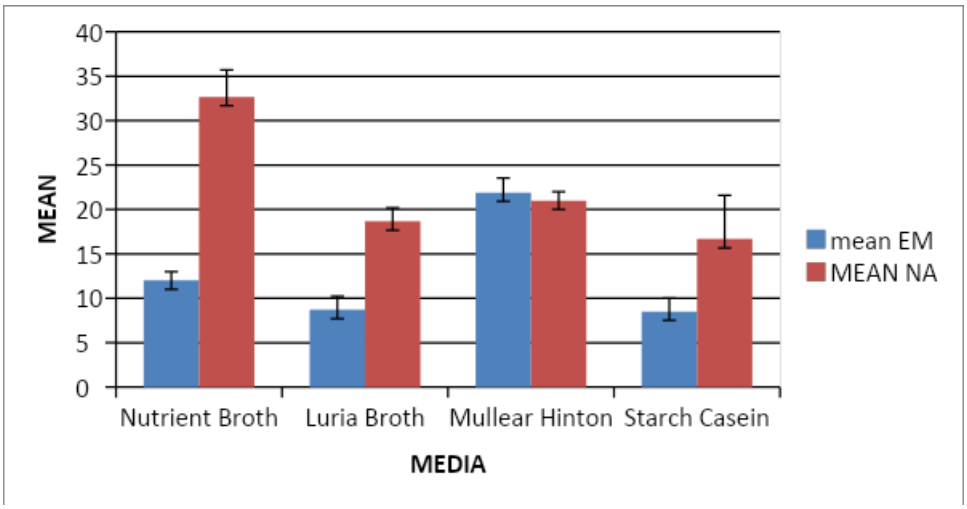



**Fig12: Effect of different media on the growth of the isolates NAA and EMBA**

**Fig 13:Effect of different carbon sources on activity (diameter of zone of inhibition) of EMBA and NAA in mm against *B. subtilis*. Data is presented as mean ±SD of three replicates.**

| Carbon Source | Zone of inhibition in mm | | | |
|---|---|---|---|---|
| | EMBA | | NAA | |
| | Mean±SD | OD(nm) | Mean±SD | OD(nm) |
| Mannitol | 16.00 ±1.28 | 0.306 | 28.00 ±1.00 | 0.219 |
| Glycerol | 13.36±0 .57 | 0.398 | 16.00± 1.00 | 0.186 |
| Sucrose | 12.87± 1.28 | 0.201 | 17.00 ±0.57 | 0.188 |
| Glucose | 22.00± 0.57 | 0.593 | 23.00± 1.00 | 0.198 |
| Fructose | 16.00 ±0.57 | 0.470 | 15.00 ±1.00 | 0.178 |
| Dextrose | 12.00± 1.89 | 0.301 | 14.00 ±0.58 | 0.156 |
| Maltose | 12.00 ±0.57 | 0.291 | 16.00 ±1.25 | 0.180 |

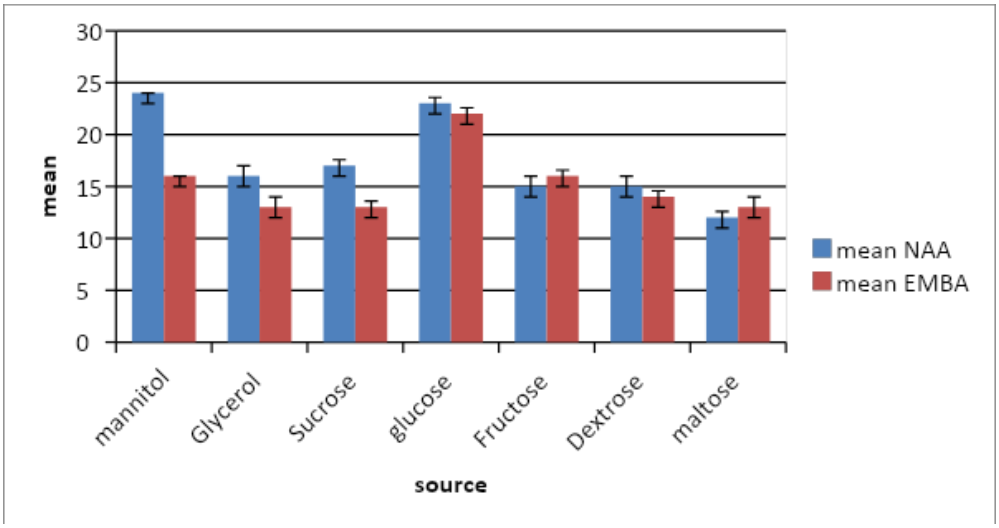



**Fig14: Effect of different carbon sources on activity of EMBA and NAA against B.subtilis**

**Fig 15:Effect of different pH range on the activity (diameter of zone of inhibition) of EMBA and NAA in mm against *B. subtilis*. Data is presented as mean ±SD of three replicates.**

| pH of Media | Zone of inhibition in mm | | | |
|---|---|---|---|---|
| | EMBA | | NAA | |
| | Mean±SD | OD(nm) | Mean±SD | OD(nm) |
| 4 | 13.00±1.28 | 0.564 | 20.00±0.66 | 0.798 |
| 5 | 17.67 ±.1.08 | 0.876 | 27.00±1.33 | 0.989 |
| 6 | 19.67 ±2.00 | 0.901 | 24.00 ±1.28 | 0.905 |
| 7 | 28.00 ±3.05 | 1.108 | 21.00 ±0.66 | 0.801 |
| 8 | 16.67±2.08 | 0.654 | 20.33 ±1.52 | 0.776 |

| | | | | |
|---|---|---|---|---|
| **9** | 15.33± 1.52 | 0.543 | 16.00 ±2.00 | 0.586 |

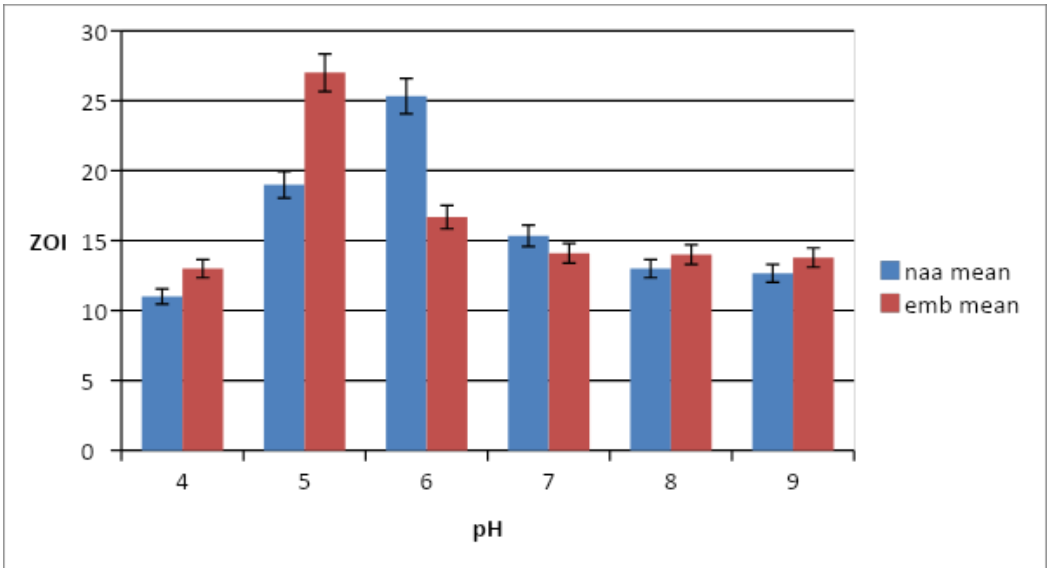



**Fig16: Effect of different pH level on activity of EMBA and NAA against B.subtilis**

**Fig:17 Effect of different temperature on activity (diameter of zone of inhibition) of EMBA and NAA against *B. subtilis*. Data presented as mean ±SD of three replicates**

| **Temperature** | **Zone of inhibition in mm** | | | |
|---|---|---|---|---|
| | **EMBA** | | **NAA** | |
| | **Mean±SD** | **OD(nm)** | **Mean±SD** | **OD(nm)** |
| **20** | 14.12±1.28 | 0.378 | 16.88±0.57 | 0.552 |
| **25** | 19.33 ±1.52 | 0.419 | 25.66 ±1.00 | 0.765 |
| **30** | 22.00± 1.00 | 0.788 | 30.33± 1.52 | 0.874 |
| **35** | 26.33±1.52 | 0.829 | 31.00± 2.00 | 0.889 |
| **40** | 29.46±1.00 | 0.985 | 22.00±1.00 | 0.701 |
| **45** | 25.33±1.28 | 0.801 | 19.00±1.28 | 0.681 |

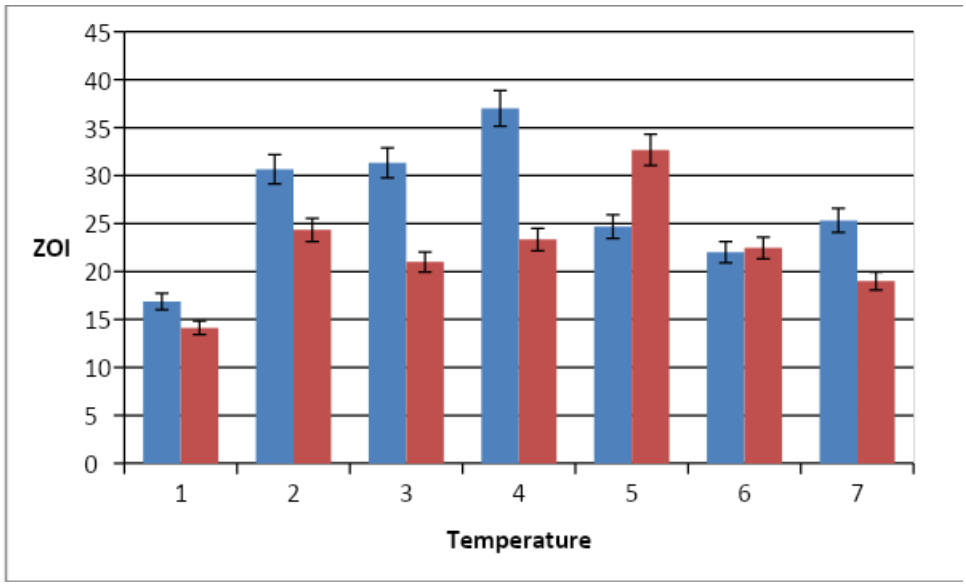



**Fig:18 Effect of different temperatures on activity of NAA and EMBA against B.subtilis**

**Fig:19 Maximum extraction of extract from culture broth using different solvents at 1:1:**

| Solvents (1Litre) | NAA (crude extract) | EMBA(crude extract) |
|---|---|---|
| Ethyl acetate | **16.7mg** | **10.1mg** |
| Hexane | 8.1 | 6.4 |
| Benzene | 11.6 | 8.2 |
| Chloroform | 10.4 | 6.4 |
| Petroleum ether | 9.5 | 5.3 |

**Fig20:Antimicrobial activity of extract of isolates NAA and EMBA against 4 MTCC strains**

| Isolate | MTCC strains | | | |
|---|---|---|---|---|
| | ***B.subtilis*** **MTCC-121** | ***S. cerevisiae*** **MTCC-3090** | ***E. coli*** **MTCC-40** | ***Pseudomonas aeruginosa*** **MTCC-4673** |
| **NAA** | 29.66±1.00 | 28.33±0.36 | 26.33±1.52 | 29.33±0.57 |
| **EMBA** | 12.00±0.57 | 14.00±0.33 | 12.00±1.00 | 14.00±1.00 |

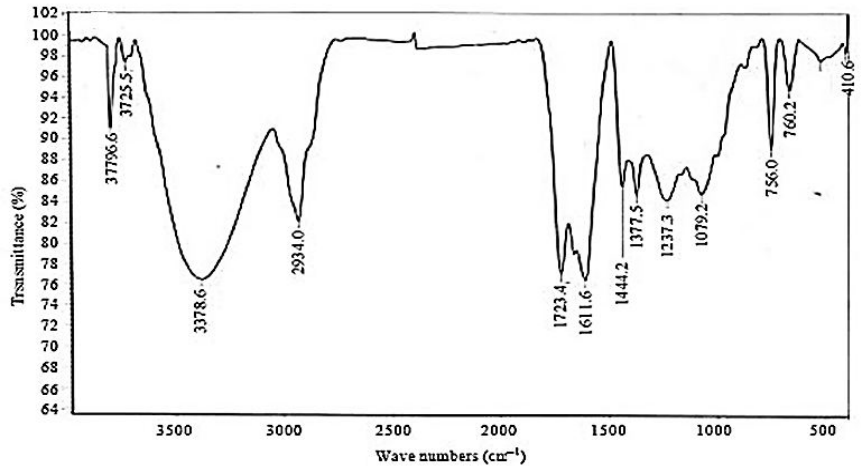


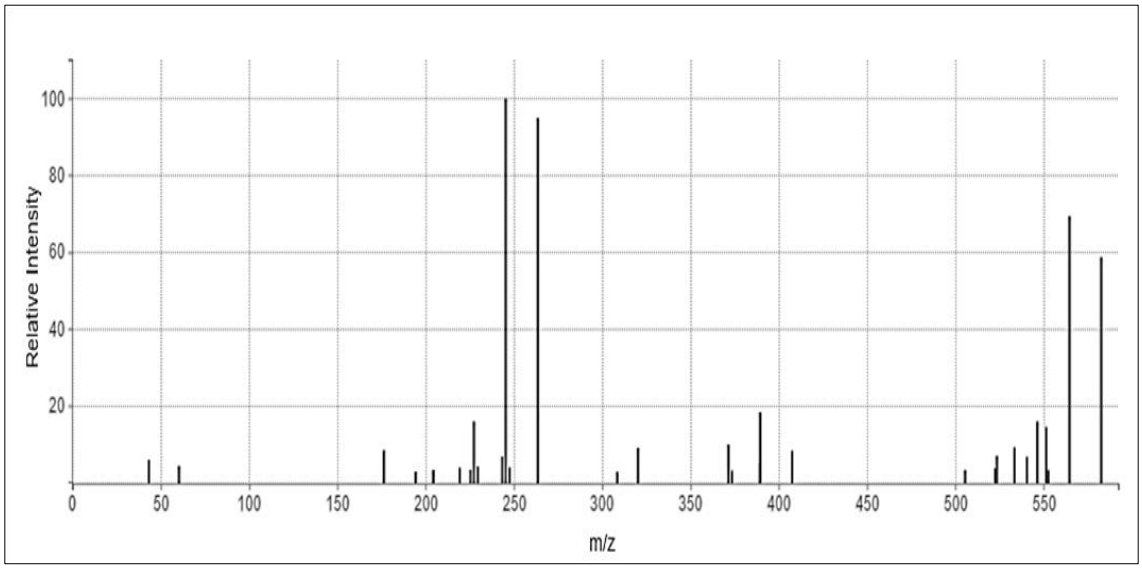

**Fig:21 FTIR analysis of active TLC spot (Rf=.72cm) of isolate NAA'**

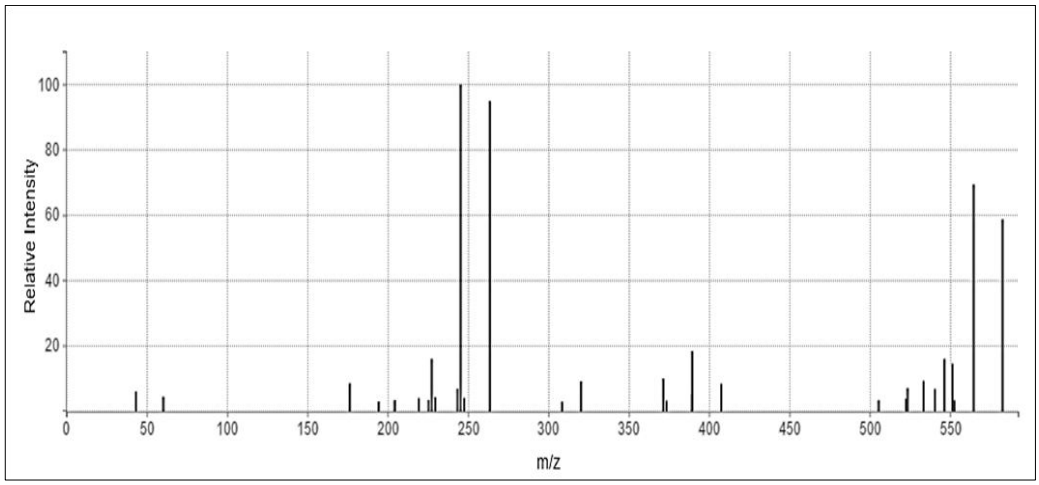
**Fig:22 Table for FTIR data of Bioactive compound of NAA**

| | | |
|---|---|---|
| 3378, 2934, 1723 | Carboxylic acids C=C-CO-OH | OH dimer |
| 1611 | Alkene (five ring structure) | C-H Stretch |
| 1444 | Aromatic (C-C ring) | Aromatic C-C stretch |
| 1377 | Alkane ($RCH_2CH_2$) | C-O Stretch |
| 1237, 1079 | Carboxylic acid (RCO-OH) | C-O Stretch |
| 760 | Aromatic (Trisubstituted) | C-H out of plane |

**Fig23: LC-MS spectral analysis of isolate NAA**

| |
|---|
| 43.0 |
| 60.1 |
| 176.1 |
| 245.1 |
| 263.1 |
| 320.1 |
| 371.2 |
| 389.2 |
| 407.2 |
| 551.2 |
| 552.3 |
| 564.3 |
| 582.3 |



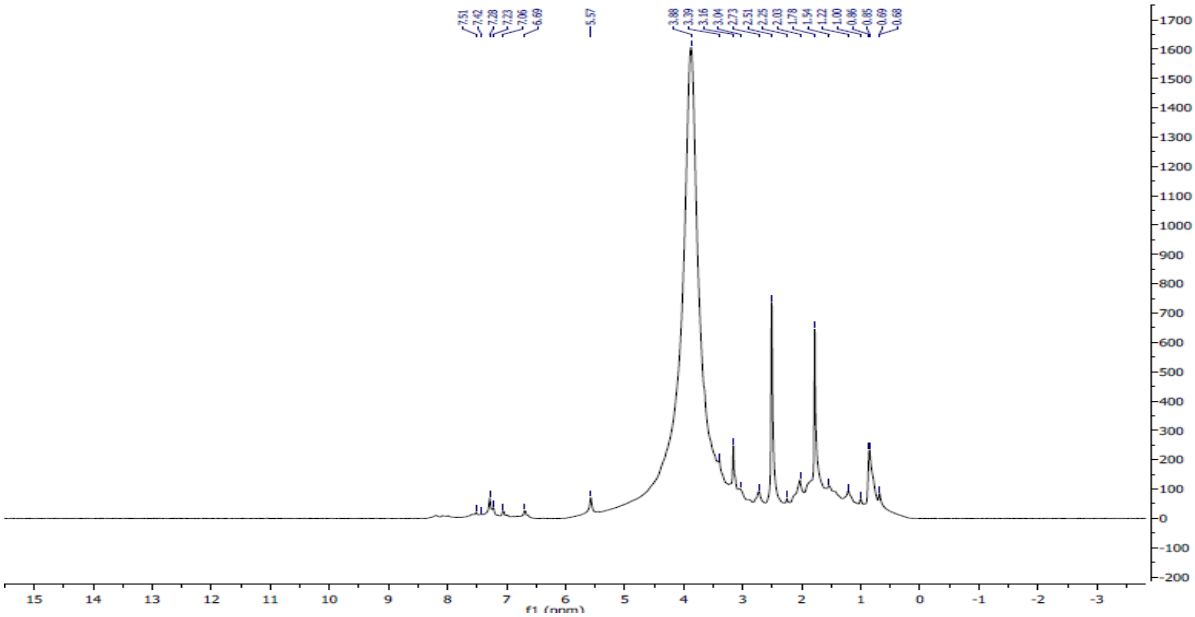


**Fig24:$^{1}$H NMR spectra of bioactive compound of NAA**

**Fig: 25 $^{1}$H NMR data of bioactive compound of NAA**

| Proton | δ | Multiplicity |
|---|---|---|
| 17/32 | 0.86 | (d,3H) |
| 7/9/25/42 | 3.16 | (s,12H) |
| E=41/37 | 1.78 | (s,3H) |
| 13/30 | 2.04 | (dd,1H) |
| 8/26 | 2.72 | (dd,1H) |
| 12/29 | 2.51 | (dq,1H) |
| 10/29 | 6.66 | (d,1H) |
| 23/5 | 7.06 | (s,1H) |
| 20/2 | 7.28 | (s,1H) |

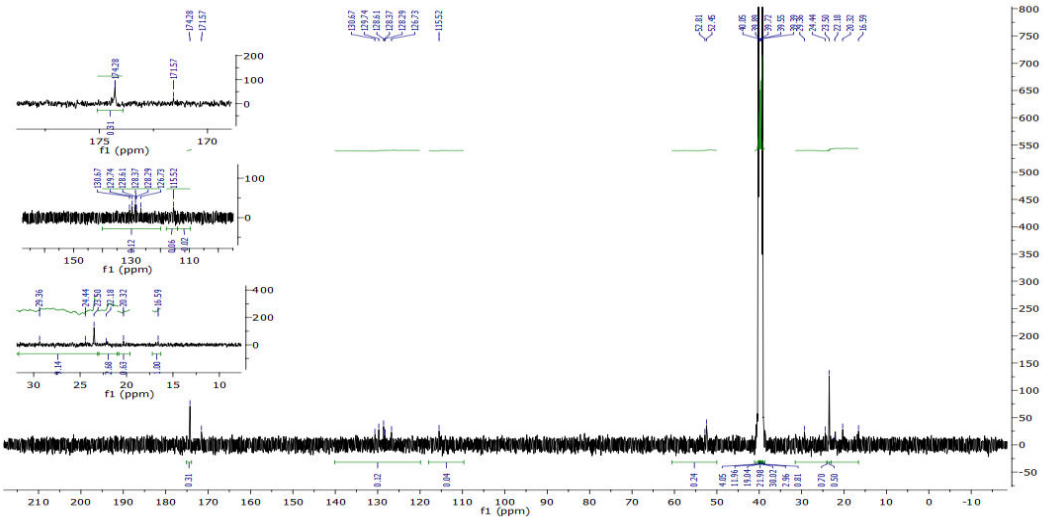


**Fig:26 $^{13}$C NMR of metabolite of isolate NAA**

**Fig:27 $^{13}$C NMR data of the bioactive compound**

| Proton | Δ |
|---|---|
| 42/7 | 16.59 |
| 9/25 | 20.32 |
| 41/37 | 22.18 |
| 8/26 | 29.36 |
| 13/30 | 52.45 |
| 12/29 | 52.81 |
| 10/27 | 115.52 |
| 20/2 | 126.73 |

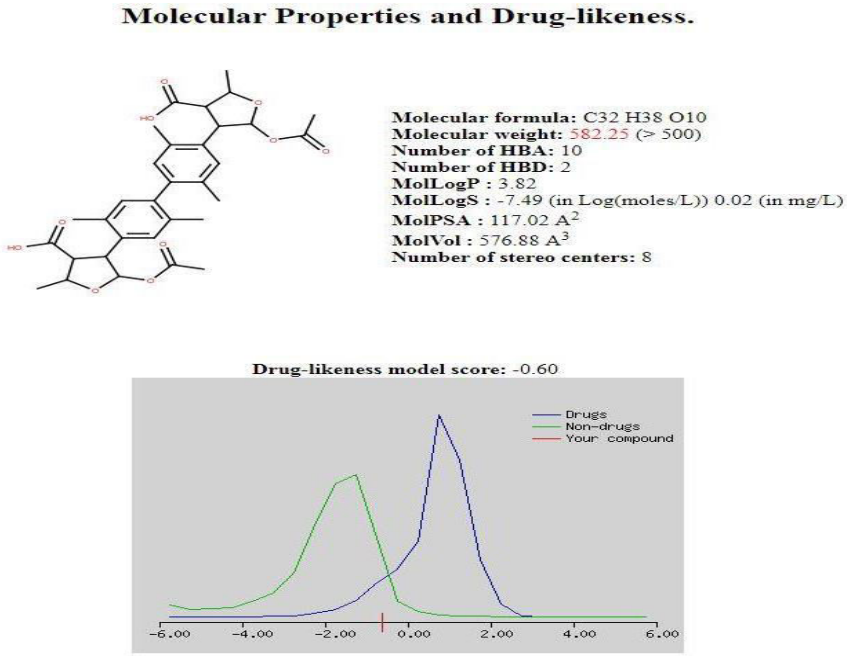



**Fig :28 Drug Likeness Screening using MolSoft Server**

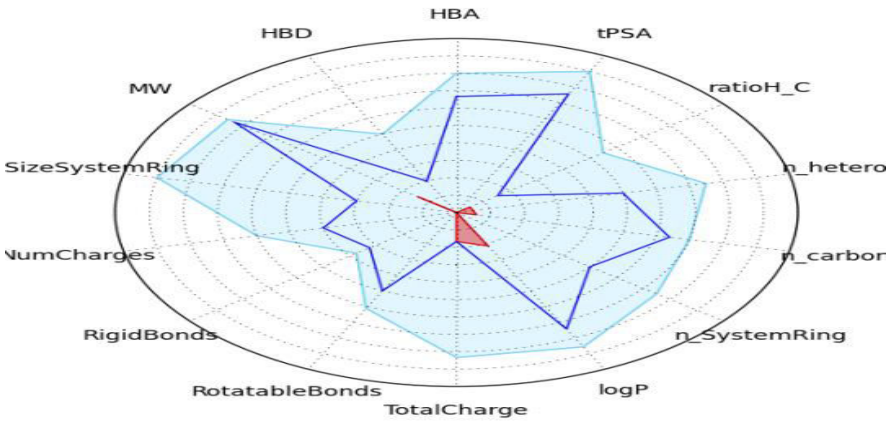



**Fig:29 PhysicoChemical Filter positioning (The compound value (Blue Line) should fall within the light blue area)**

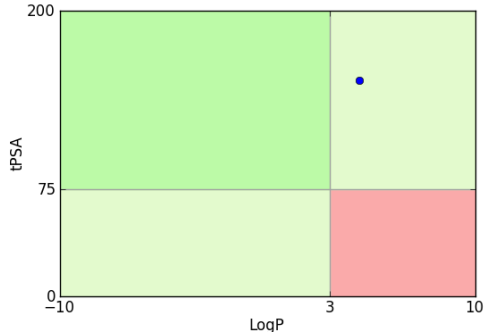



**Fig: 30 Pfizer 3/75 Rule Positioning (The compound (Blue dot) should not fall within the red area.)**

PharmMapper

Introduction | Submit Job | Check Job | Get Result | Help Document

**Result of 18068110521**

Top 300 targets ranked by normalized fit score in descending order

Ligand: *****

| | Rank | PDB ID | Target Name | Number of Feature | Fit Score | Normalized Fit Score | z'-score |
|---|---|---|---|---|---|---|---|
| + | 1 | 225L | Lysozyme | 3 | 2.996 | 0.9987 | 0.54359 |
| + | 2 | 1IVE | NONE | 3 | 2.995 | 0.9984 | 0.752031 |
| + | 3 | 3BDA | Glycogen phosphorylase, muscle form | 3 | 2.994 | 0.9981 | 0.731067 |
| + | 4 | 1ACJ | Acetylcholinesterase | 3 | 2.993 | 0.9978 | 0.650089 |
| + | 5 | 2PG2 | Kinesin-like protein KIF11 | 3 | 2.993 | 0.9976 | 0.43703 |
| + | 6 | 1AD2 | 50S ribosomal protein L1 | 3 | 2.99 | 0.9968 | 0.151847 |
| + | 7 | 1TTM | Carbonic anhydrase 2 | 4 | 3.985 | 0.9962 | 2.46313 |
| + | 8 | 1YA8 | Liver carboxylesterase 1 | 3 | 2.986 | 0.9953 | 0.545281 |
| + | 9 | 1QJP | Outer membrane protein A | 3 | 2.982 | 0.994 | 0.361313 |
| + | 10 | 1DVZ | Transthyretin | 3 | 2.982 | 0.9938 | 0.371703 |

**Fig: 31 Target Fishing analysis using Pharm Mapper**

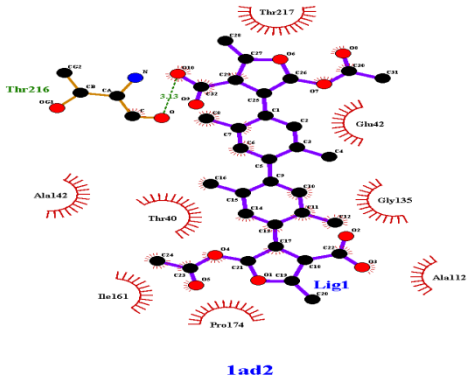



**Fig:32 Best docking pose showing H-bond and weak interactions**

**Quantitative screening of antimicrobial activity of potential isolates by well diffusion method:** The isolates showing positive results against at least 2 of test organisms were screened further for antimicrobial activity using agar well diffusion method( Aneja 2014) and was carried against the same test organisms Biochemical tests were carried out by using commercially available HiBacillus KB003™ identificationt kits (HiMedia India Pvt. Ltd.,India,as well as by using standard methods.

**Extraction of genomic DNA:**

Genomic DNA was isolated from the most potential bacterial isolates by detergent lysis method(Harisha 2007).25 ml overnight bacterial broth in LB media was centrifuged at 6000rpm for 10 minutes. 5 ml of lysis buffer (composition: 50 nM Tris HCL ,20 nM EDTA,1.25% SDS)was added to the pallet and vortexed. The tube was incubated at 65$^0$C for 30 mns with occasional shaking. 3 ml of 3M sodium acetate was added to it and incubated for 30 mns under ice cold condition and then centrifuged at 10,000 rpm for 10 mns. After centrifugation double volume of chilled ethanol was added to the supernatant to precipitate DNA. It was then centrifuged at 12000 rpm for 10 mns to precipitate the DNA and washed with absolute ethanol twice at 12000 rpm. The pallet was dissolved in 20 µl of 1× TE buffer (composition: Tris base (4.82gL$^{-1}$),0.5 M EDTA,pH=8.0).its quality was evaluated by performing agarose gel electrophoresis and the best resolution was observed at 1.2% concentration of agarose.

Genomic DNA of the isolate was outsourced to Eurofins Labs Pvt.Ltd.for 16s rDNA sequencing. Fragment of 16S rDNA gene was amplified by 27F and 1492R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon

was carried out with forward primer and reverse primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
BLAST of sequences were done with BLASTn software using NCBI database.10 Consensus sequence was generated from forward and reverse sequence data using aligner software. The 16s rDNA sequence was used to carry out BLAST with the nrda tabase of NCBI genbank database The bioactive molecules from the active isolates were extracted by the process of solvent extraction following the method of de Melo et al.,2009.

**Thin-Layer Chromatography:**
The method of Touchstone and Dobbins(1978) was employed for TLC of crude extract of the potential isoltes.

**Active Site Prediction:**
The possible target structure as identified by Pharm Mapper server was obtained from RCSB-PDB. The molecular docking analysis was performed using Auto Dock Vina (Trott and Olson, 2010). MGL Tools was used as user interface for docking using Auto Dock Vina. The protein was loaded in the interface and a configuration file was prepared in such a way that the grid box covers the predicted active site.

**Results and Discussion:**
All the 147 bacterial isolates were tested for their ability of having antimicrobial potential using 4 MTCC strains as test organisms viz. *E. coli* (MTCC -40*), B. subtilis* (MTCC-121), *Pseudomonas aeruginosa* (MTCC- 4673), *S. cerevisiae* (MTCC-3090) using disk diffusion method.
Only 6 isolates namely NAA,NAA1,NAA8, MNS1,MNS15 and EMBA are able to show significant antimicrobial activity against all the 4 test organisms used. Among these 6 isolates the NAA was found to be having maximum antibacterial activity against all the test organisms used as compared to other isolates. Antimicrobial activity of the isolates showed following trend NAA>EMBA>NAA8>NAA1>MN1>MNS15.(Fig:2)
The isolates NAA and EMBA were subculture and pure culture in glycerine stock was prepared and stored in -40$^0$ C for further use.

**Colony morphology of the potential isolates NAA and EMBA:**
The colony morphology of the two selected potential isolates NAA and EMBA were evaluated and was found that isolate NAA colony bears white colour, raised, waxy characteristics with smooth surface on Nutrient agar plates whereas the active isolate EMBA showed white, flat, waxy margin with smooth surface on Eosin Methylene Blue agar (EMB) media.(Fig:3)

**Stress tolerance test of the most potential isolates NAA and EMBA:**
**i)Salt (NaCl) tolerance:**
NAA showed luxuriant growth at 0.5% and 1% NaCl concentration. On the other hand isolate EMBA shows luxuriant growth at salt concentration of 1.0% and 1.5%. (Fig:4)

**ii)Thermo tolerance**.
Thermo-tolerance assay of the isolates were carried out in terms of determination of its thermal death point(TDP).The isolate NAA showed luxuriant growth at temperature ranging from 25$^0$ C to 35$^0$C. In case of isolate EMBA,the isolate shows luxuriant growth at temperature 35 $^0$ C,40$^0$ and 45$^0$C. Therefore TDP for isolate NAA was determined to be 75 $^0$C and for isolate EMBA the TDP was determined to be 80 $^0$C.(Fig:5)

**iii)Acid tolerance(pH):**
Isolate NAA showed luxuriant growth at pH 4 and 5,moderate growth at pH 3,6,7,8, poor growth at pH 8 and no growth at all at pH 9. On the other hand isolate EMBA shows luxuriant growth at pH 6 and 7. Moderate growth is shown at pH 5 and 8. At pH 3,4 and 9 it shows poor growth.(Fig:6)

**iv) Morpho-biochemical identification:**
A comparison of morpho-biochemical characters of isolates NAA with *Enterobacter cloaca* and EMBA with *Klebsiella pneumonia* were done with reference to the. Bergey's Manual of Systematic Bacteriology (Logan and De Vos,2009) Out of 26 characters taken for *Enterobacter cloaca,* isolate NAA shows similarity in 24 characters. As such the isolate NAA showed 92% similarity based on morpho biochemical characters with the *Enterobacter cloaca.* Similarly the isolate EMBA showed similarity in 23 characters out of 26 characters indicating 88% similarity with the *Klebsiella*

*pneumonia* sp. suggesting isolate NAA may be a strain of *Enterobacter cloaca* and isolate EMBA a strain of *Klebsiella pneumonia.* (Fig:7)
Distance matrix of the 16S rDNA sequence of the isolates NAA and EMBA with that of ten most closely related strains from NCBI database showing evolutionary divergence between sequence is done.(Fig:8)
6S rDNA sequence of the isolate NAA showed maximum evolutionary relatedness (95%)with *Enterobacter cloaca* NR_028912.(Fig:9)The other isolate EMBA showed maximum closeness (92%) with *Klebsiella pneumonia* (KU 199000.1)(Fig:10).

The potential isolate NAA was identified as a novel strain of *Enterobacter cloacae* Likewise isolate EMBA was identified as a strain of *Klebsiella pneumonia*.16S rDNA sequence of both the isolates NAA and EMBA were deposited in NCBI database and the GenBank Accession number:KX859176 and Accession number:KY111020 were respectively obtained.

**Growth optimisation of the isolates:**
Optimisation of culture conditions for both the isolates for maximum growth and production of bioactive molecules were done and results are presented below.

**i)Selection of culture media:**
For maximum growth and production of bioactive molecules by the microorganisms, selection of nutrient media is most essential parameter. Both of the isolates were grown in different culture media. The findings of the experiments are shown in table below. From the result it can be inferred that for NAA ,nutrient broth is ideal media for both growth and activity and for EMBA Mueller Hinton broth is ideal media for growth and activity. These media were therefore used for production and isolation of the active compounds.(Fig11 &Fig:12)

ii)**Selection of carbon sources:**
It can be stated that glucose for EMBA and starch for NAA isolates are ideal for both growth and activity.(Fig13 &Fig14)
**iii)Selection of optimum pH:**
From the results it can be concluded that optimum pH for NAA is 5 and for EMBA it is pH 7(Fig15 &Fig16)

iv)**Selection of optimum temperature:**
From the below table, results it can be inferred that NAA has optimum temperature of 35$^{0}$C and EMBA has 40$^{0}$ C.(Fig17 &Fig:18)

**Isoation of bioactive fractions of potential isolates NAA and EMBA:**
**Extraction of crude extract of the isolates in different solvents:**
Both the isolates were grown in optimised and were extracted using different solvents like ethyl acetate, hexane, benzene, chloroform, petroleum ether. Ethyl acetate showed maximum recovery of bioactive molecule(crude extract) as compared to other solvents used.(Fig:19)

| **Solvents (1Litre)** | **NAA (crude extract)** | **EMBA(crude extract)** |
|---|---|---|
| Ethyl acetate | **16.7mg** | **10.1mg** |

Antimicrobial activity of crude extracts of both the active isolates done against four test microorganisms are given in the table 4.15. The results showed that crude extract of isolate NAA had more prominent activity against all the test organisms used viz. *B.subtilis*(29.66±1.00), *S. cerevisiae*(28.33±0.36),*E. Coli(*26.33±1.5),*P.aeruginosa* (29.33±0.57) as compared to the crude extract of isolate EMBA. Hence ethyl acetate extract of isolate NAA was used for fractionation. (Fig:20)
Thin layer chromatography resolved the crude extract of isolate NAA and single spot which was found to possess antimicrobial activity detected by bioautography with overlaying of plates with B. subtilis with Rf value **0.72 .**
Corresponding spot from several TLC palates, having Rf values 0.72 were scrapped, pooled that showed antimicrobial activity against the test organism used. The scrapped TLC spot having significant antimicrobial activity was outsourced for spectral analysis.

| **Isolate** | **MTCC strains** |
|---|---|

| | ***B.subtilis*** **MTCC-121** | ***S. cerevisiae*** **MTCC-3090** | ***E. coli*** **MTCC-40** | ***Pseudomonas aeruginosa*** **MTCC-4673** |
|---|---|---|---|---|
| NAA (Rf=0.72) | 29.33±1.00 | 28.00±1.28 | 31.33±1.00 | 28.66±1.00 |

**Antimicrobial activity of silica gel scrap of isolates NAA and EMBA against 4 MTCC strains.**

**FTIR analysis:**

The IR spectrum showed absorption band $V_{max}$ = 3379, 3378, 2934, 1723, 1611, 1444, 1377, 1237, 1079, 760 $cm^{-1}$ indicating the presence of aromatic system with O-H bond. (Fig:21&Fig:22)

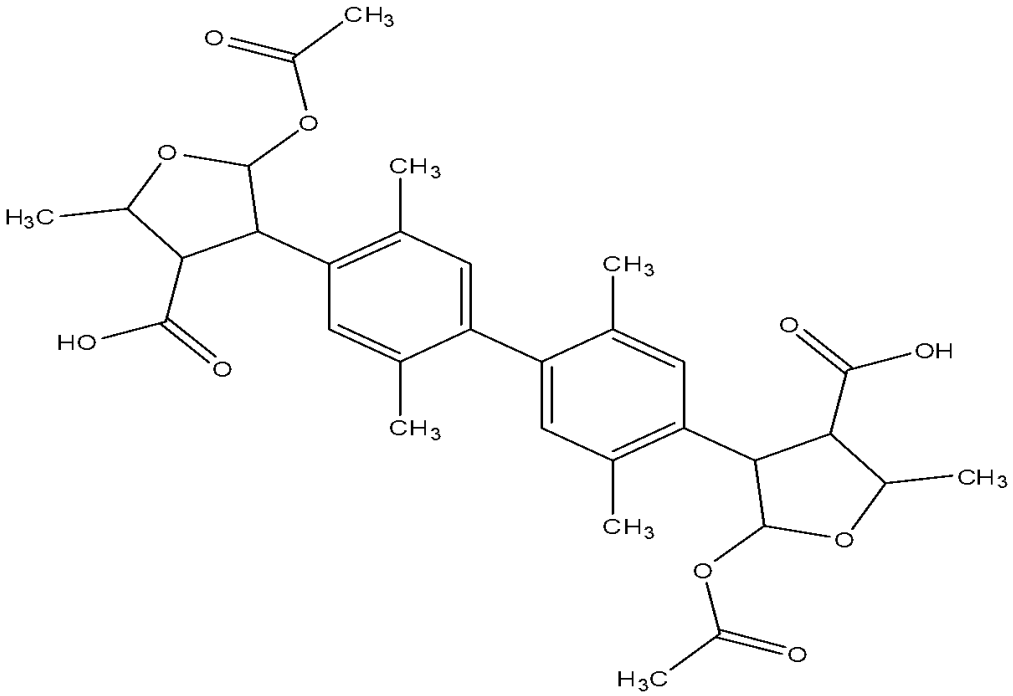
**LC-MS analysis**

ESI-MS gave molecular ion peak at m/z 582.3. The mass spectrum further gave ions at m/z 582.3, 564.3, 552.3, 551.2, 407.2, 389.2, 371.2, 320.1, 263.1, 245.1, 194.1, 176.1, 60.1, 43.0.(Fig:23)

**Result of $^{1}$H and $^{13}$C NMR:**

In $^{1}$H NMR spectra of the compound ($CDCl_3$, 400 MHz), it shows that there are $CH_3$ protons (d, δ 0.86 ppm, 3H, C-17 & C-32), $CH_3$ group attached to benzene ring (s, δ 3.16 ppm, 12H,C-7,C-9,C-25,C-42)), $CH_3$ group attached to the carbonyl group (s, δ 1.78 ppm, 3H,c-41,C-37), C-C-H (dd, δ 2.04 ppm, 1h,C-13,C-30 ), =C-C-H (dd, δ 2.72 ppm, 1H,C-8,C-26), C-C-H (dq, δ 2.51 ppm, 1H,C-12,C-29), -O-CH-O (d, δ 6.66 ppm, 1H,C-10,C-27)), aromatic C=CH (s, δ 7.06 ppm, 1H,C-23,C-5), aromatic C=CH (s, δ 7.28 ppm, 1H, C-2 & 20) groupAfter analysing the $^{1}$H and $^{13}$C NMR spectra of the active compound it is confirmed and its structure is given below.(Fig:24,Fig:25,Fig:26 &Fig:27)

**PREDICTED STRUCTURE of the extracted molecule:**




**4,4'-(2,2',5,5'-tetramethyl-[1,1'-biphenyl]-4,4'diyl)bis(5-acetoxy-2-methyltetrahydrofuran-3-carboxylic acid)**

The active compound of isolate NAA was deduced as $\mathbf{C_{32}H_{38}O_{10}}$ by elemental analysis .

**Molecular weight- 582.6.**

**Drug Likeness and ADME/Tox Screening:**

From the Drug Likeness study, it was seen that the isolated compound is having a Drug Likeness model score of -0.60 which indicated that this compound falls between the peaks of drug and non-drug molecules. It was seen that the molecule is having a molecular weight of 582, which is slightly higher than the suggested value by Lipinski et al. (1997). However this is acceptable for natural products as suggested by Lipinski (2002). ADME/Tox screening is an important feature to study whether an orally administered drug will be absorbed (A) by the intestinal tract, and whether it will be distributed (D) throughout the body and whether it will bypass the metabolic (M) activities

inside the body. Once the function of a drug molecule is over, it must be excreted (E) out of the body. Moreover it should not have any toxic (Tox) effect(Fig:28)
The compound successfully passed the ADME/Tox screening as shown by Mobyle@RPBS Server. The Physicochemical Filter positioning showed that the compound falls within the accepted range. The compound also fulfils the Pfizer 3/75 Positioning Rule as shown in the figure 29 and Fig:30.

**Target Fishing Analysis:**
The Target Fishing analysis using PharmMapper predicted a probable 300 target proteins based on their pharmacophore similarity with the pharmacophore of isolated compound. As the isolated compound exhibited antibacterial activity in wetlab screening, the topmost bacterial protein, i.e. 50S ribosomal protein L1 at rank 6 in the Pharm Mapper result was selected as probable drug target. The 50S Ribosomal Protein L1 plays an important role in the ribosome assembly in bacteria. Inhibiting this protein will hinder the ribosome assembly and thereby inhibiting the translation process in bacteria. To further analyse this predicted mechanism of antibacterial activity of the isolated compound, molecular docking analysis was performed(Fig:31)

**Active site prediction and Molecular Docking analysis:**
The 3DLigandSite server showed that Ala112, Gly135, Pro174, Thr216 etc. are some of the amino acids present in the active site. The compound successfully binds to the active site forming a H-bond with Thr216 (Energy: 3.13 kcal/mol). Moreover it forms several Vander Waal's and other weak electrostatic interactions with Thr127, Glu42, Gly135, Ala112, Pro174, Ile161, Thr40 and Ala142.
It was seen that the compound has a total docking score of -8.5 kcal/mol in its best conformation. This shows that the isolated compound successfully inhibits the active site of ***50S*** Ribosomal protein **L1(** Fig:32).

**Conclusion and Future prospect of the study:**
Microbes as potential sources of drug agents and antibiotics, has been largely unexplored in the Northeast region of India. The present study puts forward an isolate NAA, a novel strain of *Enterobacter cloacae* having significant antibacterial properties. This novel isolate NAA, a strain of *Enterobcater cloaca* produces a molecule which holds full potential of being a potent antibiotic and could be used as lead molecule. With further studies and standardized large scale extraction method, it can be developed into a proper antibiotic that could be used in both pharmaceutical and agriculture industry. The other isolate EMBA, a strain of *Klebsiella pneumonia*, isolated in the present study, had also shown significant antibiotic properties which may also could be further explored.

**Acknowledgement**: I sincerely acknowledge the NEIST, Jorhat and DBT Advanced level Bio-tech Hub, DHSK College for allowing me to perform some of my work in their laboratories.

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