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Research Article

ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC PRODUCING BACTERIA FROM SOIL SAMPLES OF DIBRUGARH DISTRICT, ASSAM

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ABSTRACT

Antibiotics are the important secondary metabolites of almost all types of microbes. Soil microbes also produce antibiotics. The process of antibiotic production by microbes is known as antibiosis where the metabolic products of one organism directly inhibit or kill other pathogenic organisms. Northeastern region of India is well known for its biodiversity hotspot with so many unexplored economically important microorganisms. The aim of the current study is to detect the antimicrobial activity of soil isolates from Dibrugarh District, Assam, India. A total of 50 soil samples were collected from the selected areas of Dibrugarh district. 115 bacterial isolates showed potential antimicrobial activities, out of which 64 showed good antimicrobial activity, 31 showed low antibacterial activity and 21 showed antifungal activities against *B. subtilis* (MTCC 121), *Pseudomonas arginosa* (MTCC 4673), *S. cerevisiae* (MTCC 3090) and *Candida albicans* (MTCC 227), *E.Coli* (MTCC 40), *Clostridium acetobutylicum* (MTCC 11274), *Enterococcus mutans* (MTCC 3031), *Streptococcus mutans* (MTCC 497) .The most potent bacterial isolates with antimicrobial effects against the above mentioned standard strains were identified as *Klebsiella pneumonia sp* on the basis of biochemical tests as prescribed by Bergey's manual of systematic bacteriology. These results indicate that the areas of Dibrugarh District soil microorganisms could be an interesting source of antibacterial and antifungal bioactive substances.

Keywords: *Klebsiella sp.*; antibiotic producing soil microbes; Northeast India.

INRTRODUTION

Antibiotics are chemical compounds that have been used since being discovered and used as remedy for infections, inflammations and diseases. This antibiotic is prevelant in almost everywhere. Soil is biodiversified with various types of microorganisms which include mainly bacteria, cyanobacteria, microfungi, microalgae and protozoans. Among all the products produced by soil microbes antibiotics are the most important secondary metabolites produced by them. Today almost all the known disease producing bacteria have developed resistance to the existing drugs because of their extensive use . So need of new antimicrobial agents is greater than ever because of emergence of multidrug rasistance in common pathogens, rapid emergence of new infections and use of multidrug resistant pathogens in bioterrorism (Spellberg et al.2004).When resources such as nutrients are limited, a bacterium can produce an antibiotic to destroy or inhibit neighbouring bacteria, thereby limiting competition for the scarce resources.(Mindy G. Brown et al. 2009). To combat the situation there is a great demand that new antibiotics is to be developed. In case of Soil microbes they have great potential to produce effective anti-microbial product. Taking up of natural material like soil can be justified by the fact that samples from widely diverse locations are more likely to yield novel microorganisms and therefore hopefully novel metabolites as a result of the geographical variation. (Sen et

al.1993) .Moreover bacterial strains from soil and sewage are a potential reservoir for antimicrobial resistance genes(Sengupta et al,2011).Besides the important approaches helpful in discovering new microbial species which are isolated and characterized from the most extreme habitat (Lee and Hwang,2002) and relatively unknown or untouched areas (Moncheva et al.2002).In this regard Dibrugarh district region is of significant areas as it is situated in the indo-Burma biodiversity hotspot range(D.Thakur et al,2007).

Due to various temperature and climatic conditions this region is likely to harbor unusual types of microorganisms while poorly studied and explored habitation increases chance of finding novel microorganisms. Keeping this points in view ,the present study was undertaken to isolate and characterise antimicrobes from soil samples of this area.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected from different sites of Dibrugarh district. For collection of samples, the whole Dibrugarh district was divided into 10 equal zones given the codes from A to J. GPS Position and location of the sites are shown in Fig 1 and Table 1. Five samples from each zone on random basis were collected from 5-8 cm depth and kept in sterile polythene bags and were preserved -20^{o}C until use.

Isolation and screening of antibiotic producing bacteria

One gram of each soil samples from the 5 sites belonged to the same zone (as mentioned earlier) were mixed and ground properly using mortar and pestle. One gram of these mixtures were serially diluted up to 10^{-7} fold and inoculated on nutrient agar plates followed by incubation at 36^{o}C for 24 hr. All the isolates obtained after incubation were subcultured and stored until further use.

Preliminary screening of the potential antibiotic producing isolate

Preliminary screening for antibiotic production was done by screening antimicrobial activity against standard microbial cultures *viz*., *E. coli* MTCC-40*, B. subtilis* MTCC-121, *Pseudomonas arginosa* MTCC-4673 , *S. cerevisiae* MTCC-*3090, Clostridium acetobutylicum* MTCC-11274*, Enterococcus mutans MTCC 3031 Streptococcus mutans MTCC 497 , Acetobacter aceti* MTCC -3246, *Serratia sicaria MTCC 8930* , *Enterobacter aerogens* MTCC 111 by well diffusion method. Further, the screening was extended with pathogenic strains viz., *Clostridium acetobutylicum* MTCC-11274*, Enterococcus mutans MTCC 3031, Streptococcus mutans MTCC 497, Acetobacter aceti* MTCC -3246, *Serratia sicaria MTCC 8930* , *Enterobacter aerogens* MTCC 111. Standard bacterial broths were spread plated on MHA plates and 100 µL of the broth of each isolates were loaded in respective agar wells. The screening of the most potential isolates were done on the basis of the zone of inhibition given by each isolates against the test organisms after incubation. (Fig-II) and (Fig-III).

Identification of most potential isolate

The potent strains hence isolated were finally identified on the basis of 16s rRNA sequencing analysis. Genomic DNA from each of the potential isolates were extracted and purified. 16s rRNA gene was amplified by using 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) forward and 1492R (5´-TACGCYTACCTTGTTACGACTT-3´) reverse primer. The purity of the amplicons was checked by agarose gel electrophoresis analysis. The sequencing of the amplicons were carried out using ABS. Phylogenetic analysis was conducted using MEGA-6 software taking 1000 bootstrap value (Tamura et.al., 2011). The 16s rRNA gene sequence of strain EMBA was aligned using the CLUSTSAL W programme(Thompson et al.1994)against corresponding nucleotide sequence retrieved from GenBank. Phylogenetic tree (NAA, fig vi) was inferred by the maximum –likelihood method (Felsenstein 1981) with Kimura 2-parameter (Kimura 1980) model (EMBA fig vii). Tree topologies were evaluated by bootstrap analysis (Felsenstein 1985). Blast results are shown and phylogenetic analysis is shown in table.

RESULTS

Current study was carried out for the isolation and screening of antibiotic producing novel bacteria collected from different localities of Dibrugarh district of Assam. A total of 115 active isolates were screened on the basis of their anti microbial activities against at least one of the test microbes considered in this study *viz*.o *E. coli* MTCC-40*, B. subtilis* MTCC-121, *Pseudomonas arginosa* MTCC-4673 , *S. cerevisiae* MTCC-*3090, o*ut of a sample size (*n*=) 50. Out of these, 64 showed good antimicrobial activity (ZOI ≥ 10 mm). These were further tested against microbes responsible for plant diseases *viz*. *Clostridium acetobutylicum* MTCC-11274*, Enterococcus mutans MTCC 3031, Streptococcus mutans MTCC 497 , Acetobacter aceti* MTCC -3246 *Serratia sicaria MTCC 8930* , *Enterobacter aerogens* MTCC 111 and 31 of these isolates

extends activity against at least one of them. 12 of these showed activity against all the 10 microbes under consideration .

The isolate designated as NAA showed highest activity amongst all the three strains followed by the isolate EMBA . A comparison of antimicrobial activities by the respective bacterial culture (broth), cell free extract and sonicated cell extract is shown in table II. Biochemical characterization is done and presented in table III. The strain is gram negative and non sporulated. Under light microscope it is observed as rod shaped.

Finally the isolates were identified as *Enterobacter cloaca* and *klebsiella pneumonia* on the basis of 16s rRNA study. The strain NAA was found to be *Enterobacter cloaca* with 95% similarity score with *Enterobacter cloaca*_ strain_ ATCC_ 13047 and EMBA was found to be *Klebsiella pneumonia* with 92% similarity score with Klebsiella pneumonia_strain _BR1_16s_ribosomal_RNA_gene_partial _sequence . GenBank accession number for strain NAA is KX859176 and for strain EMBA is KY111029.

DISCUSSION

Among various environmental compartments, soil is considered to be one of the primary reservoir of released antibiotics (kemper,2008;seveno et al.,2002).Antibiotics released to the soil whatever means it be (animal sewage,wastewater irrigation) may cause extensive antibiotic resistance build up in soil (Agerso et al.,2006;Heuer and Smalla, 2007;Kummerer,2004;Marti et al.,2013).Numerous antibiotic resistant bacteria (ARB) have been detected and isolated from soil samples (Brandt et al.,2009;DCosta et al.,2006,Schmitt et al.,2006).Antibiotic resistant genes (ARGs) have also been widely detected in soil in past few decades. Our interest focused on microorganisms belonging to soil microflora . Present finding highlights the importance of further investigation towards the goal of obtaining novel antimicrobial agent from extreme part Northeast India's untapped habitat. These areas represent diverse and largely unscreened ecosystem and the least investigated ecosystem for the isolation of potent antibiotic producing microorganisms. In our study two strains namely Enterobacter cloaca and Klebsiella pneumonia were isolated from tea garden and industrial area of Dibrugarh district ,Assam exhibited promising antibacterial and antifungal activity against a varied range of organisms. Diversified environmental condition and high level of nutrient content in this region could trigger or favor unusual metabolite production by these isolates and thus stressing their potential as a source of novel antibiotic, which encouraged further studies to isolate and identify these novel compounds.

ACKNOWLEDGEMENT

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Table 1:

	Places of sample collection	coordinates
A	Itakhuligaon	27°46"N,95°92"E
	ajarguri	
	Rangrangia	
	Chakalia 1	27°12"N,94°82"E
	Chakalia 2	27°12"N,94°82"E
B	Bamunbari	27°20"N,94°97"E
	Moran	27°17"N,94°91"E
	Kherni	
	Nachanigaon	
	Jamuguri	26°59"N,91°98"E
C	Nachanigaon 2	27°16"N,94°82"E
	dighaliya	27°16"N,94°82"E
	Keseruguri	27°13"N,94°77"E
	Dhadumia2	27°13"N,94°77"E
D	Balimara	27°13"N,95°33"E
	Namrup	21°18"N , 95°34"E
	Parbatpur	23°17"N,88°10"E
	Jaipur 1	27°26"N, 95°39"E
	Jaipur 2	27°26"N, 95°39"E
E	Balimara	27°13"N,95°33"E
	Duliagaon	27°35"N,95°32"E
	Duliajan	27°35"N,95°32"E
	Bhadoi	27°47"N,94°91"E
	Dhadumia 1	27°13"N,94°77"E

F	Bishmile	27⁰48″N, 95⁰17′E
	Chabua	27⁰48″N, 95⁰17′E
	amguri	26⁰81″N, 94⁰52″E
	Khajua	26⁰81″N, 94⁰52″E
	Tengakgat	27⁰38″ N,95⁰17″E
G	Chaolkhowa	27⁰38″ N,95⁰17″E
	Puranigaon	27⁰45″N, 95⁰08″E
	Dikom	27⁰45″N, 95⁰08″E
	Mohanbari	27⁰48″N, 95⁰02″E
	Lahoal	27⁰45″N, 95⁰00″E
H	Amolapotty	27⁰47″N, 94⁰89″E
	Dibrugarh university	27⁰47″N, 94⁰91″E
	Borboruagaon	27⁰47″N, 94⁰91″E
	Jamirahgaon	27⁰41″N, 94⁰85″E
	Majgaon	26⁰96 N, 94⁰89″E
I	Rohmoria 1	27⁰51″N,95⁰10 E
	Bogoritoli	
	Charukolia sapori	
J	Rohmoria 2	27⁰51″N,95⁰10 E
	paltanbazar	27⁰48″N,94⁰94″E
	panitola	27⁰49″N,95⁰24″E
	Maijanbeel area	27⁰32″N,94⁰58″E

Sample collection Area

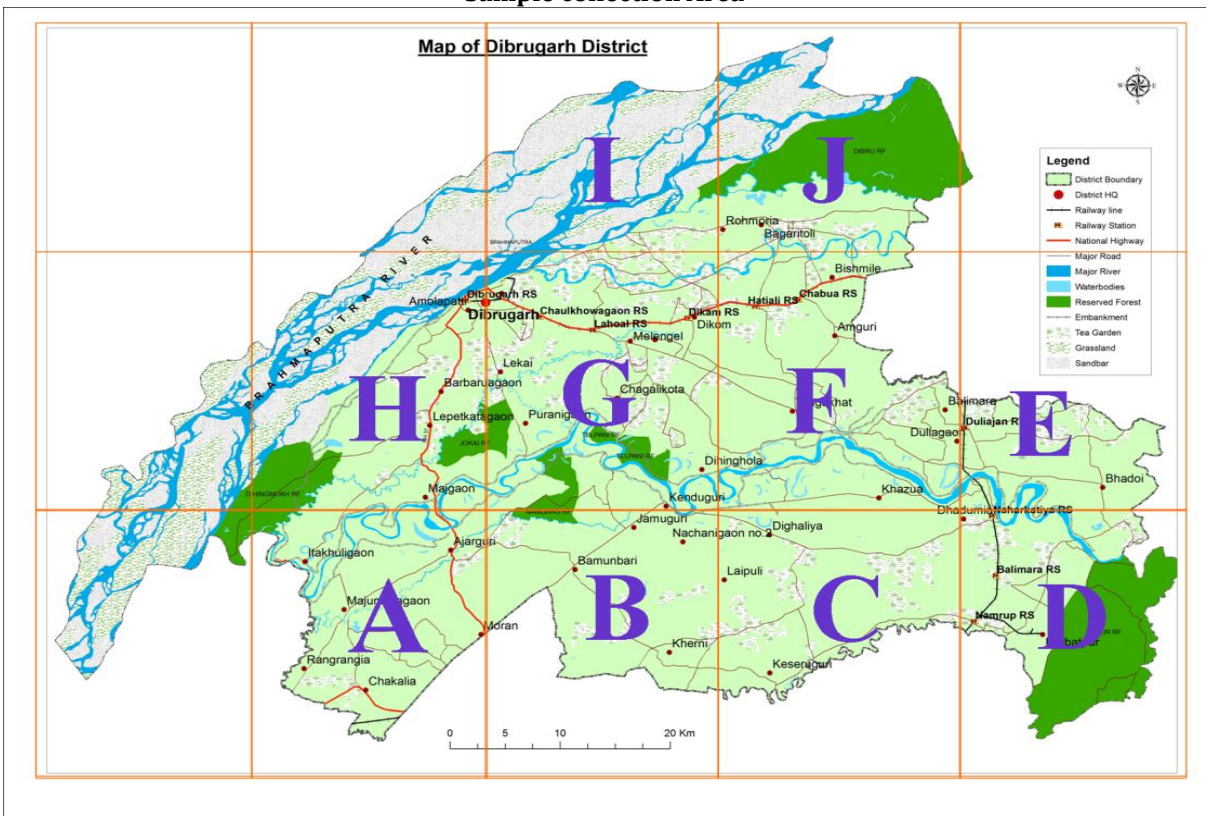

Fig. I:

Flow chart for isolation of active isolates

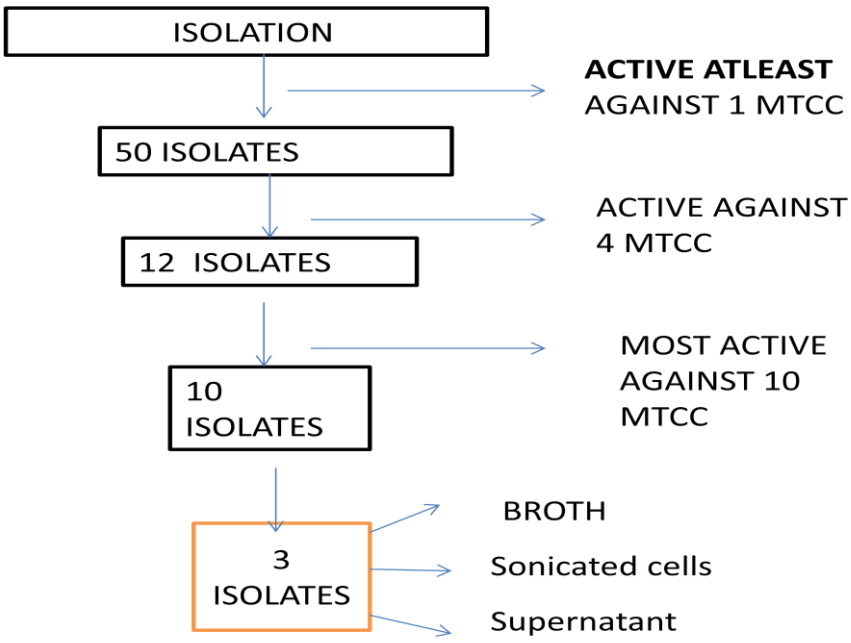

Fig. II:

Samples showing activity against MTCC

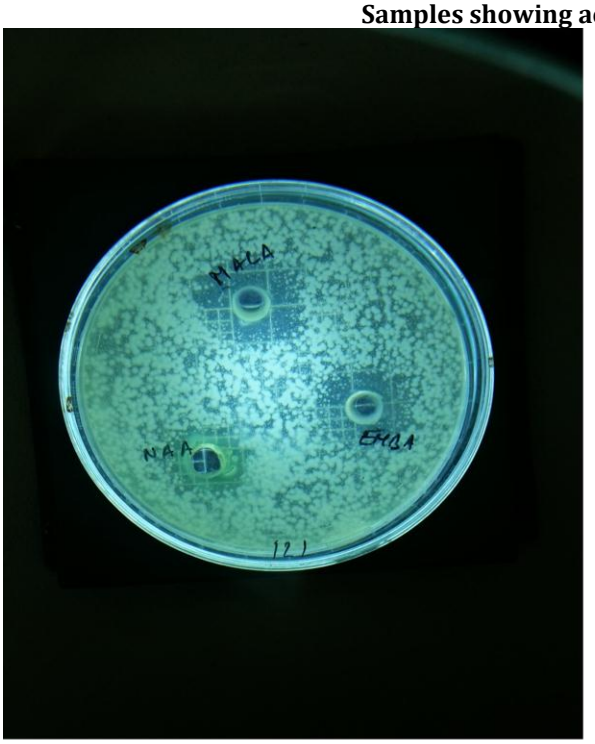

Fig: samples NAA,MACA& EMBA from table IV showing activity against 121

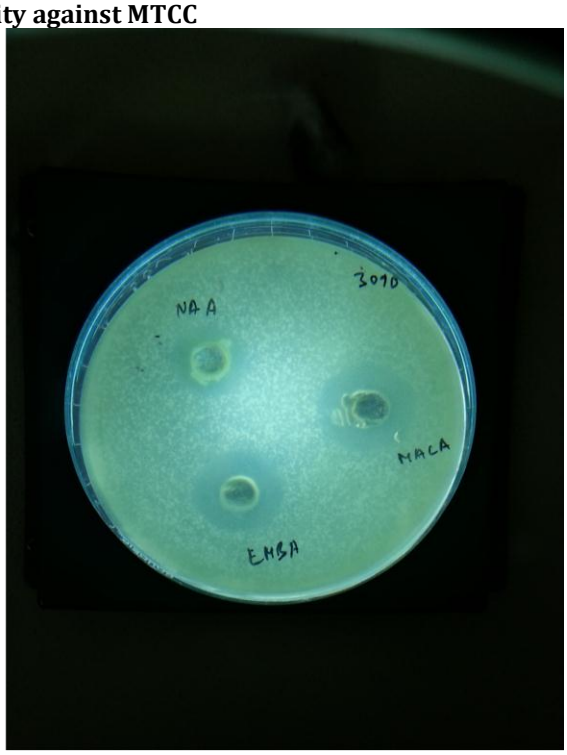

Fig: samples NAA,MACA &EMBA showing activity against 3090

Fig. III:

Table II:
Summary for sample EMBA (MEAN &SD)

MTCC	BROTH	C.F.	SUPERNATANT
8930	11.3±.577	14 ±2.64	12±1
497	16.6±1.5	21.3±2.08	14.3±.577
3246	18.66± 1.52	20±4	12.6±.577
3031	16.33 ±1.52	19.66± .577	14.33±1.57
11274	17.66±1.52	20.66±1.52	15.33±4.93
121	12.33±.577	16.66±1.52	12±2

Summary for sample NAA (MEAN &SD)

MTCC	BROTH	C.F.	SUPERNATANT
3031	14±1	10±1	16±1
8930	19±1	14.66±.577	20±1
11274	17±1	14.6±.577	18±1
497	15.33±.577	14.33±.577	18±1
3246	15±1	--	17.33±.577
111	14±1	--	18.6±.577
121	21.66±1.520	15±1	16±1
40	19±2.64	18±2.64	16±1
3090	18±1	22.6±.577	14.33±2.08

Table III: Biochemical study of both the strains

Name of tests	STRAIN NAA	STRAIN EMBA
Gram Staining	negative	negative
Spore	Non spore	Non spore
Catalase	positive	positive
Oxidase	negative	negative
Gas production	positive	positive
Citrate utilisation	positive	positive
Acid production	positive	positive
Gelatine liquefaction test	Positive	positive
Lipase test	Negative	negative
ONPG	Positive	Positive
Sucrose	Positive	positive
Mannitol	Positive	positive
Sucrose fermentation	Positive	positive
Lactose	Positive	positive
Inulin	Positive	positive
Glycerol	Positive	Positive
Sorbitol	positive	Negative
Cellobiose	Positive: negative	Positive:negative
Esculin hydrolysis	Positive	Positive
Melonate	Positive	Positive
Nitrate	Negative	Positive
H_2S production	Negative	Negative
Lipase	Negative	Negative

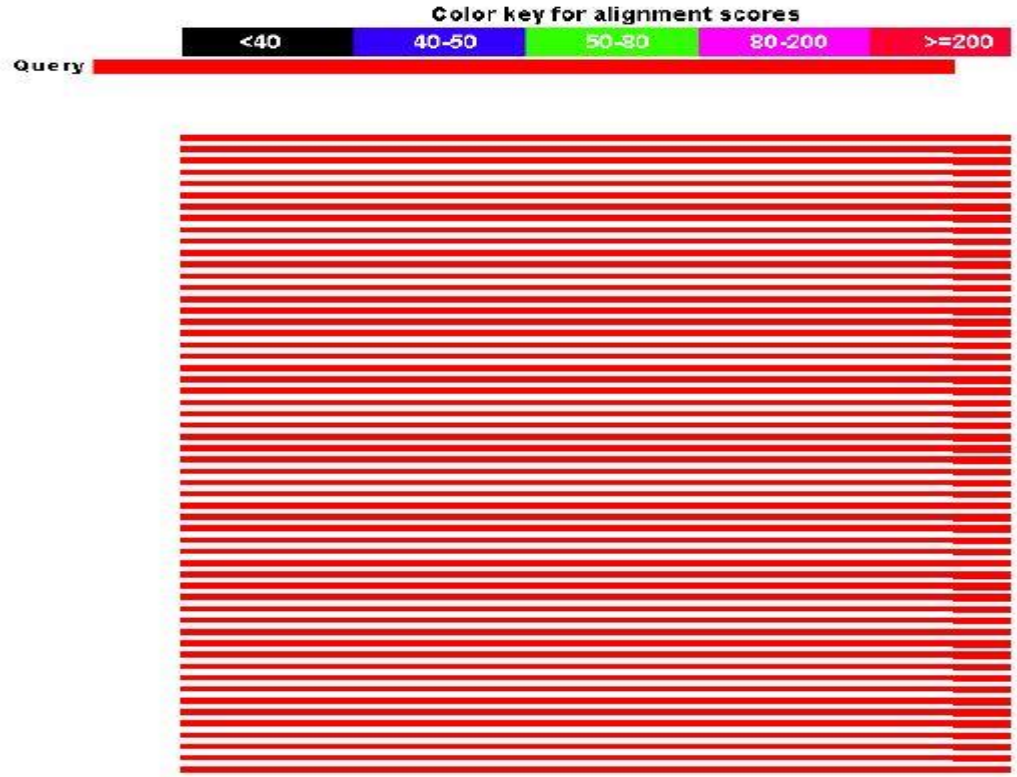

Fig. IV: BLAST result for EMBA

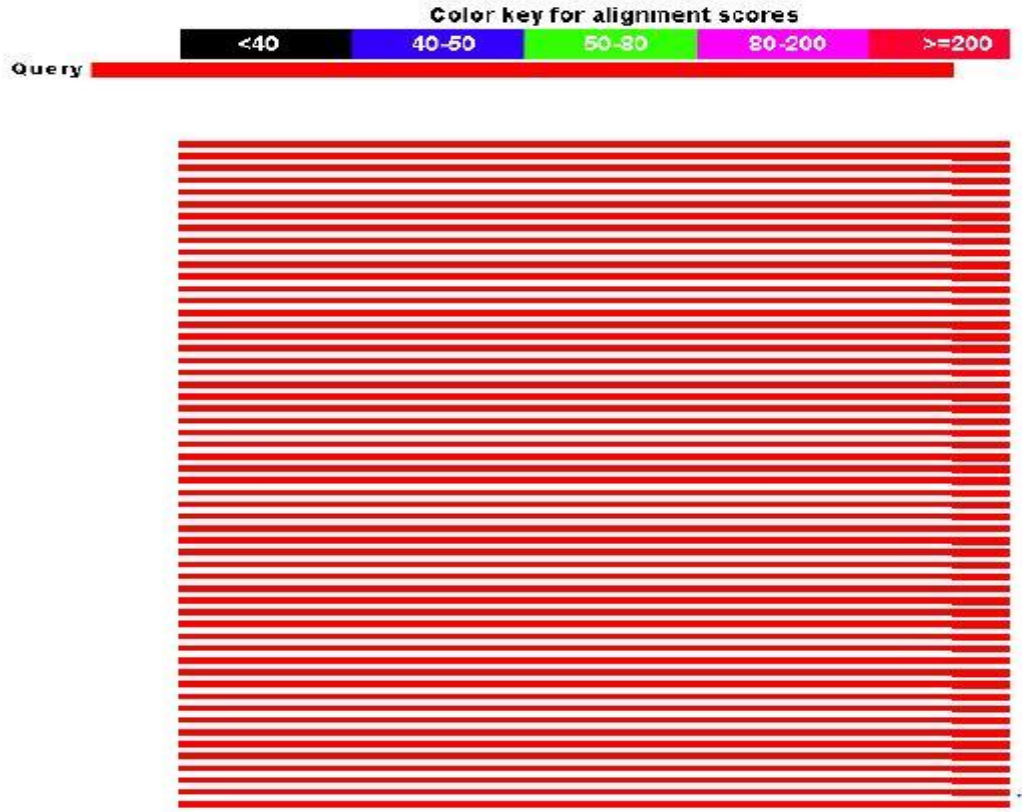

Fig. V: BLAST result for NAA

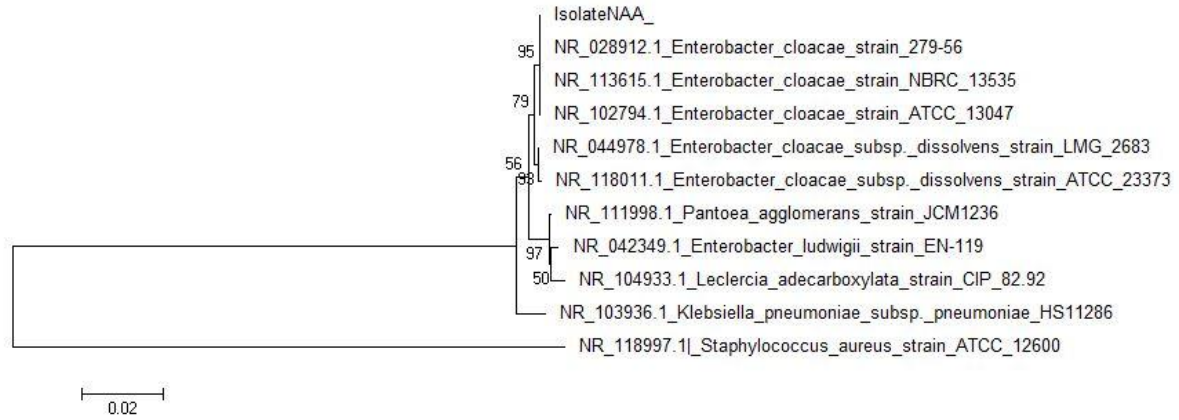

Fig. VI: Phylogenetic relationships of the new potent isolate (NAA) Enterobacter sp. with ten other most closely related Enterobacter species based on 16S rDNA sequencing. The data set resembled 1000 times using the bootstrap option, and percentage values are given at the nodes

Fig. VII: Phylogenetic relationships of the new potent isolate (EMBA) Klebsiella sp. with ten other most closely related Klebsiella species based on 16S rDNA sequencing. The data set resembled 1000 times using the bootstrap option, and percentage values are given at the nodes

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