

Chloramphenicol acetyl transferase producing bacteria

Jenny Anne Tharian, Padmapriya R. and Thirunalasundari T.*

Department of Industrial Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

ABSTRACT

Chloramphenicol acetyl transferase (CAT) is an enzyme encoded by plasmids that detoxify the antibiotic chloramphenicol. It is responsible for chloramphenicol resistance in bacteria. Chloramphenicol acetyl transferase covalently attaches an acetyl group from acetyl-CoA to chloramphenicol which prevents chloramphenicol from binding to ribosomes. CAT is used as a reporter gene marker for the successful uptake of the gene of interest. It is used to measure chloramphenicol in body fluids and also to inactivate chloramphenicol where the antibiotic has been added as potential reversible inhibitor of protein synthesis. Production of CAT enzyme is a rarely frequented area and hence it will be of great use if an indigenous methodology is developed to produce CAT enzyme with cost effectiveness. Commercial synthesis of this enzyme is complicated and is expensive too. Hence the aim of the study is to isolate CAT producing microbes from soil by inoculating the soil in nutrient agar containing chloramphenicol as substrate, thereby only those organisms that can make use of chloramphenicol will survive and multiply. This screening resulted in 44 isolates and all of them were characterized by macroscopy followed by microscopy and biochemistry. Of these 44 isolates 4 were shortlisted and were gene sequenced. The short listed strains were coded as BDU2, BDU3, BDU4 & BDU5 and their sequence was deposited in gene bank.

Key words: chloramphenicol acetyl transferase, chloramphenicol, gene sequence.

INTRODUCTION

The enzyme chloramphenicol acetyl transferase detoxifies the antibiotic chloramphenicol and gives resistance to chloramphenicol in bacteria. Chloramphenicol is the first antibiotic to be synthetically manufactured on a large scale. Chloramphenicol acetyl transferase covalently attaches an acetyl group from acetyl-CoA to chloramphenicol which prevents chloramphenicol from binding to ribosomes [1]. The biochemical mechanism of bacterial resistance to chloramphenicol is the inactivation by *O*-acetylation of the antibiotic, a reaction catalysed by chloramphenicol acetyl transferase (CAT) (EC 2.3.1.28) with acetyl-CoA as the acyl donor. Bacterial resistance to the antibiotic chloramphenicol, an inhibitor of the peptidyltransferase activity of prokaryotic ribosomes, is commonly conferred by the enzyme chloramphenicol acetyl transferase [2]. CAT is used as a reporter system to measure the level of a promoter or its tissue specific expression. Bacterial resistance to chloramphenicol is mediated through the enzymatic action of CAT [3]. The enzyme is cytoplasmic and tetrameric in all bacterial species examined to date, consisting of four identical catalytic subunits, the molecular weight of which is approximately 25000 Da [4]. Most of the CAT characterized to date constitute a family of proteins that catalyse the acetylation of chloramphenicol with variable efficiency. Each purified CAT preparation was shown to exist as tetrameric protein with a native molecular weight of 80,000 Da and 4 identical subunits size of 20,000 Da [5]. The optimum P^H needed for CAT production is 7.8 [6]. It was found that *E.coli* strains carrying transmissible elements for chloramphenicol resistance were able to inactivate the drug rapidly and completely. Chloramphenicol resistant *Staphylococci* were screened for the presence of CAT and were found to contain inducible chloramphenicol inactivating enzyme. It has been found that not only *E.coli* and

other gram negative bacteria with R factor, but also that some naturally occurring isolates of the gram positive pathogens like *Staphylococcus aureus* can inactivate chloramphenicol [3]. It is learnt that naturally occurring strains of chloramphenicol resistant staphylococci also contained CAT. Plant mitochondria are also sensitive to chloramphenicol suggesting that CAT may be a good selectable marker for plant mitochondria transformation [1]. All CAT variants behave on poly acrylamide gel electrophoresis as same. Bacterial resistance to chloramphenicol was demonstrated in various strains of bacteria. Few of them are *Escherichia coli*, several multiple resistant *Staphylococcus epidermis*, *Haemophilus parainfluenzae*, *Staphylococcus faecalis* and *Morganella morganii*.

MATERIALS AND METHODS

Five different soil samples were collected from different locations i.e hospital waste dumped soil, petrol bunk, Trichy distilleries effluent, soil containing laboratory waste and garden soil. Collection of the soil sample and its physical nature assessment were done by standard methods [7, 8]. Soil microbes were isolated by serial dilution method using nutrient agar enriched with chloramphenicol and isolates were identified by macroscopy, microscopy, and biochemical methods. Macroscopy was mainly on looking for the isolate's colony morphology, size, colour odour and appearance [9]. Microscopic observations like size, shape and motility was done by simple staining, Gram's staining and motility of the isolates [10]. Physiological and metabolic characteristics of the microorganisms were assessed through biochemical tests. The following tests were done to identify gram negative isolates: growth on Manconkey's agar, Manitol motility salt agar, Triple Sugar Iron agar, Indole reaction, Methyl red, Voges Proskauer, Citrate utilization, Nitrate reduction, Decarboxylation of lysine, ornithine and arginine, Phenylalanine deaminase. In addition, tests for the production of enzymes like Urease, Oxidase, Catalase, Gelatinase and Coagulase were done. The isolates were also looked for their Carbohydrate fermentation ability. The following tests were done to identify gram positive isolates. Biochemical tests done were Peptone water with 6.5% NaCl, Catalase, Gelatinase and Carbohydrate fermentation test [11].

Extraction of DNA, 16s rRNA sequencing and Phylogenetic analysis

Bacteria were grown for 48 hrs at either 28°C or 37 °C on LB agar. Whole cell DNA was extracted using the method of Gillings and Fahy [12]. Amplification of 16S rRNA gene was done using universal primers. PCR amplification products were electrophoretically separated on 1.5% agarose gel prepared in 1x TAE. The gel was run for 2 hrs. at 50V. Staining was done with ethidium bromide and photographed.

The PCR product of the amplified region was directly sequenced by 16S rRNA sequencing. It was done by di-deoxy chain termination method and the sequences were aligned using chrome software and homology was identified with NCBI BLAST tool with genetic analyzer at EUROFINS Pvt. Ltd., Bangalore. The Phylogenetic trees were constructed using the neighbor-joining, minimum evolution and maximum parsimony methods from MEGA Version 3.1. The genetic distance was calculated with Kimura's two-parameter model. The resultant rooted tree topologies were evaluated by bootstrap analyses based on 5000 replications [13].

RESULTS

Physical nature

A total of five different sites were selected at Tiruchirappalli city for collection of soil samples. All sites were exposed to different kinds of environmental pollution. A site at which the soil sample was collected is Kauvery Medical Center, Tiruchirappalli that was dumped with hospital wastes. Another site was at Petrol Bunk, Tiruchirappalli exposed to Petroleum products. Soil sample collected from Trichy Distilleries is rich in effluent containing dirt, organic waste and chemicals. The fourth site at which the soil sample was collected is Laboratory Waste dumped with organic wastes. Fertile cultivation land is the next site which is rich in humus. The collected soil samples were coded as JKM, JPB, JTD, JLB and JCG. Physical natures of the samples were of sandy, semisolid or clayey. Colour of the sample varied from dark brown to reddish brown and the smell was earthy or with effluent or sewage smell or unpleasant (Table 1).

Table 1 Physical nature of soil samples collected

S. No	Site	Sample code	Nature of the environment	Soil characteristics
1	Kauvery Medical center, Trichy.	JKM	Exposed to various organic wastes and environmental stress	Clay, dark brown, Wet & earthy .
2	Petrol bunk soil, Trichy.	JPB	Exposed to sediments of various petroleum products	Clay, dark brown, Wet & earthy.
3	Trichy Distilleries effluent	JTD	Exposed to contaminated with dirt, organic waste and chemicals	Semisolid, dark brown, Wet liquid & unpleasant.
4	Laboratory waste	JLB	Exposed to various organic wastes and environmental stress	Clay, dark brown, Wet & earthy.
5	Garden soil	JCG	Fertile cultivation land rich in humus	Sandy, reddish brown, free powdered, earthy.

Biological analysis

All the 5 samples were rich in bacterial population from which 44 individual isolates were chosen based on their uniqueness in colour, texture, shape etc. for further identification (Table 2).

Table 2 Bacterial population in the soil samples

S. No.	Sample code	No. of isolates chosen	Bacterial population
1	JKM	08	Innumerable
2	JPB	05	Innumerable
3	JTD	05	Innumerable
4	JLB	11	Innumerable
5	JGS	15	Innumerable
Total		44	

JKM-Kauvery Medical Center: JPB- Petrol Bunk: JTD –Trichy Distilleries: JLB – Laboratory Waste : GS - Garden Soil

Macroscopic nature of the isolates

The macroscopic natures of isolates vary slightly. Colony morphology of 24 isolates were circular, followed by 10 irregular, 8 irregular raised and 2 circular raised. All isolates were pale yellow in colour. 29 isolates had pungent odour and 15 were odourless.

Microscopic nature of the isolates

Microscopic study of the isolates showed the shape, motile nature and gram's nature. The shape of the isolates was of rod or cocci. 54% of the isolates were of rod shaped. 81% of the isolates were motile. The isolates were of both gram positive (39%) and gram negative (61%).

Biochemical nature, Enzyme analysis and sugar fermentation

The isolates were identified by various biochemical tests, enzyme analysis and sugar fermentation. Genus level identification of the selected isolates was made by their biochemical nature and it was observed that most of the isolates showed the biochemical nature of the genus *Staphylococcus*, *E.coli* and *Streptococcus*. Some of the isolates showed the characteristic features of *Bacillus* and *Enterobacter*.

Table 3 Details of short listed & gene sequenced isolates

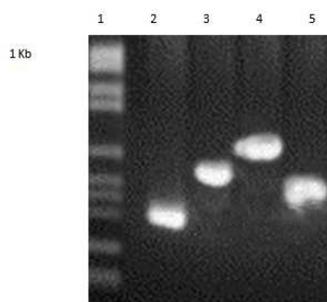
S. No.	Soil source code	Isolate code	Binomial name of the identified isolates	Gene Bank Accession No.
1.	JKM - 1	TJBUD2	<i>B. cereus</i>	JX503931
2.	JPB - 5	TJBUD3	<i>Staphylococcus hominis</i>	JX503932
3.	JTD - 3	TJBUD4	<i>Ochrobactrum intermedium</i>	JX503933
4.	JLB - 7	TJBUD5	<i>Enterobacter sp</i>	JX503934

Extraction of DNA, 16s rRNA sequencing and Phylogenetic analysis

DNA was extracted from the four shortlisted isolates (BDU2, BDU3, BDU4, BDU5) and the region was amplified using universal primers and subjected to nucleotide sequencing by gel electrophoresis (Fig.1). The amplified product was found to have 614, 1424, 1454, 1354 base pairs for BDU2, BDU3, BDU4, BDU5 respectively. The phylogenetic tree was constructed (Fig. 2, 3, 4 & 5). The aligned sequence was deposited in GenBank. They were identified and coded as TJBDU2, TJBDU3, TJBDU4, and TJBDU5 with the accession number JX503931,

JX503932, JX503933, and JX503934 (Table 3) and they were identified as *B. cereus*, *Staphylococcus hominis*, *Ochrobactrum intermedium*, *Enterobacter sp.* respectively.

Fig. 1 Gel electrophoresis of isolated DNA



Lane 1: Marker, Lane 2: BDU2, Lane 3: BDU3, Lane 4: BDU4

DISCUSSION

Soil samples were collected from the sites that were exposed to different kinds of environmental pollutants as they may be enriched with varied group of microorganisms. Soil is a rich source of diverse group of microbes and their characters are influenced by physical, physicochemical and chemical nature of the soil. Soil microorganisms are responsible for the breakdown of organic matter including hydrocarbons, conversion of inorganic components from one form to another and the production of humus. Soil microorganisms play an important role in maintaining soil quality [14, 15]. To find out a novel source organism for CAT enzyme, a total of five different samples were collected from different environment viz. Kauvery Medical Center, Petrol Bunk soil, Laboratory waste, Trichy Distilleries effluent and Garden soil and their physicochemical characters were analyzed.

The results revealed that the samples taken from Kauvery Medical Center, Petrol Bunk soil, Laboratory waste are clayey, dark brown, wet & earthy (Table 1). The reason may be the presence of moisture and much of organic wastes. The sample from Trichy Distilleries effluent is semisolid, dark brown, wet liquid with unpleasant smell and it could be due to the chemical nature of the effluent and the action of microbes on it. Small lumps were observed in the sample collected from Trichy Distilleries and it could be due to the presence of distillery waste materials. Garden soil sample was sandy, reddish brown and finely powdered which may be due to the organic content and weathering process. Colour of the samples varied from dark brown to reddish brown depending upon the site from where the samples were collected (Table 1). The colour of the samples JKM, JPB, JLB may be due to the presence of organic matter and humus. Dark brown colour of the Trichy Distilleries may be due to its continuous exposure to the effluents. The sample taken from the garden soil was reddish brown. The richness of decomposed vegetable wastes and other materials may be the reason for this. Presence of innumerable bacterial population indicates the richness of soil in terms of nutrient. Gans [16] reported that soil microbial communities are known to be remarkably complex, and the estimates of soil diversity are as high as 8.3×10^6 unique genomes per 30g of soil.

From them a total of 44 isolates were selected for further study considering colony morphology, colour and macroscopic nature. The different pigments produced by the bacterial isolates may be the reason for their pale yellow colour. The reason for the pungent odour could be its metabolic products. Some of the isolates were bacilli and some were coccid in shape. A few of them were non motile and the rest were motile. Most of the isolates were gram negative and some of them were gram positive. Based on the biochemical nature, the individual response of the isolates to various chemicals and polysaccharides were exhibited. Logan and Berkely [17], reported that bacterial species are with characteristic biochemical and morphological features and the strains examined showed little variation. On the other hand [18] isolated CAT producing organism from fish also reported the isolates with transferable R plasmids. Based on macroscopic, microscopic and biochemical results 4 isolates were shortlisted and subjected to genus level identification. Based on 16s rRNA sequence the isolates were identified as *Bacillus cereus*, *Staphylococcus hominis*, *Ochrobactrum intermedium* and *Enterobacter sp.* and they were deposited in Gene bank

and their accession numbers are JX503931, JX503932, JX503933, JX503934 respectively. Roberts et.al [3] characterized three chloramphenicol acetyl transferase isolated from *Haemophilus influenzae*.

By this preliminary study 4 different genera of microbes producing chloramphenicol acetyl transferase were identified. On the basis of the data presented it can be concluded that production of chloramphenicol acetyltransferase is possible from soil microbes identified. Considering the simplicity and reliability of the biochemical methods, increased production of CAT can be done at a lower cost in India if further explorations are made. The high cost incurred in buying and importing this enzyme from foreign countries can also be lessened considerably with the production of CAT in India itself.

Fig. 2 Phylogenetic tree of 16s rRNA for TJB DU2

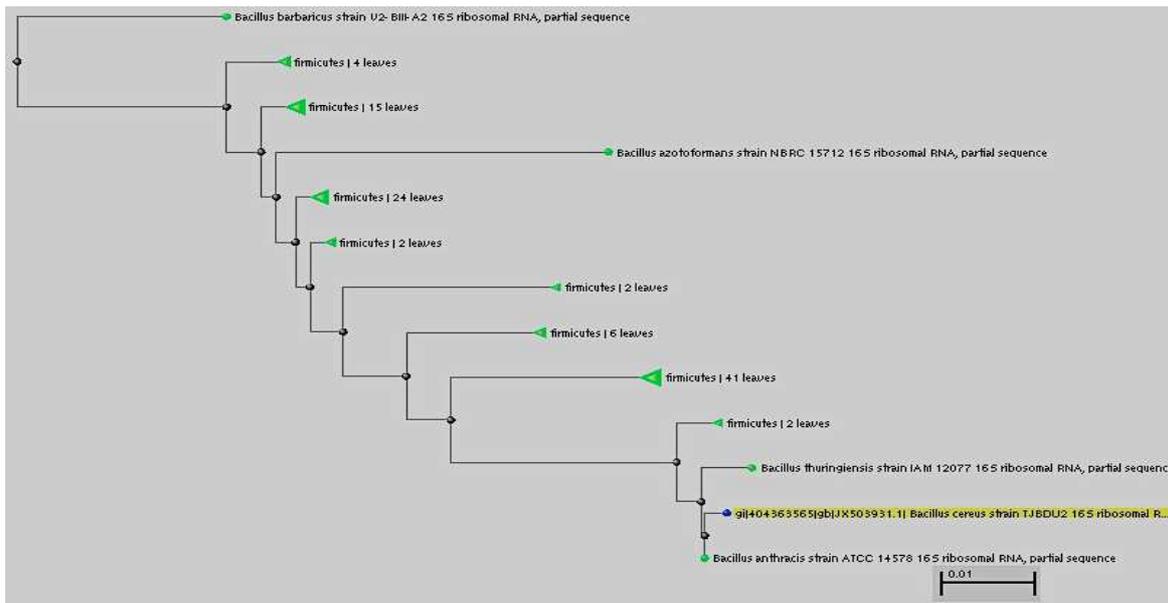


Fig.3 Phylogenetic tree of 16s rRNA for TJB DU3

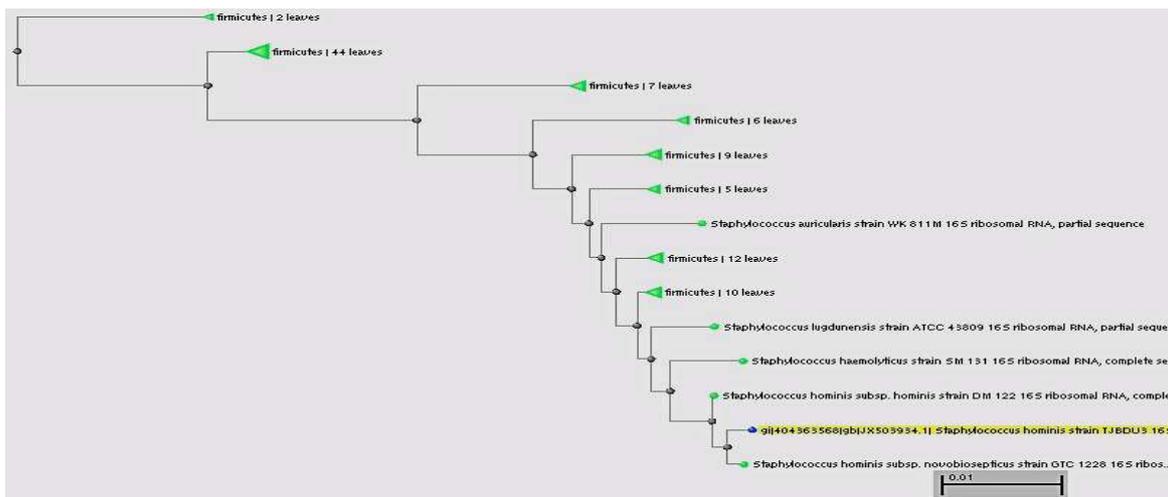


Fig.4 Phylogenetic tree of 16s rRNA for TJBDU4

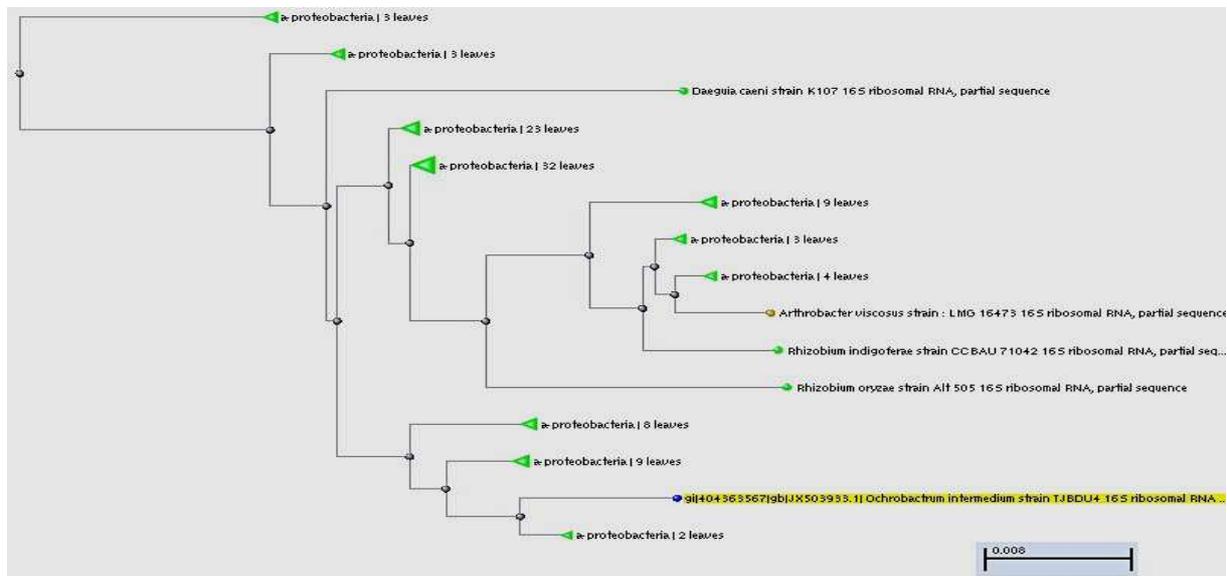
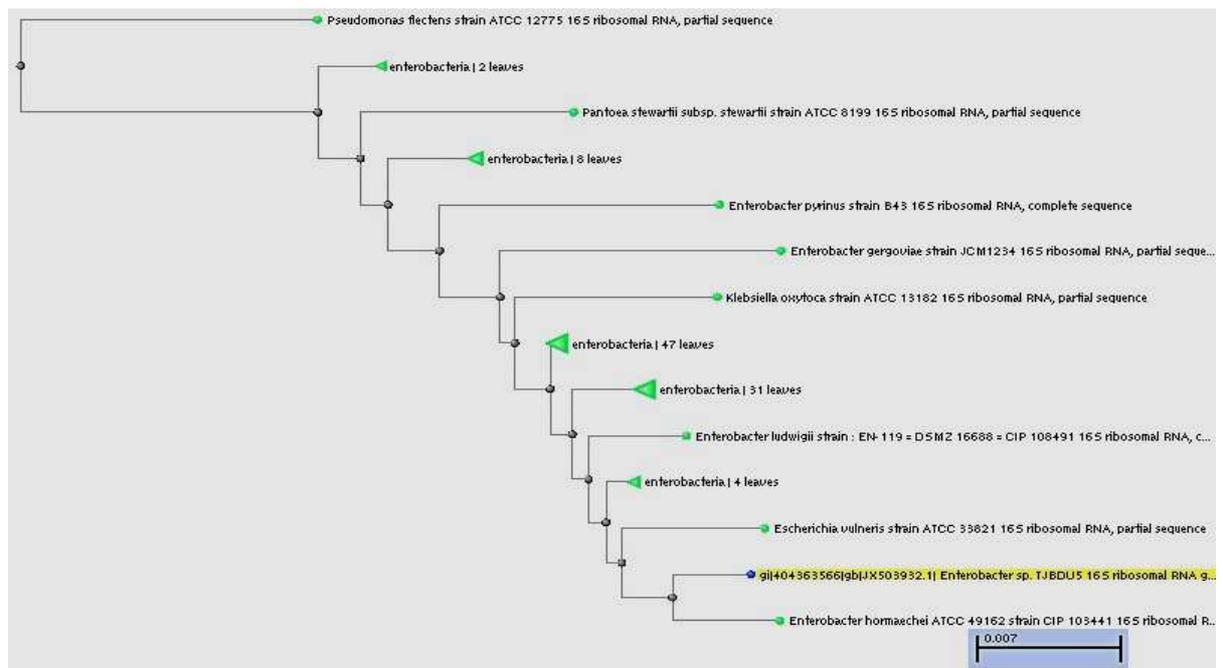


Fig.5 Phylogenetic tree of 16s rRNA for TJBDU5



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REFERENCES

- [1]. Li W, Ruf S et al. *Plant molecular biology* **2000**, 76 (3), 443-451.
- [2]. Murray I, Martinez-Suarez J, Close T, Shaw W. *Biochemical Journal*, **1990** 272 (2) 505.
- [3]. Roberts M, Corney A, Shaw W. *Journal of bacteriology*, **1982** 151 (2), 737-41.
- [4]. Kleanthous C, Shaw W. *Biochemical Journal*, **1984**, 223(1), 211.

- [5]. Sands L, Shaw W. *Antimicrobial agents and chemotherapy*, **1973**, 3(2), 299-305.
- [6]. Shaw W, Brodsky R. *Journal of bacteriology*, **1968**, 95(1), 28-36.
- [7]. Robertson G P, Coleman D C, Bledsoe C S, Sollins P. Oxford University Press; **1999**.
- [8]. Campbell D, Kinniburgh D, Beckett P. *Journal of Soil Science*, **1989**, 40 (2), 321-339.
- [9]. Prescott L M, Harley J P, Klein D A. *Microbiology*, 4th edn. WCB Mc Graw – Hill Publ, **1999**, 152 – 165
- [10]. D.I. Henry. *American society for Microbiology* **1994**, 91-94
- [11]. Bergey D H. *Bergey's Manual of Systematic Bacteriology: The Firmicutes*: Springer; **2001**.
- [12]. Gillings M, Fahy P. *Plant Pathology*, **1993**, 42(5), 744-53.
- [13]. <http://www.ncbi.nlm.nih.gov>
- [14]. Elliot L F, Lynch J M. CAS International, Wallingford, United Kingdom. **1994**, 353- 364.
- [15]. Kennedy A C. *Agric. Ecosyst. Environ.* **1999**, 74 65- 76.
- [16]. Gans J, Wolinsky M, Dunbar J. *Science*. **2005**, 309 1387-1390.
- [17]. Logan N, Berkeley R. *Journal of general microbiology*. **1984**, 130 (7) 1871-1882.
- [18]. Yoo, M H, Huh M D et al. *Aquaculture*. **2003**, 217(1) 11-21.