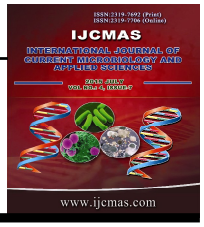
Int.J.Curr.Microbiol.App.Sci (2015) 4(7): 54-64

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 7 (2015) pp. 54-64**
http://www.ijcmas.com



Original Research Article

Identification of Novel Food Borne Pathogen, Enterobacteriaceae Bacterium from Fresh Vegetables and Egg Products

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Keywords

Enterobacteriaceae, PCR, 16sRNA, Food Borne Pathogens, Molecular methods, Qualitative assays

A B S T R A C T

Enterobacteriaceae are recognised as some of the most important food borne pathogens worldwide. These bacterial Infections made human society to suffer with wide socioeconomic and health problems. In order to improve intervention strategies and make more effective the control of production lines and single food items many efforts has been developed to increase the knowledge on the prevalence, transmission routes and persistence of these pathogens in the food chain. To fulfill this purpose, rapid and reliable detection and quantification methods are improved. The standard methods which are culture-based currently applied for identification and enumeration of *Enterobacteriaceae* are time consuming and laborious. These methods lack specificity and do not enable detection of viable but non-culturable bacteria. The focus of the present work has been development and validation of PCR-based detection methods along with Traditional culture and Rapid methods for food borne pathogens. We have

Introduction

Enterobacteriaceae are a large family of rod shaped, non spore forming and gram negative bacteria comprising of *Salmonella, Yersinia enterocolitica,* pathogenic *Escherichia coli, Shigella* spp., *Cronobacter* spp and some opportunistic pathogens such as *Klebsiella* spp, *Serratia* spp. and *Citrobacter* spp. These are facultative

anaerobes except *Saccharobacter fermentans* and some strains of *Yersinia* and *Erwinia* and are generally motile except *Shigella* and *Tatumella.* Because they are deficient in cytochrome c oxidase, it gets convenient to differentiate them from other closely bacteria except *Plesiomonas* spp. Enterobacteriaceae gives catalase positive test with the exception of *Shigella dysenteriae I* and *Xenorhabdus* species (Van Vuuren, 1966). Their ability to ferment carbohydrates is the basis of their detection and enumeration. These bacteria are widespread in soil, on plant surfaces and in digestive tracts of animals and therefore can be found in many foods. These pathogens are not only responsible for food borne illness but are also responsible for causing food spoilage, contributing to significant losses for the agricultural and food industries. For ex-Salmonella, *E. coli* and Klebsiella species are the most predominant species in all food poisoning cases associated with some meat products (Wieler *et al.,* 2011; Aleksic and Aleksić, 1979).

Several other members of the Enterobacteriaceae are responsible for spoilage of a variety of foods including fruit and vegetables, meats, poultry, eggs, milk and dairy products, as well as fish and other seafoods (Mossel *et al.,* 1963; Feldsine *et al.,* 1992). Enterobacteriacea which are fermenting carbohydrates to produce acid and gas are termed as coliforms. These are referred to as the indicator or index organisms in food and water industry, especially in the dairy sector because they are relatively quick and simple to detect. Their absence assures hygiene and good manufacturing practices have been employed while processing of the food. Many published standardised methods exist for the detection and enumeration of Enterobacteriaceae, coliforms and *E. coli* in foods including international standard methods like those published by the International Organization for Standardization (ISO). Nowadays, both Enterobactericeae and coliforms are isolated from foods fpr indication poor hygiene or inadequate processing, process failure and post-process contamination of foods. *E. coli* is commonly used to provide evidence of faecal contamination in certain foods and is used as an index organism for the presence of enteric pathogens such as *Salmonella*. Culturing of Enterobacteria can be done from a variety of raw materials depending on the origin of the raw material. Precise ambience is required for the growth and survival of particular Enterobacteriaceae which depends on various factors. Intrinsic factors like acidity (pH), water activity and natural antimicrobial substances and extrinsic factors like temperature, relative humidity, atmosphere conditions and interactions with other microbial populations, associated with a particular food product and the particular strain of bacterium play a vital role in survival of an Enterobacteriaceae member (Muytjens *et al.,* 1988). Methods for the detection and enumeration of Enterobacteriaceae rely on the growth of the bacterium in selective media along with the use of carbohydrate (e.g. glucose) as an energy source. But, several rapid methods are now available for detection of specific pathogenic members of the Enterobacteriaceae found in foods including *Salmonella* and *E. coli* (Muytjens *et al.,* 1988; Fang *et al.,* 2003).

Materials and Methods

Sample collection

Five samples from egg cuisines viz. omelets, biriyani, etc were collected from restaurants from Hyderabad, Andhra Pradesh. The samples were collected in transparent plastic bags and transported to the laboratory. Some

samples of vegetables from the supermarkets were collected in the same manner.

Preparation

Suspend 40.62 grams of dehydrated medium in 1000 ml distilled water and heated to boiling point to dissolve the medium completely. Mixed well and poured into sterile Petri plates.

Morphological characterization

Grams staining, Endospore staining test, capsulated staining test, Motility test were carried out for the morphology of cell.

Biochemical characterization

Catalase, ONPG, Lysine decarboxylase, Ornithine, Urease, Phenyl alanine deamination, Nitrate reduction, H_2S production, Citrate utilization, Voges proskaeurs, Methyl red, Indole and Ma lonate were suited for biochemical studies.

Identification of the isolated bacteria by Molecular methods

Isolation of genomic DNA from enterobacteria by using ST Buffer

1.5 ml of overnight culture was taken in Eppendorf tubes and was subjected to centrifugation at 10,000 rpm for 2mins. The supernatant was discarded and the pellet was dissolved in 200µl of T.E buffer and 300µl of ST buffer. The solution was incubated for 10mins at 65^0C. This solution was spun at 10,000rpm for 2mins. Pellet was discarded and to the supernatant 150 µl of sodium acetate was added and the solution was left for 3mins. Then the solution was transferred to fresh Eppendorf tube. 600µl of ice cold isopropanol was added to the supernatant and incubated for 30mins at -20^0C, then cooled it to room temperature. It was centrifuged at 10,000 rpm for 2mins. The supernatant was discarded. And 300µl of 70% ethanol was added to it for washing. The pellet was centrifuged at 10,000rpm for2mins and the supernatant was discarded. The pellet was allowed to air dry. The pellet was dissolved in 30µl of T.E buffer and the pellet was stored at -20^0c for further use (Lederberg and Cohen, 1974; Yves *et al.,* 1996).

Preparation of 1% Agarose gel

0.20gms of agarose powder was weighed and transferred it into a conical flask.400µl of 50X TAE was added to it to 20ml. The solution was boiled until clear solution was obtained. Then the solution was cooled to 45°C. The gel caster and comb were cleaned neatly. After the solution was cooled to around 40^oC, 2.5µl of ethidium bromide was added and mixed well. The solution was poured carefully into the gel caster without any air bubbles. The comb was placed in gel caster and allowed to solidify.

15µl of the isolated genomic DNA was mixed with 3µl of the gel loading dye (Bromophenol blue) and loaded in 1 % agarose gel well. The gel was electrophoresed at 90 volts for about 30 min and the samples were observed for genomic DNA in a gel documentation system (UV illuminator) (Jane Aldrich *et al.,* 1976; Ribot *et al.,* 2006).

16s rRNA analysis for the identification of pathogenic micro organism

A. Polymerase chain reaction rapid method

50µl of sample was taken into an Eppendorf tube. This sample was centrifuged at

10,000rpm for 3 mins. The supernatant was discarded and the pellet was dissolved in 100µl of TE buffer and 100µl of ST. It was then incubated at 95^0C for 10 mins. The tubes were cooled to room temperature and centrifuged at 10,000rpm for 2mins. Then the supernatant was taken for Rapid PCR.

PCR amplification

PCR is an in vitro biochemical synthesis of specific DNA sequences by using thermo stable taqDNA polymerase. This technique was developed by Karry Mullis in the year 1983. It is an indispensable technique used in biological and medical research labs for characterizing, analyzing and synthesizing particular piece of DNA or RNA from any organism by amplifying that particular sequence. It exploits the natural function of the polymerase present in all living things to synthesize DNA o RNA (McClelland *et al.,* 2001; Chiu *et al.,* 2005).

PCR consists of three basic steps viz.

Denaturation, annealing and extension

Initially, the reaction mixture is heated to a temperature of 94^0C, which is held for 1-3 mins. It causes the melting of DNA template by disrupting the hydrogen bonds between complementary bases of the DNA strand yielding single strand of DNA. Annealing involves joining of both primers to each original strand for new strand synthesis at 52^0C for 1 minute. Finally, extension involves synthesizes of a new DNA strand complementary to our desired sequence at 72^0C temperature for 1:30 mins (McClelland *et al.,* 2001).

B. Setting up of PCR reaction

DNA was isolated from the bacterial sample by using rapid method. The isolated DNA was used as a template for amplification of DNA by using 16s rRNA universal primers. PCR amplification of the forward 5′ AGA GTT TGA TCC TGG CTC AG 3′ and reverse primer 5′ GAC GGG C(AG)G TG(AT) GT (AG) CA 3′. The 16s rRNA was amplified in a 50 µl reaction containing 2µl of genomic DNA. 1µl of forward primer and 1µl of reverse primer was added to that template DNA. 1 unit of Taq DNA polymerase, 1 µl dNTP's, 5 µl Assay buffer, 39 µl of dd-water was added (McClelland *et al.,* 2001).

The PCR amplification was carried out by the thermal cycler under the following conditions. 94ºC for 3minutes, and then 30 cycles of at 94ºC for 1minute, 52ºC for 1minute, 72ºC for 1.5 minute, followed by final extension at 72ºC for 7minutes and Holding temperature at 4ºC for infinite times, this is the freezing state (Aabo *et al.,* 1993).

In this PCR reaction firstly the PCR product of each micro-organism is detected and then step by step pair of 2-2 micro-organism was detected.

For DNA sequencing, the sample was gone into the institute BIOSERVE BIOTECHNOLOGIES, HYDRABAD. We can obtain the 16 S rRNA of each micro-organism.

Agarose gel electrophoresis

Electrophoresis tank was taken and 200ml of 1X Tris HCL buffer was poured into the tank. The positive and negative electrodes were connected to electrophoresis tank. The gel casting tray was placed in the tank buffer. The comb was removed slowly from the gel casting tray without disturbing the agarose gel. 2µl of 6X gel loading dye was mixed with 10µl of the DNA sample

(Cooley *et al.*, 2003). That sample was loaded into the well. The power pack was switched on for electrophoresis run. After the electrophoresis run the bands were observed under UV transilluminator (Rambach, 1990).

Results and Discussion

Screening of samples for bacteria

Screening of samples resulted in 15 well-isolated colonies from 10^{-5} and 5 well-isolated colonies from 10^{-6} dilution plate. The colonies with different morphology from 10^{-5} and 10^{-6} dilution plates were selected for further study. These colonies are inoculated into nutrient broth and incubated at 37°C on an incubator shaker at 200rpm for 24 hrs. Growth obtained from these flasks was streaked onto agar media plates and after confirming purity. Theses isolates were further characterized by morphological and biochemical studies.

Morphological characteristics

Grams staining, capsule and spore staining, motility and colony morphology of the isolates were studied for identification. The results of these observations were shown in figure 1.

Fig.1 Gram negative bacteria

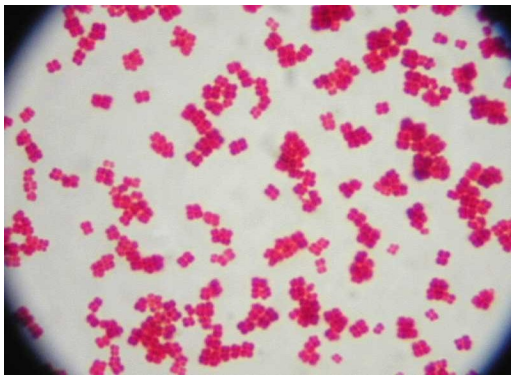

Fig.2 Biochemical test

Fig.3 DNA isolation

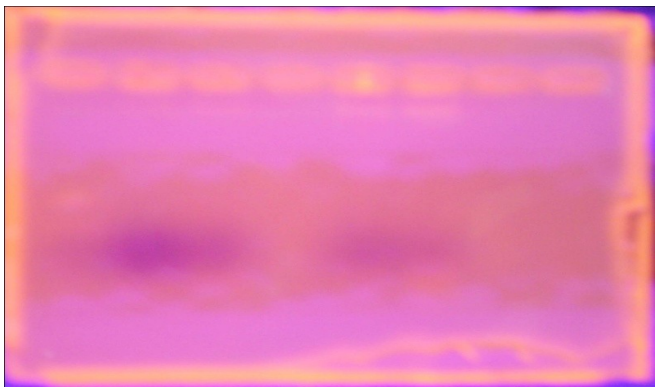

Fig.4 PCR amplification

Fig.5 sequence of 16s RNA of Enterobacteriacea

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TCGGGAATATTGCAAAATGGGCGCAAACCCGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT
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ACACCATGGGAGTTGGTTGCAAAAGATGTAGGTAGCTTAACCTTAGGGCGGGACCTTACCACATTGTGAT
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Fig.6 Nucleotide composition

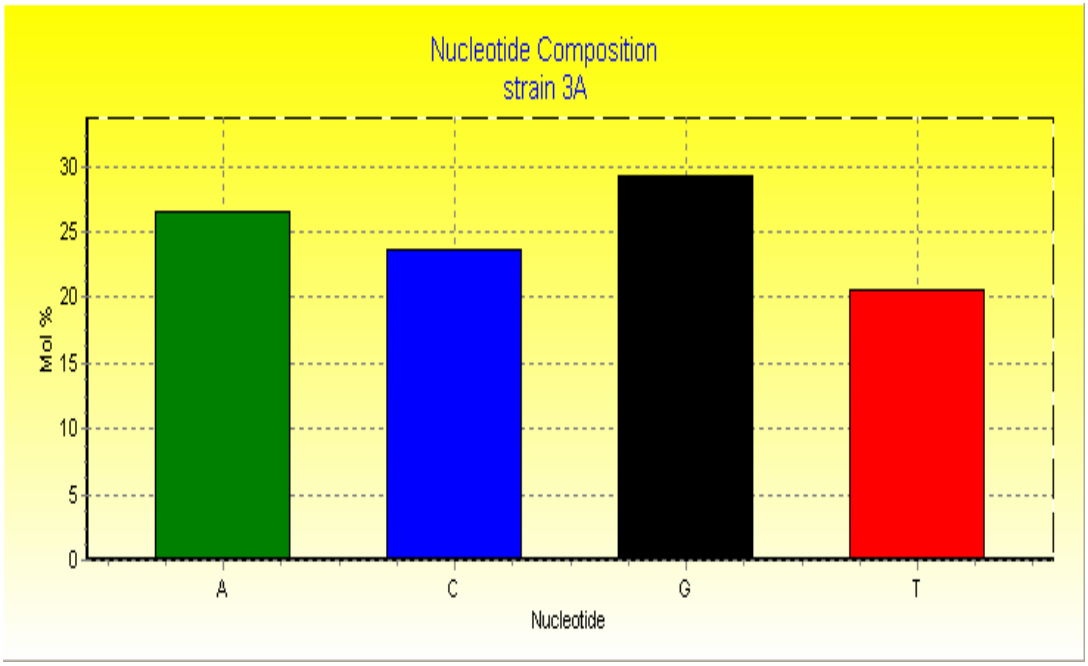

Fig.7 sequence of 16s RNA of Enterobacteriacea

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Fig.8 Nucleotide Composition

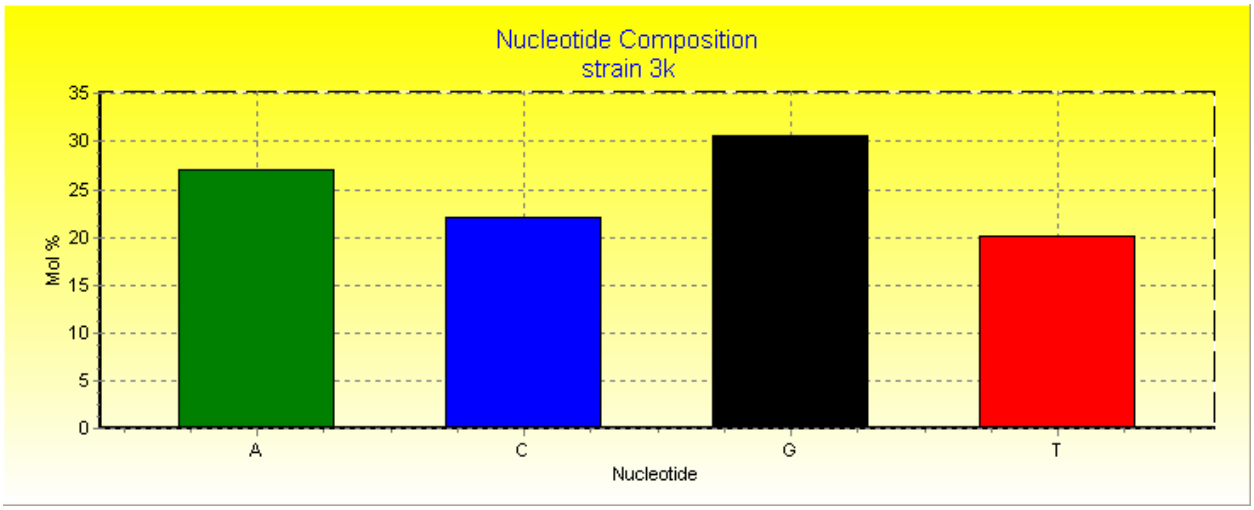

Figure.9 GenBank file of Enterobacteriaceae bacterium Pyde1

NCBI Resources How To

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Enterobacteriaceae bacterium Pyde1 16S ribosomal RNA gene, partial sequence

GenBank: KC855287.1

FASTA Graphics

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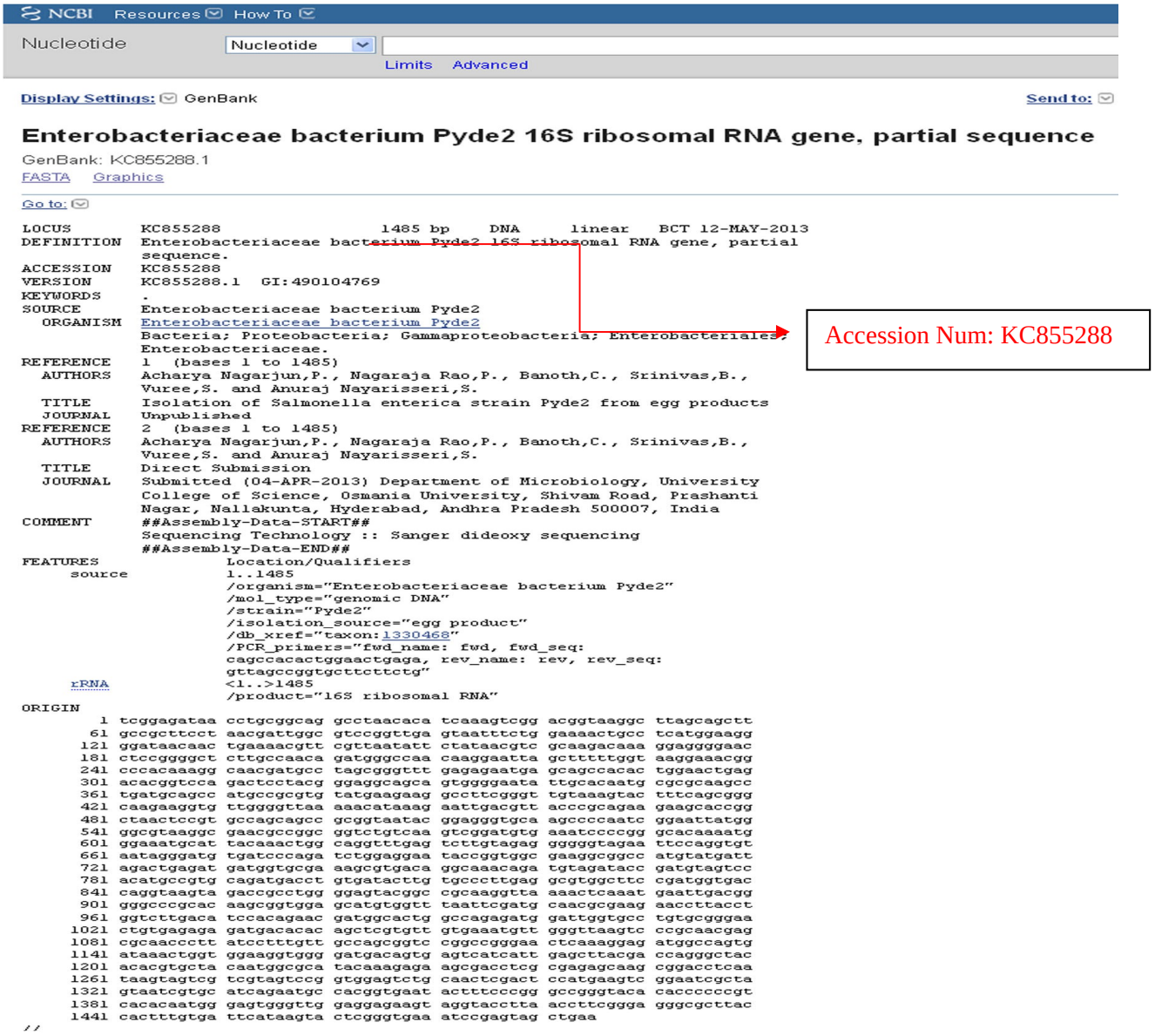
Figure.10 GenBank file of Enterobacteriaceae bacterium Pyde2



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      961 ggtcttgaca tccacagaac gatggcactg gccagagatg gattggtgcc tgtgcgggaa
     1021 ctgtgagaga gatgacacac agctcgtgtt gtgaaatgtt gggttaagtc ccgcaacgag
     1081 cgcaaccctt atcctttgtt gccagcggtc cggccgggaa ctcaaaggag atggccagtg
     1141 ataaactggt ggaaggtggg gatgacagtg agtcatcatt gagcttacga ccagggctac
     1201 acacgtgcta caatggcgca tacaaagaga agcgacctcg cgagagcaag cggacctcaa
     1261 taagtagtcg tcgtagtccg gtggagtctg caactcgact ccatgaagtc ggaatcgcta
     1321 gtaatcgtgc atcagaatgc cacggtgaat actttcccgg gccgggtaca cacccccgt
     1381 cacacaatgg gagtgggttg gaggagaagt aggtacctta accttcggga gggcgcttac
     1441 cactttgtga ttcataagta ctcgggtgaa atccgagtag ctgaa
//
```

Accession Num: KC855288

Biochemical tests

The isolates were further characterized by Bio chemical studies. The results were shown in figure 2.

Isolation of genomic DNA from bacteria

DNA was isolated by the procedure mentioned in materials and methods, and observed under UV- Tran illuminator (Fig. 3).

Polymerase chain reaction

The isolated genomic DNA and plasmid DNA samples were amplified with suitable primers mentioned in materials and methods (Fig. 4).

Sequence obtained from sequencing

Sequence analysis has been performed using Bioedit software. BioEdit is a biological sequence analysis / alignment bioinformatics tool developed for windows. An intuitive multiple document interface with suitable features create alignment and manipulation of sequences relatively simple on our desktop computer.

Several sequence manipulation and analysis options and links to external analysis programs facilitate a working environment which allows you to view and manipulate sequences with simple point-and-click operations.

Nucleotide composition of Strain includes DNA molecule with 1519 base pairs, Molecular Weight 461448.00 Daltons, Molecular Weight = 924176.00 Daltons, and G+C content 52.86%; A+T content = 47.14%.

Nucleotide composition of Strain contains DNA molecule with 1485 base pairs, Molecular Weight 450504.00 Daltons, Molecular Weight 903473.00 Daltons, and G+C content 52.79%; A+T content = 47.21%.

Identification of novel food borne pathogen

Out of 35 samples 2 strains (3f and 3k) found to be novel. The results obtained from blast were found to be a novel food borne pathogens, which were further named Enterobacteriaceae bacterium Pyde1 and Enterobacteriaceae bacterium Pyde2, after characterization the sequence of isolate was deposited in GenBank with accession numbers 'KC855287' and 'KC855288' respectively.

Conclusion

Enterobacteriaceae are recognised as some of the most important food borne pathogens worldwide. The standard methods lack specificity and do not enable detection of viable but non-culturable bacteria. To fulfill this purpose, rapid and reliable detection and quantification methods are improved. The focus of the present work has been development and validation of PCR-based detection methods along with Traditional culture and Rapid methods for food borne pathogens. We have identified new strains of E*nterobacteriaceae* based on PCR method for *Enterobacteriaceae* in egg cuisines. 16sRNA sequencing was applied to the isolated DNA and the new sequence was submitted to Genbank. Out of 35 samples 2 strains found to be novel. The results obtained from blast were found to be a novel food borne pathogens, which were further named Enterobacteriaceae bacterium Pyde1 and Enterobacteriaceae bacterium Pyde2, after characterization the sequence of isolate was deposited in GenBank with accession numbers 'KC855287' and 'KC855288' respectively.

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