Innovare
Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 8, 2014

Original Article

BIOCHEMICAL, MOLECULAR CHARACTERIZATION AND SEQUENCE ANALYSIS OF KERATINASE PRODUCING NOVEL STRAIN OF *BACILLUS LICHENIFORMIS* ISOLATED FROM POULTRY FARM

SANKARI D.[1], *KHUSRO A.[2]

[1]Department of Biotechnology, Faculty of Science and Humanities, SRM University, Kattankulathur, Chennai (India), [2]Department of Plant Biology and Biotechnology, (PG Biotechnology), Loyola College, Nungambakkam, Chennai (India)
Email: armankhan0301@gmail.com

Received: 29 Jun 2014 Revised and Accepted: 01 Aug 2014

ABSTRACT

Objective: To isolate and characterize a novel strain of keratinase producing bacterium from poultry farm.

Methods: Preliminary study of bacterial isolation from poultry faeces soil sample was done by serial dilution followed by microscopic analysis, morphological characteristics and biochemical tests of pure isolated culture. Further, the identification of bacterium as novel strain was confirmed by subjecting its amplicon to 16S rRNA gene sequence analysis and pairwise alignment through BLAST tool. RNA Secondary structure of 16S rRNA gene sequence was predicted through RNAfold Web server and RNA structure tool. Phylogenetic tree based on taxonomic positions was inferred using the Neighbor-joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0. MAR index value of this strain was determined against six antibiotics using disc diffusion method.

Results: The bacterium was identified as *Bacillus licheniformis* strain 018. Different parameters of RNA Secondary structure of 16S rRNA gene sequence was obtained through Bioinformatics tools. Minimum Free Energy and G+C content were calculated as -247.40 kcal/mol and 54.7% respectively.MAR index of this novel strain was found to be zero.

Conclusion: The isolation of bacteria from different sources offers novel bacteria as their potential applications in different biotechnological processes. The present investigation clearly indicates the isolation, molecular characterization and sequence analysis (through Bioinformatics tools) of *Bacillus licheniformis*s train 018 and its industrial applications.

Keywords: *Bacillus licheniformis*, MAR index, Phylogenetic tree, RNA secondary structure, RNA structure tool, RNA fold Web server.

INTRODUCTION

Keratin is an insoluble and highly stable protein found mostly in feathers, hair, nails and wool [1]. Keratin fibers from feathers are bio-degradable, insoluble in organic solvents and have good mechanical properties. This property is due to the molecular configuration of the constituent amino acids, high degree of cross-linkages of disulphide bridges, hydrogen bonds and hydrophobic interactions [2, 3]. Insoluble fibrous keratin protein is the primary substance found in chicken feathers, and cannot be degraded by common proteases. Keratinases belong to the group of extracellular serine proteases capable of degrading keratin. Keratinolytic enzymes have found important utilities in biotechnological processes. Feather hydrolysates produced by bacterial keratinases have been used as additives for animal feed [4].

Keratinase has potential applications in the poultry industry and for conversion of feathers into a feed protein. A diversity of microorganisms has been reported to carry out keratin degradation. Keratinolytic bacteria include *Actinomycetes* sp., *Bacillus* sp., *Micrococcus* sp., *Clostridium* sp., etc. [5-7]. The use ofcrude enzymes from *Bacillus* species have been extensively studied due to their effectiveness in terms of feather degradation [8].

B. licheniformis is a multipurpose organism and has gained popularity along with *B. subtilis*. *B. licheniformis* is most commonly found in soil and as well as near the chest region of birds. With the rapid advancement in enzymology and fermentation technology, commercially different industrial enzymes are being produced by *B. licheniformis* [9]. This organism has been reported to produce some of the most important commercial enzymes especially keratinase [10].

Keeping in view the role of *Bacillus* sp. in degradation of poultry feathers, the present study was investigated to isolate and characterize the keratinase producing novel strain of *B. licheniformis* from poultry farm using 16S rRNA gene sequencing. The present study was also carried out to analyze 16S rRNA gene sequence of the novel strain using different Bioinformatics tools.

MATERIALS AND METHOD

Collection, Isolation and Screening of sample

Poultry faeces sample was collected from poultry farm of Guduvanchery, Tamil Nadu (India). Faeces soils were brought to the laboratory in aseptic condition. One gram of sample was suspended in 9 ml of saline and mixed vigorously to make uniform suspension. After that soil samples were serially diluted up to 10^{-5} and 0.1 ml of aliquots were spread over nutrient agar plates from 10^{-5} dilution. The plate was incubated at 37ºC for 24 h. Pure strain was picked out and purified by repeated streaking on nutrient agar slants. The culture was streaked on slants and kept in incubator at 37°C for 24 h and were preserved in slants at 4±2°C.

Organism identification

Purified isolate was characterized by Biochemical analysis using Indole test, Methyl Red test, Voges Proskauer test, Citrate utilization test, Catalase test, Urease test, Oxidase test and Amylase test (according to the Bergey's Manual of Systemic Bacteriology). Gram staining and Motility test were performed under Morphological test

Genomic DNA isolation

Two ml of bacterial culture were centrifuged at 6000 rpm for 5 minutes. The supernatant was discarded. One ml of Uni Flex™ Buffer 1 and 10 µl of RNase were added to the pellet obtained. Mixed well by pipetting and incubated for 30 minutes at 37ºC in a water bath. To the lysed samples 1 ml of 1:1 phenol: chloroform were added and mixed well. The samples were centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layers were separated in a fresh 1.5 ml vial. To the aqueous layer 1 ml of Uni Flex™ Buffer 2 were added and mixed well by pipetting. The mixture was centrifuged at 12,000 rpm for 15 minutes at room temperature. The supernatant was discarded. To the pellet 500 µl of 70% ethanol were mixed. Again it was centrifuged at 10,000 rpm for 10 minutes at 4ºC. The supernatant was discarded. The pellet was air dried for about 10-15 minutes till the ethanol evaporate. The pellet was

resuspended in 50-100 µl of UniFlex™ Elution Buffer. DNA was stored at -20ºC.

PCR amplification and sequence of 16S rRNA

The 16S ribosomal RNA was amplified by using the PCR (ependorfep. Gradient) with *Taq* DNA polymerase and primers 27F (5` AGTTTGATCCTGGCTCAG 3`) and 1492R (5`ACGGCTACC TTGTTACGACTT 3`). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94°C for 4 min followed by 30 cycles at 94°C for 1 min, primer annealing at 52°C for 1 min and primer extension at 72°C for 1 min. At the end of the cycling, the reaction mixture was held at 72°C for 10 min and then cooled to 4°C. PCR amplification was detected by agarose gel electrophoresis and visualized by alpha image gel doc after ethidium bromide staining.

Purification of amplified product

PCR sample is taken in fresh vial and 5 µl of 3M Sodium acetate solution (pH-4.6) and 100 µl of absolute ethanol were added into it. The vial was mixed thoroughly. The vial was kept at -20º C for 30-40 minutes to precipitate the PCR product. Then it was centrifuged at 10,000 rpm for 5 minutes. 300 µl of 70% ethanol were added to the pellet, without mixing, and the centrifugation was repeated at same rpm. The pellet was air dried until the ethanol effervescence is removed. The pellet is suspended in 10 µl of sterile distilled water [11].

Sequencing of PCR product

The PCR product obtained was sequenced by an automated sequencer (Genetic Analyzer 3130, Applied Biosystems, and USA). The same primers as above were used for sequencing. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST available at http:// www.ncbi-nlm-nih.gov/.

RNA Secondary structure prediction

RNA secondary structure prediction at 37ºC was performed to determine the stability of chemical or biological molecules or entities of the isolate. Folds, Prob Knot, Mountain Plot, Entropy, Partition and Minimum Free Energy (MFE) of the sequence were calculated by RNA structure tool and RNA fold Web server.FTG server tool was used to determine the % content of G+C. Pictogram was visualized to identify sequence alignment.

Neighbor-joining tree analyses of *Bacillus* 16S rRNA gene for sequence comparisons

Phylogenetic relationship of the isolate with other *Bacillus* species were inferred from phylogenetic comparison of the 16S rRNA sequences. Phylogenetic trees were inferred using the neighbor-joining (NJ) algorithm in Molecular Evolution Genetic Analysis (MEGA) software version 4.0 [12].

Multiple antibiotic resistances (MAR) index determination

The antibiotic susceptibility pattern of the test organism was performed as per standard procedure. A homogeneous bacterial lawn was prepared on Mueller Hinton Agar plates using sterile cotton swabs. The sterile discs of 6 mm diameter were soaked with 25 µl of antibiotics. Using an ethanol dipped and flamed forceps the standard antibiotic and soaked discs were aseptically placed over the agar plates sufficiently separated to avoid overlapping of zone of inhibition. Plates were incubated at 37ºC for 24 hours. After 24 hours, diameter of zone of inhibition was measured in mm and results were recorded. MAR index was calculated by the ratio of number of antibiotics ineffective over the organisms to the number of antibiotics exposed [13]. The antibiotics used in this study were Ampicillin (AMP-10 µg), Kanamycin (K-30 µg), Nalidixic acid (NA-30 µg), Streptomycin (S-10 µg), Cephotaxime (CTX- 30µg) and Penicillin (P-10 µg).

RESULTS

Morphological and Biochemical test analysis

The morphological and biochemical characteristics of the isolate were studied (Table 1). The isolated bacterial strain was identified as *Bacillus* sp. based on the taxonomical characteristics.

PCR amplification and sequencing of 16SrRNA

Genomic DNA of the isolate was visualized under UV. The amplicon of 653 bp was observed using PCR amplification (Figure not shown). In the present study, 16S rRNA gene sequencing of the isolate was investigated. The isolate was identified as *Bacillus licheniformis* strain 018 by comparing the similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST. The comparison showed that the similarities of 16S rRNA gene sequences were 100%. The identities of strain KPA were determined by comparing them with the available sequences of the strains and with high scored rRNA sequences in BLAST search. The novel isolated sequence was deposited in GenBank (Accession number-KC342225), maintained by NCBI, USA.

RNA Secondary structure prediction

The optimal secondary structure with a minimum free energy (MFE) of -247.40 kcal/mol was represented in Fig-a. Mountain plot representation and entropy for each position were also determined (Fig-b). A mountain plot represents a secondary structure in a plot of height versus position, where the height is given by the number of base pairs enclosing the base at given position i.e. loops correspond to plateaus (hairpin loops are peaks), helices no slopes. Fig-C, D, E, F and G represent Folds, Prob Knot, Partition, % G+C (54.7%) content and Pictogram of the sequence respectively. Folds predict the lowest free energy structure in a set of low free energy structures for a sequence. Prob Knot predict a secondary structure of base pairs including pseudo knots. Partition performs a partition function calculation on a single sequence to calculate base pair probabilities.

Phylogenetic tree of strain KPA 16S rRNA

A Neighbor- joining tree of *Bacillus* 16S rRNA sequences, including different strains of *Bacillus* species, clustered all the isolates belonging to the previously identified species to the corresponding species (Fig-H).

MAR Index Determination

The MAR index value of the test organism was reported in Table 2. The MAR value is a ratio of the number of ineffective antibiotics to the number of antibiotics exposed. The MAR value of the test organism was found to be zero.

Table 1: Shows the Morphological and Biochemical test report

S. No.	TESTS	RESULT
1	Morphology	Rod shaped
2	Gram Staining	Positive
3	Motility	Positive
4	Indole	Negative
5	Methyl Red	Negative
6	Voges–Proskauer	Positive
7	Citrate utilization	Positive
8	Urease	Positive
9	Catalase	Positive
10	Amylase	Positive
11	Oxidase	Positive

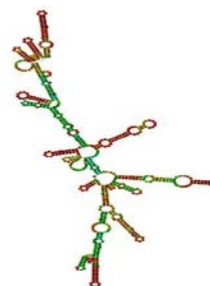

Fig. A: Shows RNA Secondary structure of *Bacillus licheniformis* strain 018 with minimum free energy of -247.40 kcal/mol

Table 2: Shows the MAR index determination of the bacterial isolate

Bacteria	MAR value	No. of ineffective antibiotics	Name of ineffective antibiotics
B. licheniformis strain 018	Zero	Zero	None

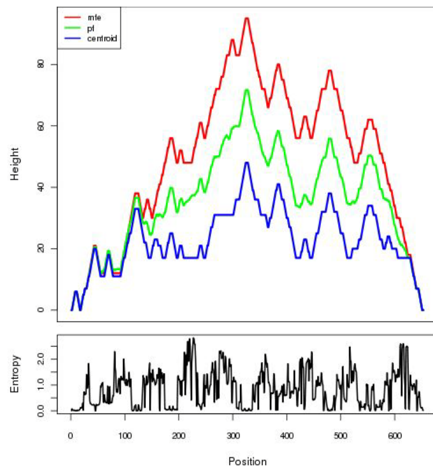

Fig. B: Shows Mountain plot and sequence Entropy for each position

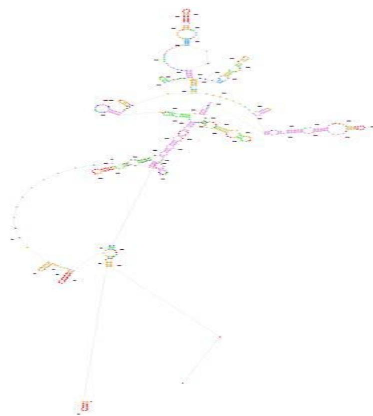

Fig. C: Shows Folding of the 16S rRNA

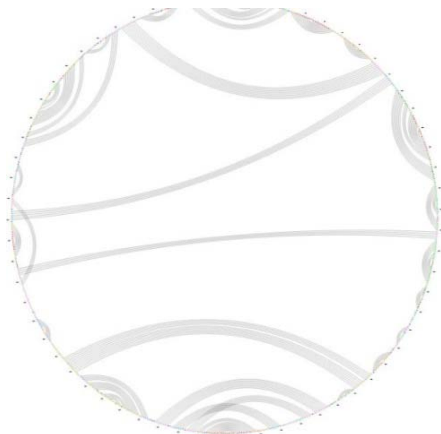

Fig. D: Shows Secondary structure of base pairs, including pseudo knots

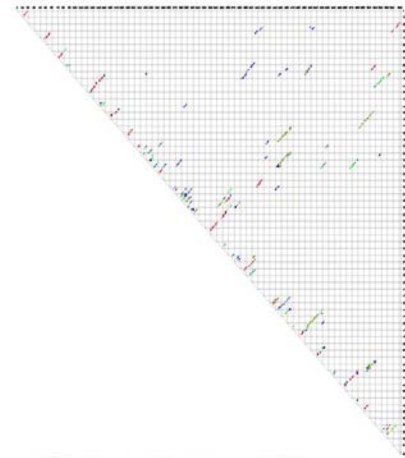

Fig. E: Shows Secondary structure Partition

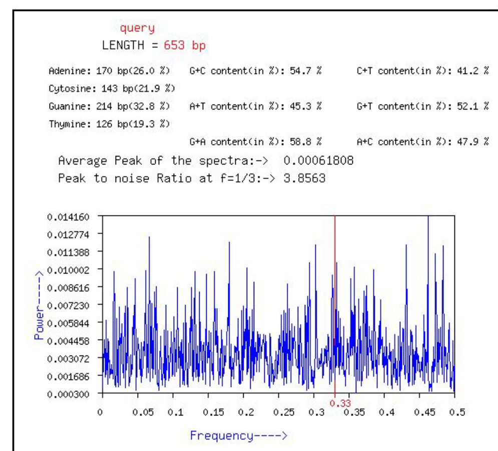

Fig. F: Shows total % G+C content

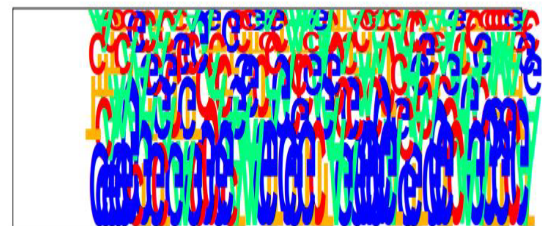

Fig. G: Shows Pictogram analysis

DISCUSSION

Bacteria have now become the choice of selection for various Biotechnological applications. They can be genetically modified to produce the desired activity and enhanced production of enzymes. Screening of a large number of microorganisms is an important step in selecting a highly potent microbial culture for multipurpose utilization. Many studies have been conducted previously in order to search and select new bacterial isolates that can substitute eukaryotes by bacterial isolates for the production of various enzymes at industrial scale. In the present study a novel bacterial strain of *Bacillus licheniformis* producing industrially important enzymes especially keratinase was isolated from poultry farm using 16S rRNA gene sequencing. Bacteria are the most dominant group of enzyme producer. Bacteria belonging to *Bacillus* sp. are by far the

most important source of several commercial microbial enzymes. They can be cultivated under unfavorable conditions to give rise to products that are in turn stable in a wide range of harsh environments. Previous researches had been done to identify the bacteria, producing various enzymes based upon the traditional methods. There are two major drawbacks of traditional methods of bacterial identification. First, they can be used only for organisms that can be cultivated *in vitro*. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species.

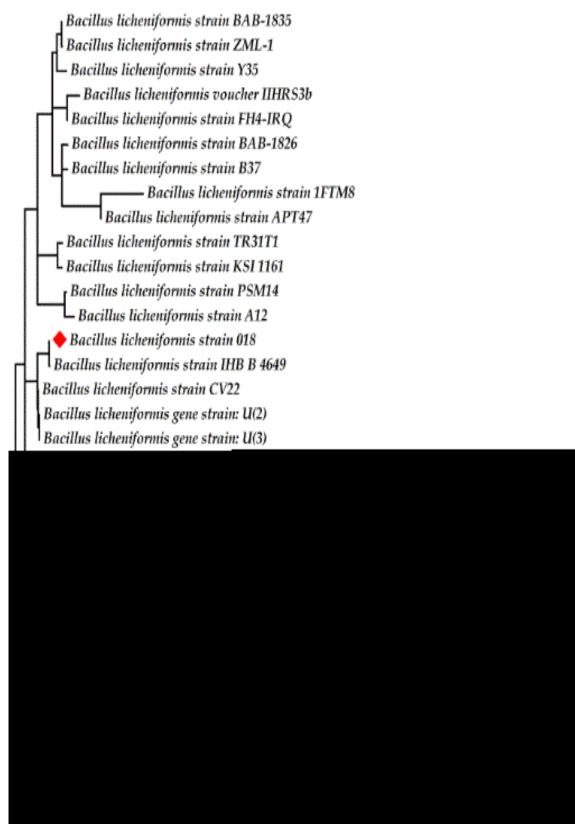

Fig. H: Shows Dendrogram depicting the phylogenetic relationship of strain 018

The traditional identification of bacteria on the basis of phenotypic characteristics is generally not as accurate as identification based on genotypic methods [14]. Comparison of the bacterial 16S rRNA gene sequence has emerged as a preferred genetic technique. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes [15]. 16S rRNA gene consists of highly conserved regions which describe the level and occurrence of phylogenetic relationships. 16S ribosomal RNA (rRNA)-based molecular identification could achieve identification because of the presence of species-specific variable regions. This molecular approach has been extensively used for bacterial phylogeny, leading to the establishment of large public-domain databases and its application to bacterial identification, including that of environmental and clinical uncultured microorganisms, unique or unusual isolates and collections of phenotypically identified isolates [16].Previous studies demonstrated that all these newly discovered isolates of *B. Licheniformis* have great potential for producing high amount of different extracellular enzymes of commercial importance. As it is clear from the previous reports that *Bacillus licheniformis* is an important source of industrially important enzymes so in view of this the first part of this study was dedicated to find the position of new strain of bacteria isolated from poultry farm via morphological and biochemical identification. Then the potential of 16S rRNA sequencing was evaluated to rapidly identify *B. licheniformis* strain 018. In the present investigation the novel strain of *Bacillus licheniformis* was isolated from poultry farm with similarity percentage of 100% from other strains of the same genus and species. Mehta *et al.* (17) isolated novel strain of *Bacillus* species with keratinolytic activity using microscopic, biochemical and 16S rRNA gene sequencing. The 16S rRNA gene is now used as a framework for the modern classification of bacteria including *Bacillus* sp. Minimum free energy for the prediction of optimal secondary structure is the method for searching the structure with stable energies. First a dot matrix analysis is carried out to highlight complementary regions (diagonal indicates succession of complementary nucleotides). The energy is then calculated for each predicted structure by summing negative base stacking energies. Using one sequence structure of complementary regions that are energetically stable can be determined. Minimum free energy value determines it as a stable model. The isolate was examined as *Bacillus licheniformis* strain 018 depending on their taxonomic positions. The phylogenetic and similarity analysis of the novel strain undoubtedly reflects diversity within the targeted DNA regions.

CONCLUSION

It is clear from the present and previous reports that 16S rRNA gene sequence information has an expanding role in the identification of bacteria. Economically valuable enzymes can be commercially produced on large scale from novel isolated strains of *Bacillus* species. In future, 16S rRNA gene sequencing will continue to be the standard and accurate method for identification of most bacteria which will produce a new era for the production of industrially important enzymes such as keratinase at large scale. Further research is necessary to determine the potential of valuable extracellular enzymes produced by strain 018 in the poultry field.

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGEMENT

The authors wish to acknowledge Department of Plant Biology and Biotechnology, Loyola College for fully supporting this research activity.

REFERENCES

1. Shih JCH. Recent development in poultry waste digestion and feather utilization:a review. J Poultry Sci 1993;72:1617-20.
2. Bradbury JH. The structure and chemistry of keratin fibers. J Adv Prot Chem 1973;27:111-211.
3. Carlos M-H. Ana Laura Santos. Keratin fibres from chicken feathers Structure and advances from polymer composites. Keratin Structure Properties and Applications Nova Science Publishers Inc Chapter. 2012. p. 149-211.
4. Williams CM, Lee CG, Garlich JD, Shih JCH. Evaluation of a bacterial feather fermentation product, feather-lysate as a feed protein. J Poultry Sci 1991;70(1):85-94.
5. Kim JM, Lim WJ, Suh HJ. Feather-degrading *Bacillus* species from poultry waste. J Process Biochem 2001;37:287-91.
6. Lucas FS, Broennimann O, Febbraro I, Heeb P. High diversity among feather-degrading bacteria from a dry meadow soil. J Microb Ecol 2003;45:282-90.
7. Böckle B, Galunski B, Müller R. Characterization of a keratinolytic serine protease from *Streptomyces pactum*. J Appl Environ Microbiol 1995;61(10):3705-10.
8. Zerdani I, Faid M, Malki A. Feather wastesdigestion by new isolated strains *Bacillus* sp. *In Morocco*. Afr J Biotechnol 2004;3:67-70.
9. De-Boer AS, Priest F, Diderichsen B. On the industrial use of *Bacillus licheniformis*:A review. J Appl Microbiol Biotechnol 1994;40:595-8.
10. Hmidet N, Ali NE, Haddar A, Kanoun S, Alya SK, Nasri M. Alkaline proteases and thermostable α-amylase co-produced by Bacillus

licheniformis NH1: Characterization and potential application as detergent additive. J Biochem Engineer 2009;47:71-9.

11. Shah KP, Chandok HK, Rathore P, Sharma MV, Yadav M, Nayarisseri SA. Screening, isolation and identification of Polygalacturonase producing *Bacillus tequilensis* strain EMBS083 using 16S rRNA gene sequencing. Europ J Biol Sci 2013;5(1):09-13.
12. Tamura K, Dudley J, Nei M, Kumar S. MEGA4:Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. J Mol Biol Evol 2007;24:1596-99.
13. Krumperman PH. Multiple antibiotic resistance indexing *E.coli* to identifying riok sources of faecal contamination of foods. J Appl Environ Microbiol 1983;46:165-70.
14. Krieg NR, Garrity GM. On using the manual, In:Bergey's manual of systematic bacteriology;Second edition ed. Bergey's Manual Trust:Springer;2001.p. 15-9.
15. Patel JB. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. J Mol Diagnos 2001;6:313-21.
16. Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D. 16S Ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 2000;38 (10):3623-30.
17. Mehta RS, Jholapara RJ, Sawant CS. Isolation of a novel feather-degrading bacterium and optimization of its cultural conditions for enzyme production. Int J Pharm Pharm Sci 2014;6 (1):194-201.