International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Impact Factor (2012): 3.358

Molecular Characterization of Cellulose Degrading *Klebsiella variicola* RBEB3 (KF036184.1) by 16S rRNA Technique

Gopinath. S. M[1], Ismail Shareef[2], Ashalatha[3], Shreya Ranjit[4]

[1]Professor & HOD Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India

[2]Professor and HOD, Department of Biotechnology,
Acharya Institute of Technology, Bangalore-560107, Karnataka, India

[3]Assistant Professor, Research scholar in Department of Biotechnology,
Acharya Institute of Technology, Bangalore, Karnataka, India

[4]2nd year M.tech, Department of Biotechnology,
Acharya Institute of Technology, Bangalore-560107, Karnataka, India

Abstract: ***The cellulose degrading bacteria was isolated from soil samples of paper industry waste, cloth industry waste, kitchen waste, garden, earthworm and characterized by morphological and biochemical analysis. The bacteria isolated were grown on Carboxy Methyl Cellulose (CMC) agar media and checked for cellulase activity by adding grams iodine, observing for zone of clearance and identified the organism by gram staining technique. Molecular characterization of cellulose degrading bacteria was determined by the phylogenetic analysis of the 16S rRNA sequence by bioinformatics tools like BLAST. The results showed 99 % resemblance with Klebsiella variicola RBEB3 and the sequence submitted to NCBI with accession number KF036184.1. Cellulase will be used as a possible food supplement or in pharmaceutical industry to degrade cellulose molecule into its monomer called glucose.***

Keywords: Cellulose degrading bacteria, Cellulase, Molecular characterization, Phylogenetic analysis, *Klebsiella variicola*

1. Introduction

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$, a polysaccharide is the most abundant renewable polymer available on earth and a major constituent of plant matter. 100 billion dry tons/year is produced in the biosphere [1]. 70% of the plant biomass found in lignocellulosic biomass comprised of mainly cellulose, lesser hemicelluloses and lignin [2]. Cellulose is a crystalline polymer of D-glucose residues connected by β-1, 4 glycosidic linkages [3]. Recently, the accumulation of cellulosic waste is becoming an environmental problem. Cost of cellulose is a major factor [4].

Cellulases are the enzymes that are produced by bacteria and fungi that catalyze the hydrolysis of cellulose. Cellulase enzymes have diverse applications. Cellulases are used in the production of animal feed, formulation of detergents, juice clarification, paper industry and wine production, it is used in the fermentation of biomass into biofuels, fibre modification and in pharmaceutical industry. The major industrial applications of cellulases are in textile industry for bio-polishing of fabrics.

Bacterial cellulases, is the opportunity for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. The final product of cellulose degradation by cellulase enzyme is glucose which is soluble sugar.

The 16S rRNA is a conserved gene, it is universally found in all bacterial cells and so relationships, bacterial phylogeny, and taxonomy of bacteria like multigenic family or operons can be measured by this technique. The comparison of the 16S rRNA gene sequences shows differentiation between organisms and thus the cellulose degrading bacteria was characterized on the basis of this gene. The function of the gene has not changed, suggesting that random sequence changes are a more accurate measure of evolution and it is large enough for bioinformatics purpose. The explosion in the number of recognized taxa is directly attributable to the ease in performance of 16S rRNA gene sequencing studies [5].

Universal 16S rRNA gene primers were used to amplify gene from the genomic DNA isolated. PCR products were then sequenced and BLAST analysis was done with nucleotide sequence databases.

2. Literature Survey

Cellulolytic *Bacillus subtilis* strains from agricultural environment were isolated [6]. Cellulolytic bacteria were screened and isolated from soil, compost, and animal waste slurry in Jeju Island, South Korea. Among the isolates, three strains, SL9-9, C5-16, and S52-2, showing higher potential for practical uses were purified on carboxy methyl cellulose (CMC) agar plates and identified as *Bacillus subtilis* strains by morphological, physiological, and biochemical characterization and 16S rRNA gene analysis. The production patterns of cellulose or hemicellulose-degrading

enzymes were investigated during cell culture. All three isolated strains produced CMCase, Avicelase, *β*-glucosidase, and xylanase enzymes, which suggested synergic cellulolytic systems in *Bacillus subtilis*. The enzymes showing CMCase, Avicelase, and xylanase activities existed in cell-free culture supernatant, meanwhile *β*-glucosidase activity was detected in cell debris suggesting that three of the enzymes, including CMCase, Avicelase, and xylanase, were extracellular, and *β*-glucosidase was cell membrane bound. The three isolates, SL9-9, C5-16, and S52-2, were not the same strains, presenting slight differences in biochemical characteristics, 16S rRNA gene sequences, and cellulolytic enzyme activities.

Characterized thermostable cellulases were produced by *Bacillus* and *Geobacillus* strains. The composition of thermophilic (60 °C) mixed cellulose-degrading enrichment culture initiated from compost samples was examined by constructing a 16S rRNA gene clone library and the presence of sequences related to *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deinococcus-Thermus*, *Firmicutes*, and *Proteobacteria* were identified. Eight isolates capable of degrading cellulose, carboxymethyl cellulose (CMC), or ponderosa pine sawdust were identified as belonging to the genera *Geobacillus*, *Thermobacillus*, *Cohnella*, and *Thermus*. A compost isolate WSUCF1 (*Geobacillus* sp.) was selected based on its higher growth rate and cellulase activity compared to others in liquid minimal medium containing cellulose as a source of carbon and energy. Strain WSUCF1 and a previously isolated thermophilic cellulose-degrading deep gold mine strain DUSELR13 (*Bacillus* sp.) were examined for their enzyme properties and kinetics. The optimal pH for carboxymethyl cellulase (CMCase) activity was 5.0 for both isolates. The optimum temperatures for CMCase of WSUCFI and DUSELR13 were 70 and 75 °C. WSUCF1 and DUSELR13 retained 89 % and 78 % of the initial CMCase activities. These thermostable enzymes would facilitate development of more efficient and cost-effective forms of the simultaneous saccharification and fermentation process to convert lignocellulosic biomass into biofuels [7].

Collected culturable mesophilic (37°C) and thermophilic (60°C) cellulose degrading bacterial flora from weathered wood-like sample from the moist place of institute campus, were isolated. Cellulase activities were checked on plate by Congo red staining method. Molecular characterization was determined by phylogenetic analysis of the 16S rRNA sequence by various bioinformatics tools like BLAST, ClustalW, Drawtree etc. The results showed 93% resemblance with *Enterobacter luduwigi*, *Leclercia adecorboxylata*, *Enterobacter cloacae* and Endophytic bacterium [8].

3. Methodology/ Approach

3.1 Isolation of cellulose degrading bacteria

Cellulose degrading bacterial strain was isolated from soil samples of garden, earthworm, paper industry waste, cloth industry waste, kitchen waste. Samples were collected from Bangalore district in sterile containers and were immediately processed in laboratory. Isolation of cellulolytic bacterial species was done by serial dilution and spread plate method. Taking 1 g of above mentioned sample and dissolve in 10 ml of sterile distilled water and serially dilute it to get the concentration of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Take 1 ml solution from each of the serially diluted samples and spread on CMC agar media plates and kept it overnight at 37 ^{0}C for growth. Colony was observed on next day, subculture this colony for screening.

3.2 Screening of cellulose degrading bacteria

For screening of bacterial strains, freshly grown cultures of isolates were streaked on sterilized CMC agar media plates containing 1% CMC. The plates were then incubated at 37ºC overnight. After the completion of incubation period, the plates were flooded with 1 % Grams iodine solution and a clear zone was observed around the colonies which indicated cellulose degradation by cellulase activity.

3.3 Identification of cellulose degrading bacteria

The bacterial isolates were identified using morphological tests.

3.3.1 Morphological test

The plates were examined by Gram staining for identification [9]. Gram staining was discovered by Christian Gram in 1884. It is a useful staining technique for identifying and classifying bacteria into two major groups: the gram-positive and the gram-negative. The fixed bacterial smear is subjected to four different reagents in the order listed: crystal violet (primary stain), iodine solution, alcohol (decolorizing agent) and safranin (counter stain). The bacteria which retain the primary stain appear dark blue or violet are called gram-positive, whereas those that lose the crystal violet and counter stained by safranin appeared and are referred to as gram-negative.

3.4 Secondary screening and production of cellulase enzyme

The potential isolates were then evaluated for enzyme productivity. The isolate showing maximum cellulase production were then considered for the further study. For preparation of standard inoculum, the isolate that showed a maximum zone of hydrolysis were cultured in 100 ml LB broth medium and incubated at 37 ºC for 24 hours. This was used as inoculum for the production medium.

3.5 Isolation of bacterial genomic DNA

The standard protocol was followed for the isolation of genomic DNA from the bacterial cells which were grown in LB broth. The broth was centrifuged at 6,000 rpm for five minutes, the supernatant was discarded and the pellet was resuspended, 4 µl of lysozyme was added and was incubated for 30 minutes at 37 ºC. 850 µl of cell lysis buffer was added to the vials and kept in water bath at 65 ºC for half an hour and the mixture was centrifuged at 10,000 rpm for ten minutes at room temperature. The supernatant was transferred to clean eppendorf tube, 850 µl of isopropanol was added and this mixture was centrifuge at 10,000 rpm for ten minutes. The supernatant was discarded and the pellet was dried at room temperature for ten minutes. 50 µl of TE

buffer was added to the pellet and resuspended. After isolation of genomic DNA, the DNA was run on agarose gel electrophoresis for qualitative analysis.

3.6 Polymerase Chain Reaction

The genomic DNA isolated was amplified using a standard PCR protocol as mentioned here. The mastermix of 40 µl was prepared, in which 25.4 µl of distilled water, 3 µl of DNA (isolated genomic DNA), 4 µl of buffer, 1.6 µl of dNTP, 2 µl of forward primer, 2 µl of reverse primer (both the primers used are universal 16S rRNA gene primers), 2 µl of taq polymerase was taken in a 0.5 ml tube and kept into the thermocycler. Prepare a control tube with no template DNA but instead 20 µl of double distilled water. The following programme was run, initial denaturation at 94 ºC for 3-5 minutes, denaturation at 94 ºC for 30 seconds, annealing of primer at 58 ºC for 1 minute, extension of primer at 72 ºC for 1 minute, final extension at 72 ºC for 10 minutes and instrument is set for 30 cycles. The amplification reaction was carried on Corbett Research PCR machine (CG1-96). The amplified sample was then stored at 4 ºC for further studies.

3.7 Phylogenetic Analysis of 16S rRNA gene

The sequence of PCR product was used for phylogenetic analysis by bioinformatics tool that include BLAST. The following procedure was followed. PCR product obtained was gel purified and quantitated and sent for sequencing. This sequence obtained was subjected to BLAST with NCBI genbank database. Based on maximum identity score, first ten identities were considered and the given culture was identified. Phylogenetic tree was also constructed for these sequences.

4. Results and Discussion

4.1 Isolation and screening of cellulose degrading bacteria

Nineteen isolates were obtained from soil samples (as shown in table.1) and maintained in pure culture in CMC agar slants. Cellulolytic bacterial colonies were isolated on the basis of their ability to grow on cellulose containing media. The cellulases producing bacterial strain were identified by the zone of clearance. This was revealed by adding Gram's iodine solution (as shown in fig: 1).

Table 1: Details of isolates isolated from different soil samples

S. No	*Source of soil sample*	*Representative isolates*
1	Cloth waste	C_5, C_9
2	Kitchen waste	K_{25}, K_{31}, K_{38}
3	Garden soil	G_1, G_{14}, G_{17}, G_{18}, G_{19}, G_{22}, G_{29}, G_{30}
4	Paper waste	P_1, P_4, P_5, P_8
5	Earthworm	E_5, E_{18}

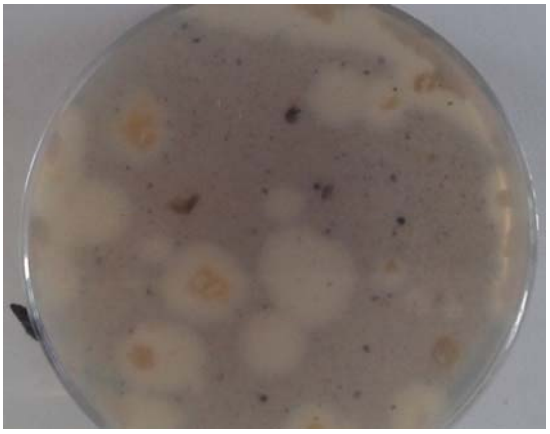

Figure 1: Screening of bacterial colonies after treatment with Gram's iodine solution showing a clear zone around bacterial colonies.

4.2 Identification and Morphology of the bacterial strain

The isolated bacterial strain was gram negative, because it takes pink colour with safranin with gram staining method. The isolated bacterial strain was rod shaped showing gram negative rod morphology.

Figure 2: Microscopic view of gram negative rods

4.3 Isolation of bacterial genomic DNA

For the PCR amplification of 16S rRNA gene, isolation of genomic DNA was carried out and the qualitative analysis of the isolated DNA is shown in fig 3.

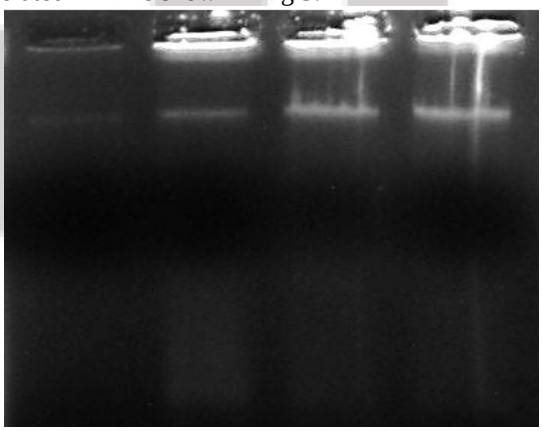

Figure 3: Lane 1, 2, 3 and 4 showing genomic DNA after running on agarose gel
Lane 1- sample G_{30}, lane 2 -sample P_5, lane 3- sample G_{29}, lane 4- sample P_4

4.4 Amplification of 16S rRNA gene by PCR

The PCR of 16S rRNA gene was carried out in a Corbett Research PCR machine (CG1-96) thermocycler by using universal primer specific to 16S rRNA gene. The size of the amplified PCR product was found to be 1.5 kb.

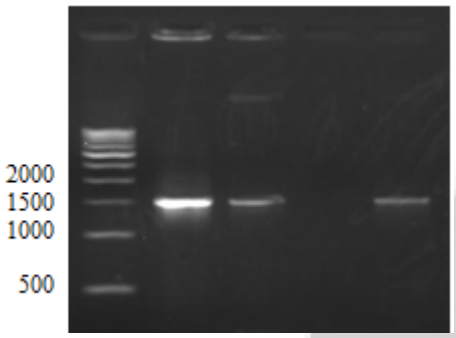

Figure 4: Lane 1- 1 kb ladder, lane 2- sample G_{30}, lane 3- sample P_5, lane 4- sample G_{29}, lane 5- sample P_4

4.5 Phylogenetic analysis of 16S rRNA gene sequence

The culture that was labeled as K-31 was identified as *Klebsiella variicola* based on sequence homology and phylogenetic analysis. The 16S rRNA sequence of *Klebsiella variicola* was deposited at NCBI with accession number KF036184.1.

Forward primer sequence

>V108_STRAINK31_16SF-NF_E11.ab1
CGCTGACGGCAGCTACACATGCAGTCGAGCGGTAG
CACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGA
CGGGTGAGTA
ATGTCTGGGAAACTGCCTGATGGAGGGGGATAACT
ACTGGAAACGGTAGCTAATACCGCATAACGTCGCA
AGACCAAAGT
GGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCC
AGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCA
CCTAGGCGA
CGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGA
GGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTA
AAGCACTTT
CAGCGGGGAGGAAGGCGGTGAGGTTAATTACCTTT
TCGATTGACGTTACCCCCCTAATATTCCCCGGCTAA
CTT

Figure 5: 16S rRNA sequence of *Klebsiella variicola* RBEB3 (KF036184.1)

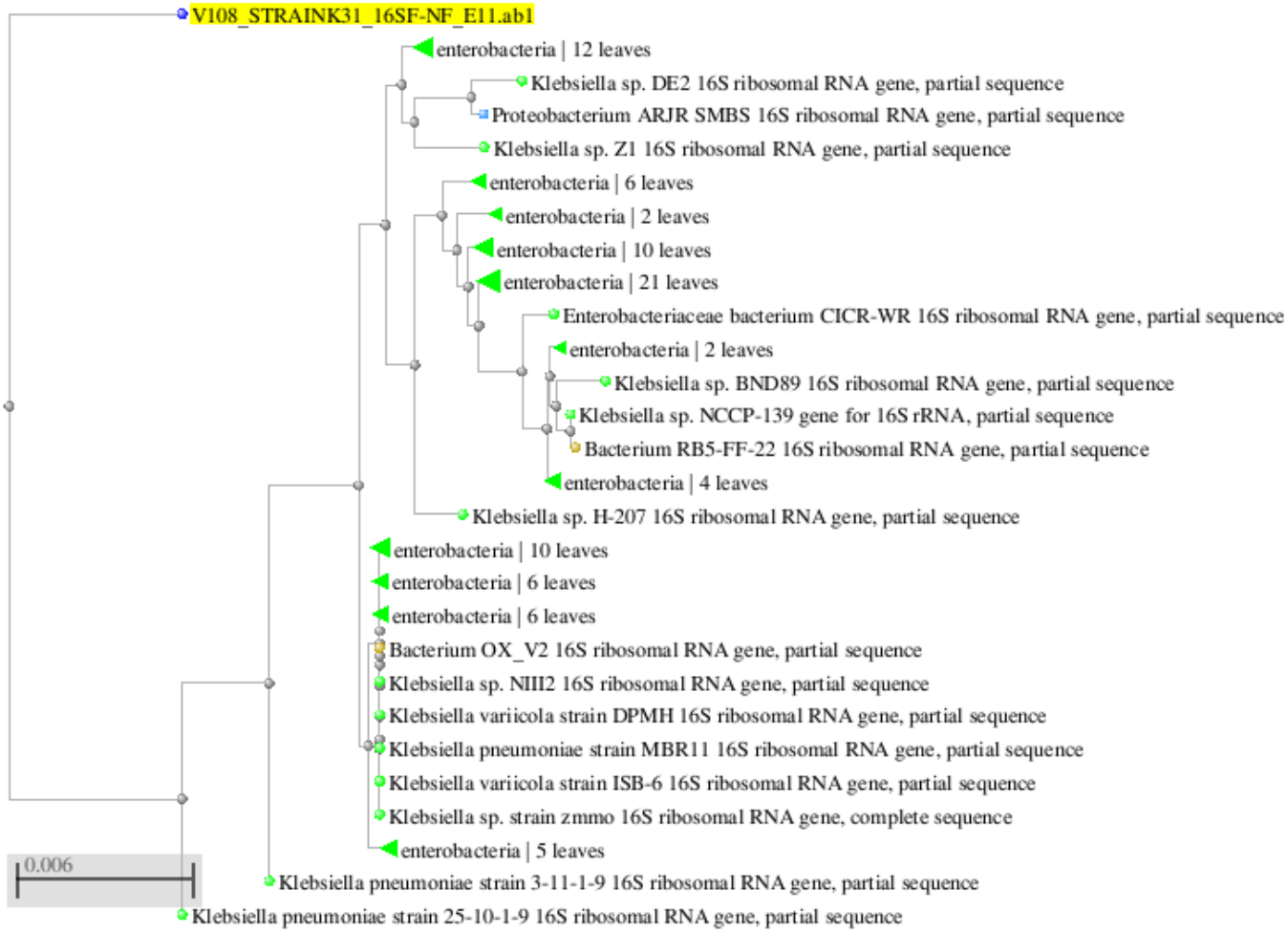

Figure 6: Phylogenetic analysis of 16S rRNA gene

5. Discussion

The present work was undertaken to isolate cellulose degrading bacteria from soil samples of garden, earthworm, paper industry waste, cloth industry waste and kitchen waste and characterized on the basis of 16S rRNA gene. The isolates were screened to see the cellulolytic activity by growing on CMC agar media which revealed the production of cellulase enzyme by hydrolyzing cellulose to its monomer ie.,glucose and thus showing clearance zone after adding grams iodine solution.

The isolated bacterial colonies were identified by morphological characterization [9]. The bacteria was tested negative for Gram staining as the outer layer of Gram negative bacterial cell wall is made of protein and lipopolysaccharide. It covers a few thin layers of peptidoglycan. In gram staining, they do not retain the crystal violet colour in their cell wall. The bacterial cell wall holds the pink dye once a counter stain chemical is used.

Genomic DNA was isolated from the bacteria. PCR amplification of this DNA was done with universal primer specific to 16S rRNA gene. The amplified product was run on 1% agarose gel with 500 bp DNA ladder. After running the gel for one hour, the size of amplified PCR product was around 1.5kb with respect to DNA ladder. It was confirmed that the region amplified was 16S rRNA region because size of 16S rRNA lies in this range.

For analyzing the sequence, direct sequencing of the PCR product was carried out. The sequence of the 16S rRNA gene was analyzed by bioinformatics tools like BLAST. The results showed 99 % resemblance with Klebsiella variicola. The results showed that the isolated organism was different from the already known organism. The cellulose degrading bacteria has the ability to degrade the cellulose molecule into glucose which is the major energy source for all kinds of organisms. Characterization of the cellulose degrading bacteria is a good strategy to obtain energy from the cellulose molecule.

6. Conclusion

The introduction of cellulose degrading bacteria has an advantage to support energy recovery from degraded ecosystems. After screening, one isolate was found to have the highest cellulase activity which indicated the presence of cellulose decomposer that can break down large organic molecules into smaller molecules that can be used by the biotic community. This will provide some medium to use the renewable sources of energy with the help of microorganisms and the processes that they undergo during the degradation of the complex polymer to simple sugars [10]. The cellulase producing bacterial species was isolated from soil samples of garden, earthworm, kitchen waste, paper industry waste, cloth industry waste and characterized by various staining procedures, biochemical analysis. Genomic DNA

was isolated from the bacteria. PCR amplification of this DNA was carried out using universal primer specific to 16S rRNA gene. The size of the amplified PCR product was 1.5 kb. The sequence of the 16S rRNA gene was analyzed by bioinformatics tools like BLAST. The results showed 99 % resemblance with *Klebsiella variicola.* The results showed that the isolated organism was different from the already known organism. The cellulose producing bacterial species showed a potential to convert cellulose into reducing sugars which could be readily used in animal foods and as a feed stock for producing valuable organic compounds. The microorganisms are used to produce an enzyme that offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry [11].

7. Future Scope

The isolated strain can be manufactured by fermentation in larger scale for the production of cellulase enzyme for conversion of complex cellulose into simpler glucose.

8. Acknowledgement

I would like to thank Aristogene Biosciences private limited for giving an opportunity to work in their institute and directly and indirectly helping for my work.

References

[1] L. R. Lynd and Y-H. P. Zhang, 2004, Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Non complexed cellulase systems, *Biotechnology and Bioengineering* **97**, 797-824.
[2] S. Sadhu, T. K. Maiti, 2013, Cellulase production by Bacteria: A Review, *British Microbiology Research Journal* **3**, 235-258.
[3] K. Ray, R. Roy, S. K. Sen, S. Saha, 2006, Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes), *Aquaculture Research* **37**, 380-388.
[4] J. Siripa, S. Kanokphorn, V. Piyaporn, 2011, Isolation of novel cellulase from agricultural soil and application for ethanol production, *Internat Journal of Advanced Biotechnology and Research* **2**, 230-239.
[5] E. Ahmed, S. S. I. Abdelnasser, 2007, Isolation and Identification of New Cellulases Producing Thermophilic Bacteria from an Egyptian Hot Spring and Some Properties of the Crude Enzyme, *Australian Journal of Basic and Applied Sciences* **4**, 473-478.
[6] Kim Yu-Kyoung, Ko Young Hwan, Oh Hyun-Jeong, 2012, Comparison of nucleotide sequences of Endo-B-1,4-Glucanase genes from *Bacillus subtilis* strains, *International Journal of Biotechnology Applications* **4**, 130-133.
[7] Gurdeep Rastogi, M. Kenneth Bischoff, R. Stephen Hughes, 2010, Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains, *Bioresource Technology* **101**, 8798-8806.
[8] K. Kaulesh Yadav, Mohammad Shariq, K. Sarad Maurya, 2012, Molecular Characterization of cellulose degrading bacteria on the basis of 16S rRNA.
[9] C. Jong, K. Apun, M. A. Salleh, 2000, Screening and isolation of a cellulolytic and amylolytic *Bacillus sp*

from sago pith waste, *Journal of Genetics and Applied Microbiology* **46,** 263-267.
[10] J. Niranjane, P. Madhou, T. W. Stevenson, 2007, The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantean*, *Enzyme and Microbial Technology* **40**, 1464-1468.
[11] J. Weber, M. Mandels, 1969, The production of cellulases: Cellulases and their Applications, *Advances in Chemistry Series* **6**, 391-414.

Author Profile

Dr. S. M. Gopinath received the B.Sc. and M.Sc. degrees in Microbiology from Kuvempu university, M. phil from Gulbarga university and Doctor of philosophy from Kuvempu university, Shankaraghatta, Shimoga district, Karnataka, India. As an experience in teaching for more than 2 decades and has published over 100 professional research paper in International and National refereed journals in various field of life science. He has been included as editor in various International and National journals and recognized in many professional bodies and have few DBT funded projects under him. He now working as Professor & HOD Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India.

Dr. Ismail Shareef. M., M.Sc., in Biotechnology from Bangalore University, Ph.D., from DOS in Botany, University of Mysore, is working as Assistant professor in the department of Biotechnology, Acharya Institute of Technology, Bangalore, India since 2004. Has 13 years of teaching and research experience, teaching both UG & PG students. He has published more than 30 International research papers in peer reviewed journals. Is a life member ISTE, NESA and IAENG. Ismail Shareef. M is been honored with Karnataka Suvarna Shree award for excellence in Education and also been bestowed with Junior and Senior Scientist awards for his research on Rheumatoid Arthritis (RA). Has deliberated his research findings in International conferences and attended various workshops, hands-on training programs, seminars, guest lectures, pedagogy programs, FDP's and also has conducted two National conferences on recent issues in Nano science and Biotechnology. Member of many Advisory boards, Industry-Institute interaction and member of University Board of Examiners. Has published a book titled "Downstream Process Technology".

Prof. Ashalatha received the B.Sc. and M.Sc. degrees in Biotechnology from Bangalore university and pursuing Doctor of philosophy from Visvesvaraya Technological university, Belgaum, Karnataka, India. As an experience in teaching for more than 6 years and has published and presented research article in various International and National refereed journals and conferences in various field of life science. She is now working as Asst Professor and research scholar in Department of Biotechnology, Acharya Institute of Technology, Bangalore, Karnataka, India.

Shreya Ranjit, final year M. Tech student at Department of Biotechnology, Acharya Institute of Technology, Bangalore. For the partial fulfillment of M.tech the project was carried out under the guidance of Dr, S.M.Gopinath, Prof and HOD, Prof Ismail Shareef.M and under the mentoring of Prof.Ashalatha Asst Prof, Biotechnology department, Acharya Institute of Technology at Aristogene Biosciences Private Limited, Bangalore. The isolation, screening, amplification of the strain was carried out independently and the sequence of the strain *Klebsiella variicola* RBEB3 is submitted to NCBI having accession number KF036184.1