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Analysis of Microbes and their Enzyme in *Achatina fulica*

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Abstract: ***Molluscs forms one of the imperative biotic components in an ecosystem playing vital roles in the ecosystems and are often used as bioindicators of the ecosystem health. As bioindicators, they uptake environmental toxins through food chain from their surroundings. The heavy metals such as zinc, cadmium, lead and copper can be taken up by land snails, as shown by laboratory experiments wherein they were fed toxin laced lettuces. In addition, molluscs are useful indicators for biological assessment of water quality monitoring. But in more recent lab.tests, snails incorporate showed that cadmium from their surroundings when they are living in contaminated soil. Achatina fulica snails are polyphagous and along with organic detritus, preferred plant species, ranging from coffee and bananas in the tropics, potatoes and tobacco in more temperate zones. The snails are voracious eaters and competitors of native snails as well as habitat modifiers. Their importance in agricultural systems, gardens and as agents for the reduction of biological diversity is increasing at an alarming rate making the mas one of the most important pests worldwide. There are limits to the duration of aestivation that can be tolerated by land snails and mortality eventually increases as aestivation is prolonged. While physiological alterations that occur during aestivation have received much interest. Lignocellulosic biomass is degraded with the cooperation of many microorganisms, producing a variety of cellulolytic and hemi-cellulolytic enzymes under aerobic and anaerobic conditions.Glycoside hydrolases from bacteria are assembled in multienzyme complexes that provide increased synergy, stability and catalytic efficiency. Multifunctional, harbouring both endoglucanase and xylanase activities in the polypeptide isolated and identified the cellulolytic bacteria from the gastrointestinal tract of Achatina fulica and analyse strains confirmed using bioinformatics tools. Cellulose digesting enzyme production of cellulolytic bacteria isolated from the gastrointestinal tract of Achatina fulica were confirmed by zymogram and cladogram analysis.***

Keywords: Xylanase, cellulolytic bacteria, Achatina fulica, FPase, CMCase, zymogram, cladogram

1. Introduction

Achatina fulica is an arboreal and terrestrial mollusc frequently invades vegetated shores of tropical and subtropical water bodies. Lignocellulosic plant biomass is an important renewable carbon resource (Kamm & Kamm, 2004) (Lynd *et al.,* 1991). Cellulose, the major component of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes.

In nature, lignocellulosic biomass is degraded with the cooperation of many microorganisms, mainly including diverse fungal and bacterial genera producing a variety of cellulolytic and hemi-cellulolytic enzymes under aerobic and anaerobic conditions. The biodegradation of cellulosic biomass through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological application, since it avoids the problems of feedback regulation and metabolite repression posed by isolated single strains (Charrier *et al*., 2006). Bacteria have some advantages over fungi in certain aspects. They usually have a higher growth rate allowing faster production of recombinant enzymes (Maki *et al*., 2009). In addition, some glycoside hydrolases from bacteria are assembled in multienzyme complexes that provide increased synergy, stability, and catalytic efficiency (Hou *et al*., 2006; Jiang *et al*., 2006; Waeonukul *et al*., 2009), while others display modular architecture (Cann *et al*., 1999; Zhang *et al.,* 2014) or multifunctional, harbouring both endoglucanase and xylanase activities in the same polypeptide (Perez-Avalos *et al*., 2008). Finally, cellulolytic bacteria have been isolated from harsh climate conditions (Soares *et al*., 2012). In the present study we have isolated and identified the cellulolytic bacteria from the gastrointestinal tract of *Achatina fulica* and analyse strains confirmed using bioinformatics tools. We also compared the cellulose digesting enzyme production of cellulolytic bacteria isolated from the gastrointestinal tract of *Achatina fulica*.

2. Materials and Methods

Medium sized, healthy, terrestrial snail, *Acatina fulica* were collected from Kochuveli, Thiruvananthapuram (Lat. 9º96683´N & Long. 76º25´08E). They were brought to the laboratory, sorted them and were acclimatized for a week to lab. conditions. They were selected and sacrificed for performing experiment.

The anaesthetized adults of *Achatina fulica* (40.89±0.290 gm) were dissected by removing shell with bone cutter. Digestive tract was separated and the surface was sterilized with 70% ethanol. Gut content of different regions (oesophagus, stomach, intestine and rectum) were taken out with sterile syringes. The gut contents were homogenized in an isotonic saline solution for physical and biochemical analysis.

3. Isolation and Screening of Microorganisms

Enrichment of cellulolytic bacteria

The homogenized samples of gut contents were collected from *Achatina fulica.* The samples were subjected to serial

dilution and it was spread on to the surface of CMC (Carboxymethyl cellulose) agar medium. Preliminary screening was done on the Petri plates containing the CMC agar medium. Cultures were incubated for 3 days at 37°C and bacterial colonies of cellulose were isolated. The cellulose-degrading ability of bacteria was confirmed by streaking on minimal saline agar supplemented with 0.5% CMC.

Microbial Analysis
The microbial analysis like Gram's staining, Motility, Morphology of colony, and growth studies were performed.

Growth Studies and Biochemical Analysis
Growth in the liquid culture was conducted using CMC agar medium. From the culture medium, selected four bacteria and were subjected to fermentative studies in liquid medium for 144 hours. Culture broth and pH were monitored at every 24 hours and cell free culture supernatant was used for the estimation of CMCase, xylanase and FPase activity respectively. Aliquots taken from the growth medium and were subjected to optical density measured at 600 nm against blank (distilled water). The pH of the growth medium, collected at every 24 hour were measured using pH meter.

Isolation of 16 S rRNA Gene
The Genomic DNA isolation was done as per standard protocol - NucleoSpin® Tissue Kit (Macherey-Nagel).

DNA Sequencing and Identification of Bacteria up to Genus Level
The amplified DNA fragment was run in 1.5% agarose gel for size confirmation. It was purified subsequently and sequenced commercially (Scigenom, India). Approx. 550 bp long gene sequence obtained was compared with corresponding sequences of related organisms retrieved from GeneBank database with BLAST algorithm for identifying the isolated strain.

Zymogram analysis
The zymogram analysis was done by the method of Morag with some modifications.

4. Result and Discussion

4.1 Isolation and Screening of Microorganisms

Primary and secondary screening using CMCagar medium and Congo red
In the CMC agar media, primary screening test from four isolates of *A. fulica*, inspected visual colony morphologies such as shape, colour, elevation, margin etc. Each isolates was further sub- cultured at 37^{0}C incubation. Four strains named as *AS1, AS2, AS3, AS4 (isolates of *A. fulica*). (*AS1- *Achatina* Strain 1, AS2- *Achatina* Strain 2, AS3- *Achatina* Strain 3, AS4- *Achatina* Strain 4). The secondary screening using CMC agar medium and Congo red dye showed promising results. All strains had shown a clear zone around the colony. The bacterial strain AS3 [Fig. 1(a-d)] showed greater clear zone radius. Strain AS4 shows poor clear zone radius. The decreasing order of clear zone radius of strains were AS3>AS2>AS1>AS4 [Fig. 1 (e and f)].

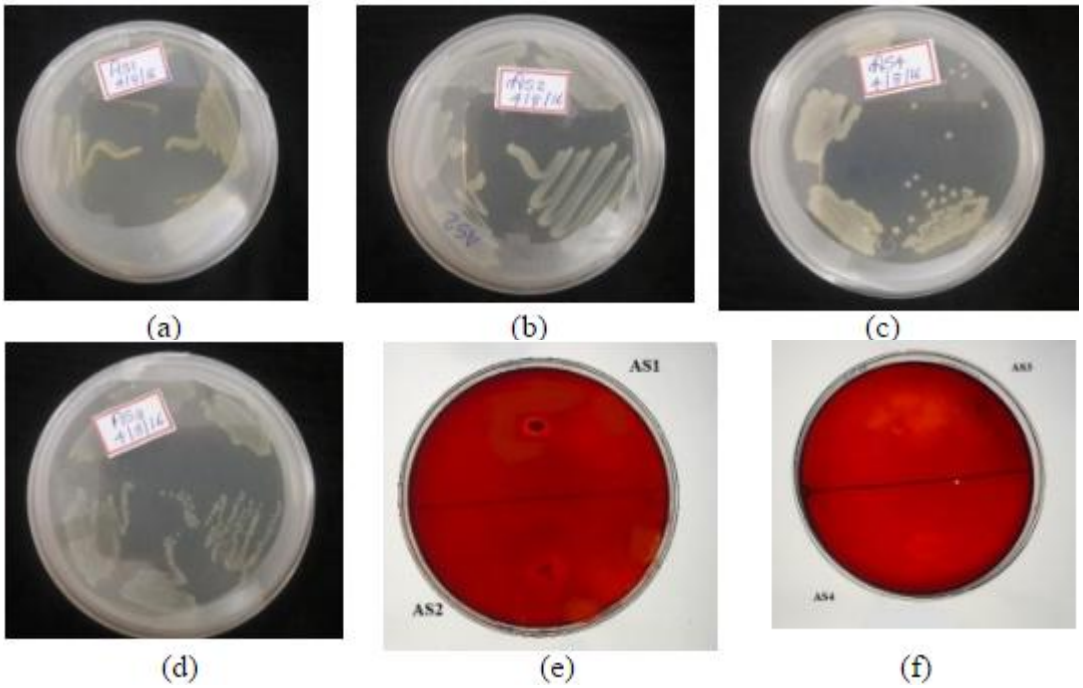

Figure 1: Four isolated strains [AS1 (a), AS2(b), AS3(c) and AS4(d)] and cellulolytic activity of [AS1and AS2(e)] and [AS3 and AS4 (f) by Congo red]. The clear zone in Congo red staining indicates cellulolytic activity

4.2 The tertiary screening with CMC liquefaction

All the isolates were subjected to tertiary screening for cellulose activities using carboxymethyl cellulose medium in the culture tubes. Each strains were inoculated into the culture tubes containing the solidified medium. AS3 showed

a greater conversion of solid CMC medium into liquid form. AS3 (Fig. 2) converted the solidified medium into clear and transparent liquid. All other strains such as AS1, AS2 and AS4 showed very poor conversion efficiency.

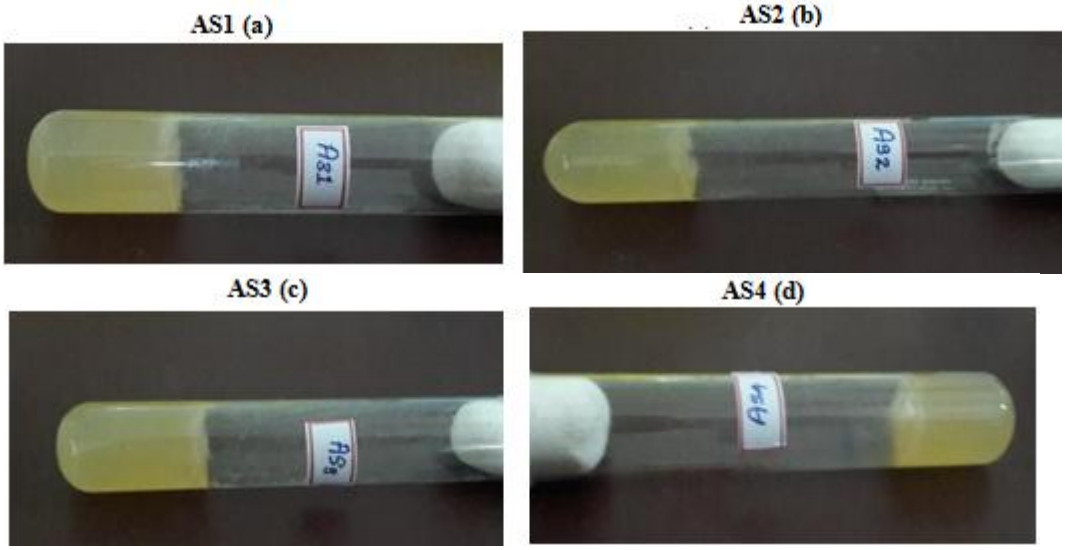

Figure 2: The cellulolytic activity of four isolated strains AS1(a), AS2 (b), AS3 (c) and AS4 (d) of *A. fulica*) in culture tubes

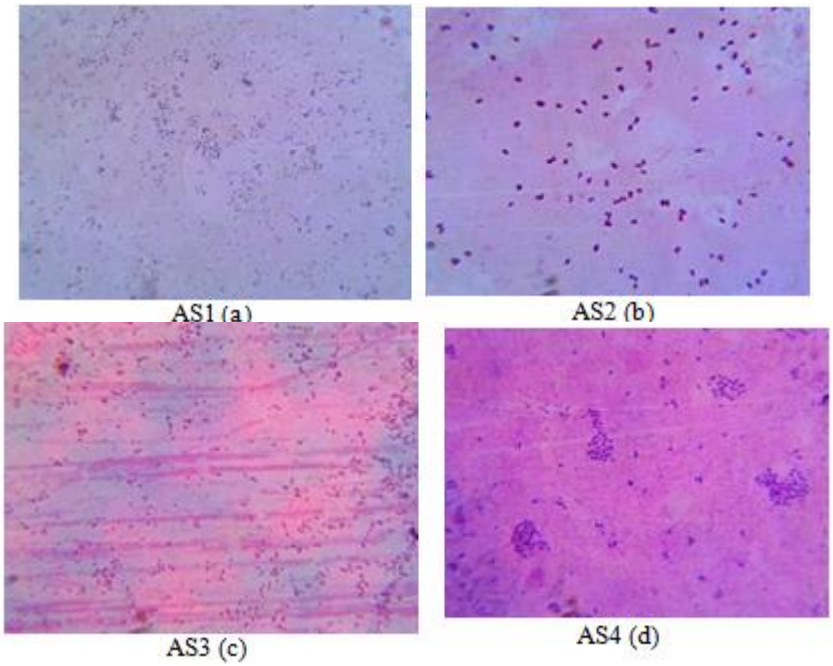

Figure 3: The smear of four isolated strains (AS1, AS2, AS3 and AS4 of *A. fulica*) after Gram's staining

4.3 Microbial Analysis

Table 1: Microbial analysis of different strains (AS1, AS2, AS3 and AS4 from *A. fulica*

S. No	Character	Bacterial Isolates			
		AS1	AS2	AS3	AS4
1	Colony colour	yellow	cream	cream	pale yellow
2	Colony margin	entire	entire	entire	entire
3	Colony elevation	flat	flat	raised	raised
4	Gram`s staining	+ive	-ive	-ive	+ive
5	Motility	non-motile	non-motile	vigorous	less motile
6	Shape of cell	cocci	rod	rod	cocci

The morphological characters were observed and recorded in Fig.1(a-d), Fig. 3 and Table 1.

5. Fermentation in Liquid Culture Medium with the Selected 2 Bacterial Isolates

The culture showed greater clear zones and better conversion efficiency of solidified medium into clear and transparent liquid were selected for further studies. Fermentation in liquid culture medium was tried to ensure the extracellular enzyme production by the selected isolates.

The bacterial isolate AS3 showed a typical growth phase (Fig.4). The highest activity of AS3 was achieved by the growth of the bacteria for 72 hours in the liquid medium. In AS2 the OD showed highest growth at 24 hour, and then both were decreased. The OD of AS3 slightly elevated from 120 hour and AS2 was from 96 hours. All the bacterial

isolates showed the pH ranging from 4 to 9. In AS2 and AS3 the initial pH was in between 4.95 to 5.91. Later on, AS3 was increased up to the level of 9.07. AS2 showed similar patterns in the level of pH (Fig.5). The pH of AS2 was slightly decreased in 24 hour and then increased from 8.43 to 8.57. All the bacterial isolates showed pH change to alkaline condition. Figure 6, 7 and 8 shows the isolates with significant changes in the enzyme production.

This may be due to the food habits (Raut and Barker 2002,). Herbivorous animals lack the capacity to degrade lignocellulose themselves and instead rely upon the gut microbial community having this repertoire. Since the gut of *A. fulica* was not associated with much more cellulolytic enzymes and reduction of higher amount of cellulose in the gut. It may be due to the inhabitance of a large set of cellulose degrading bacteria in the gastro-intestinal tract of *A. fulica*. Recent studies revealed that different regions of gastro-intestinal tract of *A. fulica* harbours high bacterial diversity (Kiran *et al.*,2014).

Growth profile of bacterial isolates from the gut of *Achatina fulica*

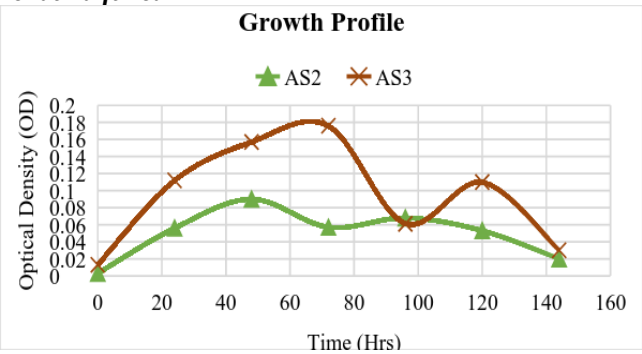

Figure 4: OD of culture broth of different bacterial isolates measured at 600 nm with dilution factor 10.

pH of the spent culture broth of bacteria

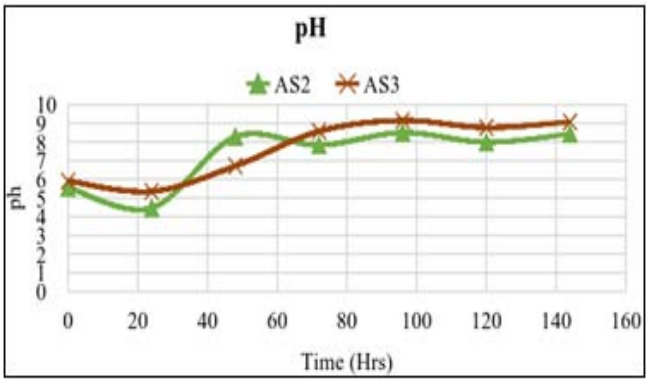

Figure 5: pH of culture broth of different bacterial isolates.

Comparison of FPase production by two isolates

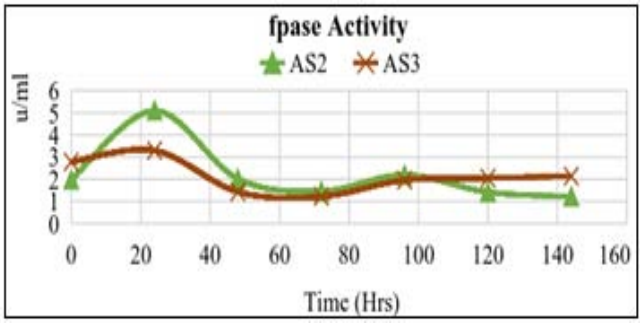

Figure 6: The FPase activity of culture broth of different bacterial isolates.

Comparison of CMCase production by 2 isolates

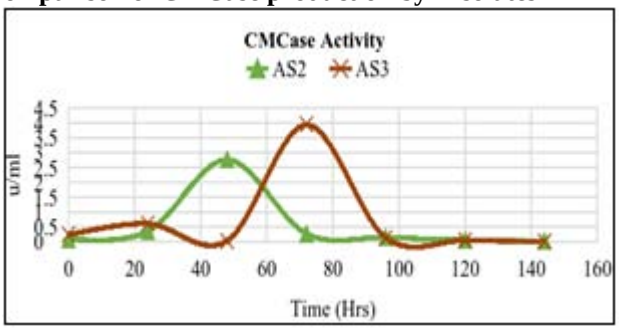

Figure 7- The CMCase activity of culture broth of different bacterial isolates

Comparison of Xylanase production by two isolate

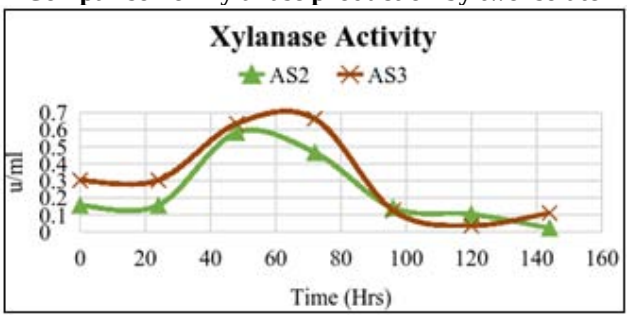

Figure 8- The Xylanase activity of culture broth of different bacterial isolates

6. Isolation and Sequencing of 16S rRNA Gene

AS1, AS2, AS3 were selected for the identification up to the molecular level. Identification up to the species level was conducted by direct comparison by Bioinformatics tools.

The 16 S rRNA Gene sequence of AS1

>AS1 SR640--16S
ACGGGTGAGTAACACGTGAGTAACCTGCCCTTAAC
TCTGGGATAAGCCTGGGAAACTGGGTCTAATACCG
GATAGGAGCGTCCACCGCATGGTGGGTGTTGGAAA
GATTTATCGGTTTTGGATGGACTCGCGGCCTATCAG
CTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAA
AGTGACGGTACCTGCAGAAGAAGCACCGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGA
GCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGT

AGGCGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGG
CTTAACCCCGGATCTGCGGTGGGTACGGGCAGACT
AGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTA
GCGGTGGAATGCGCAGATATCAGGAGGAACACCGA
TGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCT
GAGGAGCGAAAGCATGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACT
AGGTGTGGGGACCATTCCACGGTTTCCGCGCCGCA
GCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGG
CCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGC
CCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
GCAACGCGAAGAACCTTACCAAGGCTTGACATGTT
CTCGATCGCCGTAGAGATACGGTTTCCCCTTTGGGG
CGGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCG
TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTCGTTCCATGTTGCCAGCACGTCGTGGTG
GGGACTCATGGGAGACTGCCGGGGTCAACTCGGAG
GAAGGTGAGGACGACGTCAAATCATCATGCCCCTT
ATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTA
CAATGGGTTGCGATACTGTGAGGTGGAGCTAATCC
CAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCA
ACTCGACCCCATGAAGTCGGAGTCGCTAGTAATC

Figure 9: Sequence of 16 S rRNA gene of AS1in FASTA format

The 16 S rRNA Gene sequence of AS2

>AS2 SR640--16S
CCTGGGAAACTGCATTTCGAAACTGGCAGGGCTAG
AGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC
GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTT
GGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCT
AACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCG
CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGATGCA
ACGCGAAGAACCTTACCTACTCTTGACATCCAGAG
AACTTAGCAGAGATGCTTTGGTGCCTTCGGGAACTC
TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT
TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATCCTTTGTT

Figure 10: Sequence of 16 S rRNA gene of AS2in FASTA format

The 16 S rRNA Gene sequence of AS3

>SR640-AS3-16S

GGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGC
CCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC
CTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGT
CGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAAC
TCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTGGTCTTGACATCCACGGAATTTGGCAGAGA
TGCCTTAGTGCCTTCGGGAACCGTGAGACAGGTGC
TGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTT
GTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGAC
TGCCAGTGATAAACTGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGACCAGGGCTACAC
ACGTGCTACAATGGCATATAC

Figure 11: Sequence of 16 S rRNA gene of AS3 in FASTA format

7. Identification of Bacteria Using Bioinformatics Tools

Phylogenetic Tree Creation

The phylogentic tree was created with bioinformatics tool NJPLOT. The input file used is the *.phb produced by the MSA with CLUSTAL × 2.0.11. The tree output was viewed using software NJPLOT. The phylogenetic tree revealed that the bacterial strain AS1 has significantly related with the genera *Micrococcus* (Fig. 12). Hence the name of the bacterial isolate AS1 is *Micrococcus sp*. AS1. Bootstrap values obtained with 1000 resampling are indicated as percentages at all branches. The phylogenetic tree of AS2 showed that, though the strain has relation with *Yokenella*, it is more related to the genera *Enterobacter*. Thus the name of the bacterium is *Enterobacter sp*. AS2. Bootstrap values obtained with 1000 resampling are indicated as percentages at all branches (Fig.13). The phylogram revealed that the bacterial isolate AS3 has high phylogenetic affinity with *Yokenella* genus (Fig. 14). Thus the name of the bacterium is *Yokenella sp*. AS3 Bootstrap values obtained with 1000 resampling are indicated as percentages at all branches. Previous studies have shown that the isolation and identification of cellulolytic bacteria from the gut of both *A. fulica.* Kiran *et al.,* (2014) proved that more than ten different bacterial strains have been isolated. Metagenomic analysis of microbiota of crop of A. *fulica* revealed an abundance of sequences coding for oligosaccharide-degrading enzymes and many novel cellulase and xylanase (hemicellulose) coding sequences. The cellulolytic bacterial community structure in the gut of gastropods such as *A. fulica* were investigated. Cellulolytic bacterial community was enriched using CMC as cellulosic substrate and characterized their sequences (Kiran *et al.,* 2012).

Phylogram of bacterial isolate AS1

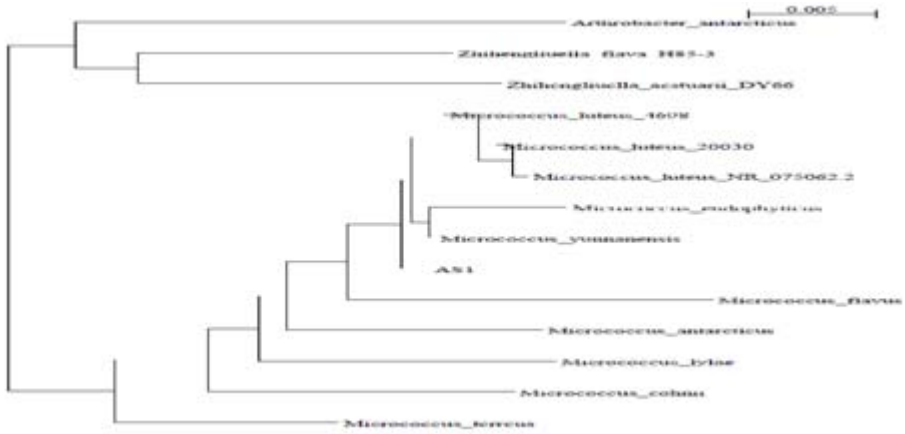

Figure 12: Phylogram of AS1 showing the phylogenetic affinity of the new bacterium with other bacteria

Phylogram of bacterial isolate AS2

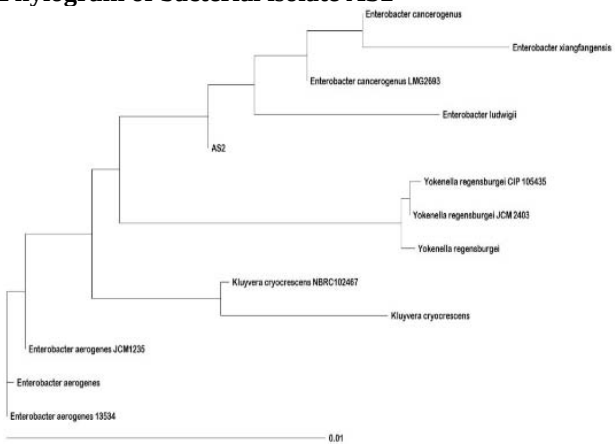

Figure 13: Phylogram of AS2 showing the phylogenetic affinity of the new bacterium with other bacteria.

Phylogram of bacterial isolate AS3

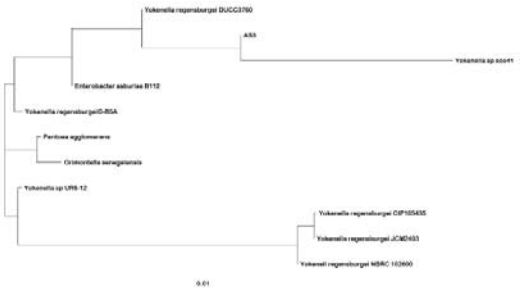

Figure 14: Phylogram of AS3 showing the phylogenetic affinity of the new bacterium with other bacteria

Figure 15: The zymogram of bacterial strains having most cellulolytic potential.

Zymogram studies

The enzyme activity was later confirmed by zymogram analysis. The cellulolytic potential of the samples were confirmed in the Zymogram. The fig. 15 shows that both the culture supernatants possess CMCase activities. Among two strains, AS3 showed a distinct clear zone with highest activity in Zymogram. There are reports regarding lower levels of CMCase with Carboxymethyl cellulose substrate. Dhillon *et al.,* (1985) proved that the simple sugars like glucose and sucrose are more effective for CMCase production. The constituent endoglucanase (CMCase) has been produced by *Bacillus licheniformis*-1 as a positive response was made with simple sugars.

8. Conclusion

The analytical study regarding the presence of microbes in various regions of gut proved that different varieties of bacteria produce different actions and helps to accelerate the process of digestion without any cumulative effect to other organs. The present study of cellulose degrading bacteria using 16S rRNA sequencing and data proved that they show similarity with higher groups of organisms. The isolated and identified bacterial strains *Achatina fulica* (AS1, AS2 and AS3) have shown as *Micrococcus sp*. AS1, *Enterobacter sp*. AS2 and *Yokenella sp*. AS3. Different strains of bacteria isolated were the first report in this generation of study and it will be confirmed with cladogram.

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