



## RESEARCH ARTICLE

## Investigation of *Aeromonas*: A Medical and Biotechnological Perspective

Zeynep Yegin<sup>1\*</sup> and Cumhuri Avsar<sup>2</sup>

<sup>1</sup>Medical Laboratory Techniques Program, Vocational School of Health Services, Sinop University, Sinop, Turkey

<sup>2</sup>Department of Biology, Faculty of Science and Arts, Sinop University, Sinop, Turkey

\*Corresponding author: Zeynep Yegin, Medical Laboratory Techniques Program, Vocational School of Health Services, Sinop University, Sinop, Turkey, E-mail: [zyegin@sinop.edu.tr](mailto:zyegin@sinop.edu.tr)



### Abstract

The genus *Aeromonas* is widely distributed in aquatic environments and certain strains are serious pathogens in poikilothermic animals and humans. Human infections related with this pathogen include gastroenteritis, urinary tract infections, pneumonia, wound infections and septicemia. In this study, both the correct medical diagnosis with taking advantages of molecular approaches and a brief investigation of the enzymatic activity potential of *Aeromonas* were aimed. *Aeromonas* spp. isolates were collected from different stream samples of Sinop, Turkey and were identified according to morphological, physiological and biochemical tests based on API 20NE (Biomeriux, France). The evaluation of total cell protein profiles and molecular identification based on 16S rRNA sequencing were conducted. Amylase, cellulase and protease enzyme activities of strains were also determined. The strains were separated into two clusters of 73% and over according to the SDS-PAGE total cell protein profiles reflecting a less discriminative power for this method in the strain identification. On the other hand, according to the 16S rRNA sequence analysis results, two isolates were identified as *Aeromonas tecta* and the other two as *Aeromonas veronii* at 99% similarity level reflecting the superiority of this approach when compared with SDS-PAGE profile. Enzyme assays showed that; three of the isolates were able to produce the amylase and only one could produce the cellulase but none produced the protease enzyme. Though our results are based on limited samples, enzyme activity potential of *Aeromonas* is recommended to be further investigated in detail in future studies. Pollution of the water by organic materials is a serious environment and health problem and thus bacterial extracellular enzyme activities degrading these pollutants in aquatic environments may reflect a bioremediation strategy in terms of a biotechnological perspective. Taking into consideration of *Aeromonas* in human infections, the reliable identification based on molecular techniques is an obligatory for medical

practice. Besides, exploiting this pathogen in search of enzyme activities may broaden our biotechnology perspective.

### Keywords

*Aeromonas*, SDS-PAGE, Molecular characterization, 16S rRNA, Protease, Amylase, Cellulase

### Introduction

*Aeromonas* species are Gram-negative, rod-shaped facultatively anaerobic bacteria and ubiquitous in aquatic environments, different types of food, also in invertebrate and vertebrate organisms [1]. Certain *Aeromonas* strains are serious pathogens in poikilothermic animals, birds and mammals. Human infections include gastroenteritis, urinary tract infections, pneumonia, wound infections and septicemia [2,3]. Potential pathogenicity of *Aeromonas* spp. stems from the expression of several virulence factors including inherent resistance to various antibiotics and ability to form biofilms using quorum sensing [1,2]. Though the effect of *Aeromonas* in medicine is clear enough, the biotechnological processes such as biodegradation of toxic organic pollutants and thus developing bioremediation strategies it can be involved in is a field needs to be further investigated. This study aimed to propose both the medical diagnosis with the comparison of molecular approaches and potential biotechnological role of *Aeromonas* with the aim of enrichment in future comprehensive studies.

Conventional biochemical tests are important for biotyping *Aeromonas* isolates however they lack of discriminatory power to correctly identify at the species

level and thus the use of various genotyping methods are inevitable. In this study, we aimed to correctly characterize this waterborne opportunistic pathogen with SDS-PAGE total cell protein profiles and 16S rRNA sequencing approaches. In addition, some enzymatic activities of the isolates were determined for the future utilization potential of these pathogens in the biotechnology area. Our preliminary results can direct future researchers to conduct comprehensive studies required in this field.

## Material and Methods

### Bacterial isolates

A total of 27 samples from surface stream sampling sites were collected over a one year period (September to November, 2016) in Sinop, Turkey and analyzed for the presence of *Aeromonas* species based on API 20NE tests for the identification and speciation of *Aeromonas*. Sampling was carried out at a depth of 15 cm. Water samples were collected in 100 ml autoclaved sterile bottles and immediately placed on ice in coolers, and delivered to the laboratory. The samples were processed within 3 h of collection time. The isolates were grown on Nutrient agar at 37 °C for 18-24 hours. Four *Aeromonas* isolates were identified from surface stream sampling sites in Sinop, Turkey.

### Phenotypic characterization

The strains were identified as *Aeromonas* according to API 20NE (Biomérieux, France) identification kit, Gram staining and reproductive abilities at physiological conditions such as different temperature, salinity and pH.

### SDS-PAGE analysis

Extraction of whole cell proteins was carried out according to the method of Laemmli [4]. Protein analysis was carried out with SDS-PAGE in gel slabs of 1 mm thickness (4% stacking and 10% resolving gels for total proteins). The molecular weight of each band was calculated with Total Lab 1D Manual R11.1, UK programme.

### Molecular identification

Chromosomal DNA extraction from *Aeromonas* strains was carried out according to the method of Sambrook, et al. [5]. DNA samples were stored at -20 °C until further molecular analysis. Sequence analysis of the 16S rRNA gene regions was performed for genotypic tests with primer pairs 519F and 1492R [6]. Primer sequences are shown in Table 1. The following PCR conditions were applied to each assay; 0.75 mM MgCl<sub>2</sub>, 100 μM dNTPs, 25 pmol of forward and reverse primers, 5 U Taq DNA polymerase (Thermo Fisher Scientific), 1 μl DNA per 50

μl reaction. The thermal parameters at Techne TC-5000 (California-USA) were as follows: Initial denaturation at 95 °C for 7 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 90 sec. A final extension at 72 °C for 6 min at the end of the cycles was included. The amplicons obtained from 16S rRNA region were resolved by 1.8% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transilluminator (Cleaver-MicroDOC, UK). The PCR products were purified and sent to sequencing analysis to MedSanTek biotechnology firm. Sequences generated from different isolates were compared to the GenBank database using the BLAST (Basic Local Alignment Search Tool) maintained by the National Center for Biotechnology Information and multiple sequence alignments were made with the Clustal W. The alignment was further analyzed using MEGA.7.0 (Molecular Evolutionary Genetics Analysis Version 7.0) program with Neighbour-Joining (NJ) method and phylogenetic tree was constructed.

### Enzyme assays

Enzyme production activities of isolates in Skim milk agar (protease), M9 agar (amylase) and CMC agar (cellulase) were performed at 25, 35 and 45 °C at different pH (5-10).

**Protease:** The isolated strains were streaked on pH: 7 skim milk agar and incubated at 37 °C for 24 h. Proteolytic bacteria use the enzyme caseinase to hydrolyze casein and form soluble nitrogenous compounds displayed as a clear zone around colonies. The nonappearance of a clear zone around colonies was evaluated as protease negative.

**Amylase:** The isolated *Aeromonas* strains were streaked on pH-7 M9 starch agar plate and incubated at 37 °C for 72 h. Following incubation, the plates were flooded by iodine to produce starch-iodine complex which is visualized by deep blue color. The appearance of clear zone at starch agar plate was used as the main indicator of amylase positivity.

**Cellulase:** The isolated strains were streaked on pH-7 CMC agar plate and incubated at 37 °C for 24 h. At the end of the incubation period, the strains were stained with iodine solution for 5 min. The appearance of clear zone around colonies was evaluated as positive.

## Results

### Phenotypic characterization

All the four isolates were able to grown in 2% NaCl at pH values 6-7 and they were positive for katalase and Voges-Proskauer reaction. All the isolates were grown

**Table 1:** Primer sequences used in the study.

Primer	Sequence	Gene	Reference
519F	5'-CAG CMG CCG CGG TAA TAC-3'	16S rRNA	[6]
1492R	5'-GGT TAC CTT GTT ACG ACT T-3'	16S rRNA	[6]

at temperatures 25 °C and 37 °C, only one isolate (Z1) were grown at 45 °C, and no growth was observed at 55 °C and over. According to the fermentation analysis results, all the four isolates were positive for sucrose, D-mannose, maltose, D-glucose, D-manitol, and D-xylose and negative for D-sorbitol.

Biochemical, temperature, and fermentation test results of *Aeromonas* isolates are shown in Table 2, Table 3, and Table 4.

### SDS-PAGE analysis

According to SDS-PAGE total cell protein profiles, the strains were separated into two major clusters between 73% and over (Figure 1). First cluster showed 78% similarity level and second cluster showed 81% similarity

**Table 2:** Biochemical analysis.

Strains	Gram Staining	2% NaCl	5% NaCl	7% NaCl	10% NaCl	pH-5	pH-6	pH-7	pH-8	pH-9	pH-10	Katalase	Voges	Nitrate	Gelatinase
Z1	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+
Z2	-	+	-	-	-	+	+	+	-	-	-	+	+	-	-
Z3	-	+	-	-	-	-	+	+	+	+	+	+	+	-	-
Z4	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+

+: Positive, -: Negative.

**Table 3:** Temperature analysis.

Strains	4 °C	25 °C	37 °C	45 °C	55 °C	65 °C
Z1	+	+	+	+	-	-
Z2	+	+	+	-	-	-
Z3	+	+	+	-	-	-
Z4	-	+	+	-	-	-

+: Positive, -: Negative.

**Table 4:** Fermentation analysis.

Strains	Glucose	Sucrose	Lactose	Gas produce	D-Gelatos	D-Mannose	D-Fructose	L-Arabinose	Maltose	D-Glucose	D-Manitol	D-Xylose	D-Sorbitol
Z1	+	+	+	-	+	+	+	+	+	+	+	+	-
Z2	+	+	+	+	-	+	-	-	+	+	+	+	-
Z3	-	+	-	-	-	+	+	-	+	+	+	+	-
Z4	+	+	+	+	+	+	+	-	+	+	+	+	-

+: Positive, -: Negative.

level. Protein electrophoretic patterns of the isolates yielded between 22-26 bands. Protein profiles were very similar among the strains and several strains exhibited dominant protein bands.

### Molecular identification

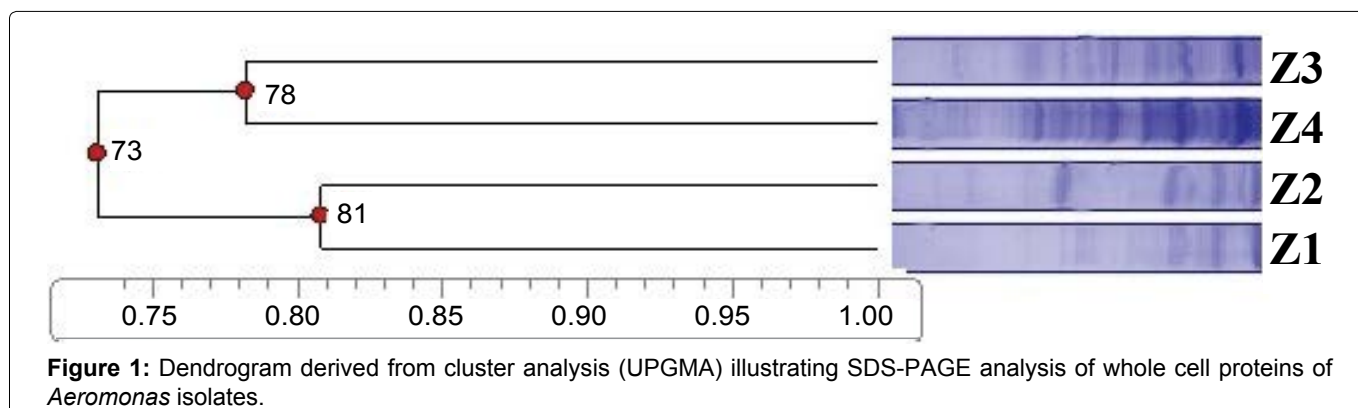
Sequence analysis of the 16S rRNA gene regions was performed for genotypic tests and compared to the sequences recorded in GenBank. GenBank accession numbers for the isolates were also determined: MH444890, MH444891, MH444892, and MH444893. Homology search carried out using BLAST (<http://www.ncbi.nlm.nih.gov>) revealed that the two isolates were similar to *Aeromonas tecta* and the other two were close to *Aeromonas veronii* at 99% similarity level. When the alignment was further analyzed using MEGA.7.0 Neighbour-Joining (NJ) method, the phylogenetic tree constructed reflected the evolutionary relationships of the isolates (Z1-Z2-Z3-Z4) with the other strains in the genus (Figure 2).

### Enzyme assays

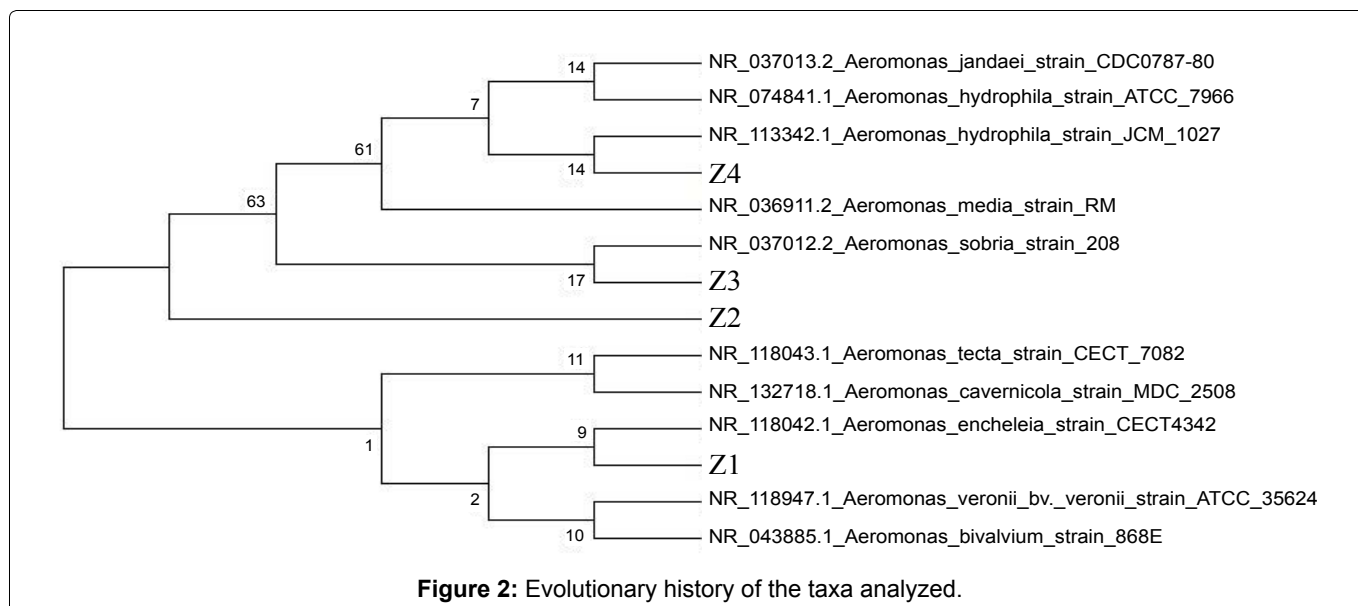
According to results obtained, three of the isolates were able to produce the amylase enzyme and only one could produce the cellulose enzyme but none produced the protease enzyme. For the amylase enzyme, in particular at 25 °C, one strain formed a 12-16 mm zone diameter in the pH range of 6-10, the second strain for 35 °C formed a 13 mm zone diameter at pH-6, and for 45 °C only the other two strains in the pH (6-10) had a low zone diameter of 2-4 mm. The cellulose enzyme producing strain was found to have zone diameters of 23 and 13 mm at pH-7 for 25 °C and 45 °C, respectively. Results of amylase and cellulose production at different temperature and pH values are depicted in Table 5 and Table 6.

### Discussion

Since *Aeromonas* is widely distributed in aquatic environments, serious cautions must be taken. Fraisse, et al., presented a retrospective study of *Aeromonas* spp. infections between years 1997-2004 and stated that ten infections were nosocomial and four were associated with medical leeches [7]. Voss, et al. stated that 13 of 28 *Aeromonas* wound and soft tissue infections during a 4-year-period were associated with a water-re-



**Figure 1:** Dendrogram derived from cluster analysis (UPGMA) illustrating SDS-PAGE analysis of whole cell proteins of *Aeromonas* isolates.



**Figure 2:** Evolutionary history of the taxa analyzed.

**Table 5:** Amylase Production Analysis.

Strains	pH-5		pH-6		pH-7		pH-8		pH-9		pH-10		
	C	Z	C	Z	C	Z	C	Z	C	Z	C	Z	
25 °C	Z1	4	6	8	8	5	11	12	26	5	18	4	10
	Z2	4	6	6	20	5	16	8	20	4	16	4	12
	Z3	4	9	5	21	5	21	9	24	6	18	4	18
35 °C	Z1	5	6	9	16	7	12	10	16	6	6	4	-
	Z2	5	6	6	19	8	15	9	14	6	6	5	-
	Z3	7	8	8	19	6	14	10	18	8	8	5	5
45 °C	Z1	9	-	10	14	10	12	9	13	8	12	7	10
	Z2	5	-	11	12	10	13	9	11	6	8	6	-
	Z3	9	-	9	-	9	-	8	-	8	-	5	-

C: Colony diameter (mm), Z: Zone diameter (mm), - : No Growth.

**Table 6:** Cellulase Production Analysis.

Strains	pH-5		pH-6		pH-7		pH-8		pH-9		pH-10		
	C	Z	C	Z	C	Z	C	Z	C	Z	C	Z	
25 °C	Z4	5	19	7	20	6	29	5	18	8	18	5	18
35 °C	Z4	6	15	6	18	10	18	7	12	7	12	7	11
45 °C	Z4	6	17	6	17	7	20	6	12	6	15	6	14

C: Colony diameter (mm), Z: Zone diameter (mm).

lated injury and lakes or rivers constituted 43% of the total [8]. Extracellular proteases allow *Aeromonas* sp. to persist in different environments and thus facilitate ecological interactions with other organisms and promote invasion by direct damage of host tissue [3]. Therefore, the investigated enzymes such as proteases are not only important in terms of biotechnological perspective, but also they can be a measure of improvement of survival and infection potential in aquatic environments. Since none of our isolates produced the protease enzyme, this can also be a measure of low infectivity potential in the aquatic areas investigated.

Using various conditions for the biochemical identification of *Aeromonas* spp. is one of the major problems

that gives unstable results in clinical laboratories. Thus, reliable method choice to correctly identify *Aeromonas* species can help to prevent the inconsistency for this complicated taxonomic genus. Though API strips have database for the genus *Aeromonas*, they are effective in the identification at the genus level since they lack the database for the entire range of the known *Aeromonas* species [9]. The correct method approaches will be beneficial to clarify the identification of *Aeromonas* spp. at the species level and support routine biochemical identification in laboratories. One of these approaches can be supporting the routine phenotyping tests with multiplex PCR though it also has some limits in final discrimination and therefore must not be used as a single identi-



fication approach [1]. Sarkar, et al., also implied the less discriminatory power of SDS-PAGE and offered Random Amplified Polymorphic DNA (RAPD) analysis as an ideal tool for molecular characterization [10]. Demarta, et al., characterized clinical and environmental *Aeromonas* isolates by a polyphasic approach including phylogenetic analysis derived from *gyrB*, *rpoD*, and 16S rRNA gene sequencing as well as DNA-DNA hybridization together with extensive biochemical tests and described a novel species named as *Aeromonas tecta* [11].

The less discriminative power of SDS-PAGE at strain level demands more specialized molecular tools alone or in combination and sequence analysis of the 16S rRNA gene regions corresponds to this requirement. The combination of phenotyping and a strong molecular approach ensures the accurate identification of *Aeromonas* spp. in medical laboratories.

*Aeromonas hydrophila* is the predominant species in freshwater and municipal drinking water supplies and in Nigeria lakes, the isolated species were *A. hydrophila* and *A. sobria* [12]. Several *Aeromonas* species having potential pathogenicity were also investigated in environmental water of Okinawa and *A. veronii* biovar *sobria* and *A. jandaei* were reported as the predominant species in dams, and *A. hydrophila* and/or *A. eucrenophila* were predominant in wells [13]. However, our limited number of isolates did not allow us to characterize the predominant species *A. hydrophila* and extended studies are certainly required.

Enzyme production activities of the isolates in Skim-milk agar (protease), M9 agar (amylase) and CMC agar (cellulase) were also determined at 25, 35 and 45 °C at different pH (5-10). Besides with other various industrial applications of microbial enzymes, utilizing these microbial enzyme systems may also be involved in the biodegradation of toxic organic pollutants. Soil and water pollution by industrial chemicals and petroleum hydrocarbons is a serious modern world problem and the use of bioremediation strategies may offer nature friendly approach to remove/reduce these contaminants [14,15]. Our results showed that three of the isolates were able to produce the amylase enzyme and one could produce the cellulase enzyme. However, none produced the protease enzyme. Though this is a preliminary analysis with limited number, our results reflect the potential of *Aeromonas* in biotechnological field besides with the widely studied bacteria and fungi. We encourage future comprehensive studies focusing on this perspective.

Since *Aeromonas* species are opportunistic water-borne pathogens and can cause health problems, continuous surveillance of water sources is an important issue to protect susceptible individuals. Also, the reliable identification methods based on molecular techniques is an obligatory in medical practice for this phenotypically and genetically heterogeneous taxon. Besides, exploiting this pathogen in search of enzyme activities

may have area of utilization in developing bioremediation strategies.

## Acknowledgments

We thank to Prof. Dr. Sumer ARAS.

## References

- Králová S, Staňková E, Sedláček I (2016) Classification of *Aeromonas* spp. isolated from water and clinical sources and distribution of virulence genes. *Folia Microbiol* 61: 513-521.
- Batra P, Mathur P, Misra MC (2016) *Aeromonas* spp.: An emerging nosocomial pathogen. *J Lab Physicians* 8: 1-4.
- Elgendy MY, Soliman WS, Abbas WT, TB Ibrahim, AM Younes, et al. (2017) Investigation of some virulence determinants in *aeromonas hydrophila* strains obtained from different polluted aquatic environments. *JJBS* 10: 265-272.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227: 680-685.
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: A laboratory manual*. (2<sup>nd</sup> edn), Cold Spring Harbor Laboratory, New York.
- Abell GC, McOrist AL (2007) Assessment of the diversity and stability of faecal bacteria from healthy adults using molecular methods. *Microbial Ecology in Health and Disease* 19: 229-240.
- Fraisse T, Lechiche C, Sotto A, Lavigne JP (2008) *Aeromonas* spp. infections: retrospective study in Nîmes University Hospital, 1997-2004. *Pathol Biol (Paris)* 56: 70-76.
- Voss LM, Rhodes KH, Johnson KA (1992) Musculoskeletal and soft tissue *Aeromonas* infection: An environmental disease. *Mayo Clin Proc* 67: 422-427.
- Awan MB, Ahmed MM, Bari A, Saad AM (2005) Biochemical characterization of the *Aeromonas* species isolated from food and environment. *Pak J Physiol* 1: 1-2.
- Sarkar A, Saha M, Patra A, Roy P (2012) Characterization of *Aeromonas hydrophila* through RAPD-PCR and SDS-PAGE Analysis. *Open Journal of Medical Microbiology* 2.
- Demarta A, Küpfer M, Riegel P, Harf-Monteil C, Tonolla M, et al. (2008) *Aeromonas tecta* sp. nov., isolated from clinical and environmental sources. *Syst Appl Microbiol* 31: 278-286.
- Bello HS, Mustapha A, Ismail HY, Isa MA, Mangga HK (2016) Detection of *aeromonas* species from water sources in North Eastern Nigeria. *IJSET* 3.
- Miyagi K, Hirai I, Sano K (2016) Distribution of *Aeromonas* species in environmental water used in daily life in Okinawa Prefecture, Japan. *Environ Health Prev Med* 21: 287-294.
- Karigar CS, Rao SS (2011) Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. *Enzyme Research* 2011: 805187.
- Singh R, Kumar M, Mittal A, Mehta PK (2016) Microbial cellulases in industrial applications. *Annals of Applied Bio-Sciences* 3.