

# Phylogenetic Relationship of Indigenous Bacteria Fermetodege based on Next-Generation Sequencing (NGS) Molecular Analysis

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**Abstract.** The objective of this study was to reveal the phylogenetic relationship of indigenous bacteria in a fermented feed called Fermetodege made from fermented water hyacinth (*Eichhornia crassipes*), corn (*Zea mays*) cobs and rice (*Oryza sativa*) bran using a phylogenetic tree. This mixture is rich in cellulose and a good source of cellulolytic bacterial isolates. Phylogenetic trees can provide important information, including the evolutionary processes, the similarity of characters, and their bio prospects. To establish the phylogenetic relationship of indigenous bacteria, fermetodege was made through a series of procedures. Afterwards, molecular analysis was carried out using NGS. Based on UPGMA analysis, there were ten definphylaylum and oindefiniteite groups. The phylum includes Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota, Desulfobacteriota, Cyanobacteria, Chloroflexi, Campilobacteriota, Acidobacteriota, and Synergistota. It was found that Proteobacteria are closely related to Actinobacteriota, which are more closely related to Firmicutes than Bacteroidota. The main characteristic of bacteria in each phase of the fermentation process is their optimum growth temperature. The optimum growth temperature in the mesophilic phase I, II and ripening phase is around 30-50 °C. Meanwhile, bacteria in the thermophilic phase grow well at temperatures around 70 °C. In addition, another characteristic of these bacterial species is its ability to degrade cellulose.

## 1 Introduction

A phylogenetic tree refers to a chart or a branching diagram that visually represents the evolutionary relationships between different species of living organisms. Phylogenetic analysis is used in studying genetic variation analysis and systematic analysis to identify relationships between different species and to find common ancestors of the species that share common features or DNA sequences. In studying the analysis of genetic variation/differentiation between populations, genetic distance can be calculated from the number of differences in polymorphic bases gene loci of each population based on DNA sequence. In evolutionary biology, these studies are often applied to trace past migration/dispersal patterns of animals and plants. The benefits of compiling a phylogenetic tree include being able to find out the similarities among living things. There are several

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advantages and purposes of a phylogenetic tree: (1) to observe how the evolution of an organism occurs and how the evolutionary path occurs in an organism, (2) to estimate the separation from the ancestor of an organism, (3) to interpret data about the origin of an organism, such as in the form of fossils or research.

In this research, we want to observe the relationship between indigenous bacteria that play a role during fermentation in making feed made from a mixture of water hyacinth, corn cob and rice bran called Fermetodege. It is a feed that utilizes cellulolytic bacteria as a starter in the fermentation process of cellulosic materials. Cellulolytic bacteria are a specific group of microorganisms that play a central role in cellulose degradation, a complex carbohydrate found in plant cell walls. During fermentation, cellulolytic bacteria work synergistically with other microorganisms, various bacteria phyla such as lactic acid bacteria and yeast, depending on the stage of the fermentation. Related species of bacteria have many similarities in their physiological and biochemical processes, which creates cooperation and competition for food resources and environment conditions.

Moreover, cellulolytic bacteria contribute to pH regulation within the fermentation environment. While some produce acids (e.g., acetic acid), others may contribute to buffering mechanisms. This regulation is crucial for creating an environment that supports the activity of cellulases and other enzymes involved in fermentation. Previously, it was observed that there is an optimal pH that will affect the cellulase enzymatic reaction stability during cellulose degradation [1].

This knowledge will facilitate the use of related bacteria for various purposes, such as using the bacteria as a starter, which speeds up the fermentation process for making feed, transforming raw plant materials into a more digestible and nutrient-rich feed for ruminants, source of isolates for isolation of cellulase enzymes, and many other bioprospecting. This balance between different microorganisms is crucial for a successful fermentation process. However, knowledge about the kinship and similarity among indigenous fermetodege bacterial species is still unknown. Understanding and managing the relationship between cellulolytic bacteria and other microorganisms during fermentation are critical factors in optimizing the nutritional quality of fermented feed for ruminants. A well-balanced microbial community ensures efficient cellulose digestion and contributes to the overall health and performance of the animals.

## 2 Method

Materials for making fermetodege, such as water hyacinth, corn cobs and rice bran were collected accordingly. The raw materials were cut to reduce their size which then steamed to soften the texture. Soft texture is easier to degrade. The fermentation process was carried out by putting the raw materials into a plastic basket with banana leaves lined in the inside of the basket. Next, the mixture of the three ingredients is fermented for 15 days. During fermentation, temperature and pH were measured every 24 hours. Temperature measurements are carried out every day throughout the fermentation process with a thermometer. Temperature measurements were taken at the top, middle and bottom of the feed. Temperature measurements were carried out 4 times (in 4 baskets containing the fermented material). Daily temperature during the fermentation process is the average temperature of the top, middle and bottom of the pile of fermented material and the average of all replicates. Feed samples were taken at the four phases of fermentation processes: mesophilic I, thermophilic, mesophilic II and maturing phase. These phases were identified based on temperature changes.

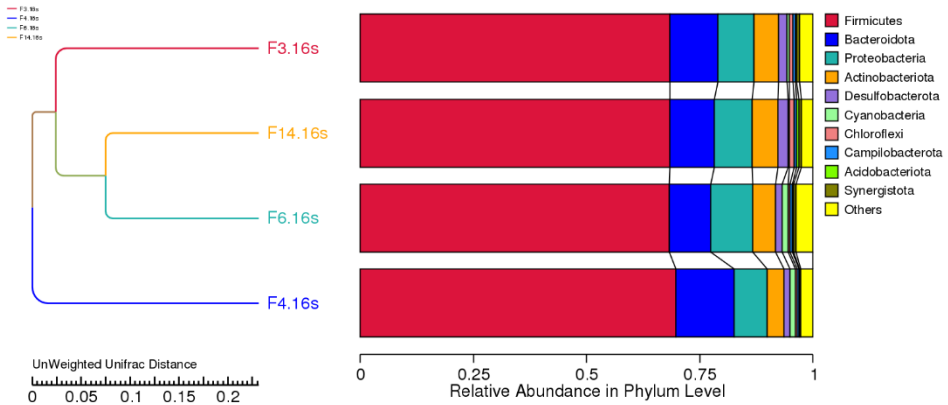
Then, DNA isolation was carried out directly from the feed sample using Presto™ Mini gDNA Bacteria Kit. The isolated DNA was then amplified by PCR and sequenced by the following generation sequencing technique. The sequences obtained were analyzed with

PhyloSNP computational software to achieve the desired goals. Distance analysis in the research based on UPGMA (Unweighted Pair Group Method with Arithmetic Means). The UPGMA method assumes a molecular clock and rooted tree. This method standard computes a defined similarity score as the total number of identical sequences and a conservative number of substitutions in the alignment of two sequences with ignored gaps. The identity score between sequences shows only that identity may be found in the alignment. For analysis, Phylogenetics uses a distance score between two sequences. The score between two sequences is the number of positions does not match/mismatch in alignment or quantity, the position of the sequence that must be changed to produce another sequence. The gap may be neglected in calculation or treated as a substitution. When a scoring or substitution matrix is used, calculations become more complex, but in principle, it remains the same.

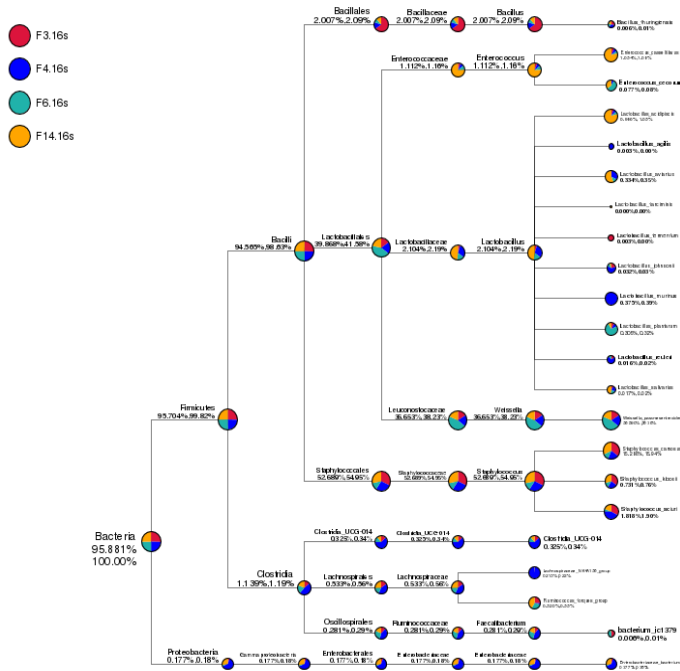
### 3 Result and Discussion

The phylum-level relationship of indigenous fermentodege bacteria in mesophilic I (F3.16S), thermophilic (F4.16s), mesophilic II (F6.16s) and ripening (F14.16s) is presented in Figure 1. Firmicutes phylum dominated the four stages of the fermentodege fermentation process, followed by the Bacteroidota, Proteobacteria and Actinobacteria phyla (Figure 1). The phylogenetic tree at the phylum level has two clades with bacterial phyla in the thermophilic phase as outgroups. This is because the bacterial phyla in the thermophilic phase are dominated by bacteria that grow at higher temperatures than the bacterial phyla in the mesophilic and cooking phases. The bacterial phyla in the mesophilic phase II and the bacterial phyla in the cooking phase are closely related. Both are more closely related to the bacterial phyla in the mesophilic phase I than to the bacterial phyla in the thermophilic phase. In terms of temperature requirements for life, bacterial phyla are found in all three phases, except the thermophilic phase, which relatively requires the same temperature.

Based on Figure 1, the number of bacteria phyla found in the four phases of the fermentation process was the same. It could be because fermentodege is made of cellulosic material, which makes the only population of bacteria with cellulase enzyme activity able to live. However, the number of each species across the four stages of the fermentation is varied. Firmicutes were observed to have the highest abundance in all four phases of the fermentation process. Members of the phylum Firmicutes that have cellulolytic activity include *Bacillus subtilis*, *Bacillus pumilus*, *Paenibacillus polymyxa*, and *Bacillus licheniformis* [2]. The next three phyla with high abundance include Bacteroidota, Proteobacteria and Actinobacteria. Examples of bacteria belonging to the Bacteroidota that have cellulolytic activity include *Bacteroides cellulosolvens* [3-4]. This bacterium was isolated from human fecal sample which can grow on cellulose and able to consume wide variation of sugars [4]. It was observed to be able to produce cellulase and to degrade cellulose into glucose and cellobiose [5]. *Acidothermus cellulolyticus*, *Pseudomonas aeruginosa* and *Pseudomonas veronii* were the examples of bacteria belonging to the Proteobacteria which have cellulolytic activities [6-7]. In addition, *Cellulomonas iranensis*, *C. persica*, and *Acidothermus cellulolyticus* are members of the species of the phylum Actinobacteria [6-7]. This diverse population of cellulolytic bacteria is essential to breakdown cellulose efficiently. Different species of cellulolytic bacteria may specialize in breaking down specific types of plant fibres, contributing to a more comprehensive degradation of the feed.



**Fig. 1.** Phylum level relationships in the four phases of the fermentodege fermentation process.



**Fig. 2.** Species level relationships in the four phases of the fermentodege fermentation process

At the species level, relationships in indigenous bacteria fermentodege as shown in Figure 2. There are 9 clades with details of one clade from the phylum Proteobacteria and eight clades from the Phylum Firmicutes. The Clostridia class from the Firmicutes phylum composes two clades and the Bacilli class composes 6 clades with the order Lactobacillales

having the largest members with various species. Figure 2 shows that members of the Proteobacteria phylum are only found in the thermophilic phase (blue circle line) and ripening phase (yellowish brown circle line). Many members of the Clostridia class are found in the thermophilic phase. From the Lactobacillales order, the Leuconostocaceae family is mainly found in the mesophilic II phase, and the Lactobacillaceae and Enterococcaceae families are often found in the ripening phase. Mesophilic phase I is dominated by the Bacillales order.

Huang and his team have conducted research on the diversity of cellulolytic bacteria from the Gut of *Holotrichia parallela* Larvae (Coleoptera: Scarabaeidae) [8]. They observed the microbial community comprises mainly of Proteobacteria (70.05%), followed by the Actinobacteria (24.15%), the Firmicutes (4.35%), and the Bacteroidetes (1.45%). Based on the result of this research was stated that scarab gut is an attractive source for the study of novel cellulolytic microorganisms and enzymes beneficial for cellulose degradation [1]. The research results of Huang and his team strengthen the results of this research that the phyla Firmicutes, Bacteroidota, Proteobacteria and Actinobacteriota have cellulolytic activity. In the fermentodege there are two classes from the phylum Proteobacteria which includes Gammaproteobacteria and Alphaproteobacteria, three other classes namely Betaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria are not present in fermentodege. Proteobacteria, which were initially known as “purple bacteria and relatives,” are characterized by a bewildering diversity of morphological and physiological types: besides rods and cocci, curved, spiral, ring-shaped, appendaged, filamentous and sheathed bacteria occur among this phylum [9-10]. The Proteobacteria group of bacteria is also found in soil that is rich in humus and has high salt levels. This indicates that Proteobacteria members have cellulolytic activity and can be used as a source of cellulase enzymes [11].

Proteobacteria are also commonly found in habitats with higher temperatures than the mesophilic group. Therefore, in the fermentation process, many Proteobacteria groups are found in the thermophilic phase [12]. Proteobacteria are present in large numbers during the ripening phase because this phylum has members whose habitat varies greatly.

Many members of the Clostridia class are found in the thermophilic phase. Some Clostridia species are common in habitats with high temperatures, such as *Clostridium perfringens* which is often a pathogen in livestock [13], *Clostridium clariflavum* that have cellulolytic activity [14]. Dumitrache and his team have tracked the conversion of cellulose by *Clostridium thermocellum* in the form of a biofilm, which turns out to have better conversion kinetics, because the cells in the biofilm are not limited in their activity by nutrient depletion [15]. Yu and his team have succeeded in increasing the degradation capability of cultures between *Clostridium thermocellum* and *Geobacillus* sp which can balance medium pH. They may provide the required anaerobic environment for *Clostridium thermocellum*, so that the cellulolytic activity of the *Clostridium* bacteria increases [16].

From the Lactobacillales order, the Leuconostocaceae family is mostly found in the mesophilic II phase. Various *Leuconostoc* species are found in materials that are undergoing lactic acid fermentation, and other materials that have a low pH. The suitable temperature for *Leuconostoc* habitat is 20-30° Celsius, so that these bacteria are mostly found in the mesophilic phase [17].

The Lactobacillaceae and Enterococcaceae families are primarily found in the ripening phase. Some kinds of species of Enterococcaceae have cellulolytic activity like *Enterococcus hirae* strain 1-1X-16 and *Enterococcus mundtii* strain ZU 26. Apart from having cellulolytic activity, it can also increase the cellulolytic activity of the group of lactic acid bacteria found in the intestines of livestock [18].

Mesophilic phase I is dominated by the Bacillales order. Members of Bacillales are widely used to fertilize agricultural land. Members of this order are able to degrade cellulosic materials into mineral nutrients that can be utilized by plants [19].

Indigenous bacteria in fermentation methods are composed of only one kingdom, namely Bacteria. There are two phyla which include Firmicutes and Proteobacteria. The Firmicutes phylum carries two classes, namely Clostridia and Bacilli. In the Bacilli class, there are three orders as indigenous fermentation bacteria, namely Bacillales and Staphylococcaceae which carry only one family. While the Clostridia class consists of three orders, each of which includes the taxon.

## 4 Conclusion

There were one undefined group (was not identified the phylum name because it consists of a mixture of organisms from various phyla) and 10 defined phylum (was identified the name of the phylum). The defined phylum included Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota, Desulfobacteriota, Cyanobacteria, Chloroflexi, Campilobacteriota, Acidobacteriota, Synergistota and other groups. Based on the results, it was found that Proteobacteria are closely relationship to Actinobacteriota, and both are more closely related to Firmicutes than to Bacteroidota. These bacteria were found to have cellulolytic bacteria. Several bacteria phyla have similar percentages in different stages of fermentation due to their similarity in the condition. However, depending on the stages, several species “come and go” depending on the fermentation stages. One of the factors that might be each phase of the fermentation process is its optimum growth temperature. The optimum growth temperature in the mesophilic phase I, II and ripening phase is around 30-50 °Celsius. Bacteria in the thermophilic phase grow well at temperatures around 70 °Celsius.

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