CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Analyzing the Spectrum of Cellulolytic Bacteria Inhabiting the Digestive Track of Termites

by

Farhat Mushtaq

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Dedicated to Allah Almighty, Hazrat Muhammad (S.A.W.W) and my family life. To my mother and father, who never stopped believing in me and their prayers have always enlightened my way throughout my life to my mother and father, who never stopped believing in me and this can't be possible without their unwavering support, endless love and encouragement throughout my pursuit for education. My parents taught me that the best kind of knowledge to have is that which is learnt for its own sake. I hope this achievement will fulfill the dream they envisioned for me.

CERTIFICATE OF APPROVAL

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(Farhat Mushtaq)

Abstract

Termites belonging to the order *Isoptera*, are cellulose-eating insects. Termites are the present abundance in both numbers and species observed in tropical rainforests. Dry-wood termites, such as those belonging to the Cryptotermes genus, thrive in small colonies within wood and can endure extended periods of dry conditions. Termites play a crucial ecological role by aiding in the conversion of plant cellulose into substances that can be recycled within the ecosystem, supporting new growth. For this study, Termite samples were collected from different locations of the Kotli, Azad Kashmir, Pakistan. Termite gut was used for the isolation of the microbiomes. Gut sample were processed and cultured on the growth mediums, CMC media and nutrient broth agar.

Colonies were observed for morphological characterization and biochemical characterization and different tests were performed such as Gram staining, Catalase test, Oxidase test, Urease test, Motility-Ornithine test, Voges-proskauer tests and TSI (triple, sugar, iron). Moleculer characterization was done by doing the 16S rRNA of the selected bacterial strains.

Morohological characterization of the tree trunk termite samples showed that all the seven strains of the were gram positive. Four samples of J3 and two samples of J2 were characterized by rod shaped colonies morphologically except J1 which was gram positive but characterized by coci shaped colony morphologically. Sample J2.1 was positive for Catalase test, Gram staining test, Urease test and was negative for Oxidase test, Motility-Ornithine test, TSI and voges-prokauer test. Sample J2.3 was positive for catalase test, oxidase test, gram staining, ans urease while negative for the motility-ornithine test, voges-proskauer test and TSI. Sample j3.2 and 3.3 was positive for Catalase test, Oxidase test, motility-ornithine test, gram staining, TSI and urease test while negative for the Voges-Proskauer test. Sample 3.4 positive for Gram staining test, Catalase test and for TSI while negative for the Motility-Ornithine test, Oxidase test, Urease test and Voges-Proskauer test.

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Abbreviations

AQS	Asexual queen succession
BHI	Brain heart infusion
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
FF	Female-female ratio
GHF9	Glycosyl hydrolyse family 9
Hyd	Hydrogenases
H_2S	Hydrogen sulphide
KOH	Potassium hydroxide
MR	Methyl red
NaCl	Sodium chloride
NifH	Nitrogenase iron protein
PH	Potential of hydrogen
rRNA	Ribosomal ribonucleic acid
TBE	Tris-borate-ETDA buffer
TSI	Triple, sugar, iron
VP	Voges-prokauer

Chapter 1

Introduction

Termites, social insects forming colonies and able of ranging from a few hundred to over a million individuals, play a crucial role in the degradation of diverse materials [1]. The identification of termites has been based on the methods outlined involving the assessment of factors such as carbon and nitrogen cycling, soil structure, and the promotion of microbial activity at different levels [2].

Termites contributes to biodiversity by creating conducive conditions for plants and other biota. [3]. Besides offering ecosystem services, various termite species also pose economic significance as pests affecting agricultural crops, forest plantations, and the structural wood components. [4] Globally, approximately 2,650 termite species have been documented, categorized into 280 genera and seven families [5].

According to reports, there are 16 genera, 53 species, and four families *Kalotermitidae*, *Hodotermitidae*, *Rhinotermitidae* and *Termitidae* within the termite fauna in Pakistan [6]. Comprehensive studies on the various species, their relative abundance, and distribution across different localities in Pakistan are lacking. Despite some subtropical species having less ecological significance, their economic value cannot be underestimated, particularly in urban areas where their extinction could impact the various sectors [7].

Termites are prevalent pest in tropical regions, posing challenges in residential, forestry, and agricultural settings [8]. While certain species are acknowledged

for their economic impact termites also contribute to ecosystem processes and influence carbon and nitrogen cycles [9]. Damp structures act as a refuge for subterranean termites. Out of the 2,650 species currently identified globally, only 300 are classified as pests [10].

The strategies employed to control termites raise various environmental concerns, as highlighted in studies. Ineffectiveness in controlling termites can be attributed, in part, to a limited understanding of factors such as their presence, host preferences, and the specific types of habitats they infest [11].

Farmers, foresters, and the general public experience undisclosed annual losses due to termites because they lack comprehensive information about the species present, preferred hosts, and habitats. Due to their involvement in the recycling of lignocellulosic biomass-a blend of cellulose, hemicellulose, and lignin—termites are essential to the terrestrial ecology [12]. Termites are one of the most common soil insects. They break down lignocellulose effectively with the help of their microbial symbionts resulting in simpler forms of carbohydrates [13]. Yeast organisms convert these sugars to produce ethanol. Termites are thought to devour a sizable amount of the hemicellulose (65–67%) and cellulose (74–99%) components of lignocellulose [14].

Lower termites host a significant number of prokaryotes in their stomach, as well as protests' (single-celled eukaryotes). More than three-quarters of all termite species fall under the category of higher termites, exclusively belonging to the apical family *Termitidae*. Unlike lower termites, higher termites lack protests but possess a diverse array of prokaryotes [15].

Termites are also prevalent in Australia, Tasmania, New Zealand, and the Cape region of South Africa. Besides naturally occurring termites, humans have inadvertently introduced numerous other species to new regions worldwide [16]. Specifically, termites such as *Cryptotermes* and *Coptotermes* have been unintentionally transported within wooden items like furniture, lumber, boat timbers, and shipping crates. Species of dry-wood termites, such as *Cryptotermes*, live in small colonies inside wood and may withstand prolonged dry spells [17]. Termites are

easily transported across long areas because they can live in seasoned wood and furniture [18].

Coptotermes and other members of the *Rhinotermitidae* family cannot survive extended dry spells and need access to moisture. Several locations have seen the introduction of this termite species, including the Pacific islands, Hawaii, California, South Africa, East Africa, and Sri Lanka (Ceylon) [19]. Being ability to live without direct soil contact as long as there is a source of hydration makes *Coptotermes formosanus*, which is found throughout Japan, Taiwan, and South China, unique among species in its family., The termite species *Reticulitermes flavipes*, which is indigenous to the United States, was first discovered in the hothouses of *Schönbrunn* Palace in Vienna. with its first documentation and description occurring there before its discovery in the United States [20]. It is hypothesized that termites were transported from North America using aesthetically pleasing potted plants placed in wooden containers. The termite society or colony operates as a highly efficient and well-functioning group [21].

A caste system, delineated by the structure, function and behavior of the colony's inhabitants, is present with a distinct division of labor. The colony primarily comprises three main castes: reproductive, soldier, and worker castes [22]. Both male and female members of the worker and soldier castes are sterile. Functional reproductives fall into two categories: primary and secondary, or supplementary [23].

Termites play a significant role in two distinct ways. Introduced species, which typically have a lower ability to adapt to changes in new environments compared to native species, often seek refuge in sheltered, man-made structures like buildings. These introduced species are more likely to emerge as significant pests, causing substantial damage to homes and wooden furnishings [24].

Certain termites are destructive as they feed on living plant material, posing a threat to wooden structures and valuable vegetable matter [25]. Termites also play a crucial role by aiding in the transformation of plant cellulose into materials that can be recycled back into the ecosystem. This process stimulates new development,

showcasing the dual nature of termites as both destructive and beneficial agents in the environment [26].

Endogenous cellulase of termite origin, such as β-glucosidase and endo-1,4-glucanase, have been shown to be produced by both lower and higher termites. These enzymes are released from the gut or salivary glands. According to molecular research, these endogenous enzymes belong to the glycosyl hydrolase family 9 (GHF9) [27]. The organic cellulose-forming process in higher termites meets their metabolic demands while the hindgut of lower termites houses cellulolytic activity [28]. The cellulose ingested by termites can undergo slight degradation through the termite-derived endoglucanase This well-known example of symbiotic symbiosis involves the relationship between lower termites and gut cellulolytic protists [29].

Termites assimilate the acetate produced through the protists' endocytosis of wood or cellulose particles in order to obtain carbon and energy. They distributed as active wood-degrading organisms and play a vital role in the carbon cycle of the environment and may serve as biochemical catalysts [30]. Through evolution and adaptation, these termites establish symbiotic relationships with diverse bacteria in their gut enhancing crucial physiological processes like reproduction, immunity, and the digestion of lignocelluloses in their diet [31].

Termites are integral to the environment's carbon cycle and may offer various benefits described as social insects exhibiting diverse morphologies [32]. Termites can be categorized into two main groups based on their reproductive capabilities: reproductive (queen castes) and non-reproductive (worker and soldier castes) [33]. Termites are vital components in intricate relationships within the termite species, classified as higher and lower termites, contributing to the overall termite ecosystem [34].

The unhydrolyzed cellulose travels to the hindgut, where symbiotic protists in lower termites can endocytose and ferment it. In the realm of gut microbial ecology and symbiotic relationships, these microbes utilize electron equivalents, often in the form of molecular hydrogen, generated during the intermediate stages of degradation [35]. This utilization of electron equivalents in the final stage of degradation, has vital role in enhancing decomposition efficiency. Methanogens usually

dominate in environments with a low supply of oxygen [36]. In the microbial fermentation that occurs in the termite gut, a distinctive and perplexing feature is the CO_2-reducing acetogenesis, acting as a "H_2 sink" reaction. This stands out due to the thermodynamic challenges associated with acetogenesis [37].

Although the process of methanogenesis is frequently seen in termite guts, acetogenesis is more important, especially in termites that consume wood [38]. Termites' ability to meet their respiratory needs is largely dependent on the gut microbiota's synthesis of acetate. The effective breakdown of lignocelluloses is significantly influenced, albeit indirectly, by nitrogen distribution [39].

The breakdown of nitrogen wastes produced during termite metabolism, such as uric acid, is facilitated by the gut symbionts. Protozoans or protists within the gut can consume cellulose and break it down into acetate, hydrogen, and carbon dioxide [40]. Bacteria then utilize the hydrogen (H_2) and carbon dioxide (CO_2) to produce more acetate. Certain methanogens are capable of using H_2 and CO2 to generate methane through inter-species hydrogen transfer, facilitating the elimination of surplus hydrogen, which in greater concentrations can be harmful to bacteria [14].

The breakdown of lignocellulose in termites has been the subject of extensive study [41]. Termites, aided by their associated microbial symbionts, play a crucial role as one of the most significant organisms in breaking down lignocelluloses into simpler forms of sugars [42].

Despite termites being highly efficient lignocellulose decomposers, there is a need to explore the anaerobic cellulolytic bacteria present in their guts. It has been suggested that the microbiome of gut of termites could potentially serve as a valuable model system for understanding the electron flow dynamics involved in the degradation of biopolymers by anaerobic microbial communities [43].

This study project's particular goals were to investigate the variety, abundance, and dispersion of termites in the study area, considering the economic importance of termites [44]. Termites naturally inhabit a broad spectrum of terrestrial environments especially in tropical regions. The symbiotic relationship between termites

and microorganisms, particularly the cellulolytic flagellates in their gut, plays a crucial role in breaking down and digesting plant biomass [45]. Termites, with the help of these microorganisms, can efficiently utilize cellulose, a major component of plant cell walls, turning it into a valuable energy source for their colonies. This process has garnered interest from researchers looking for sustainable ways to convert plant biomass into useful products [46].

1.1 Problem Statement

"Investigate the diversity of cellulolytic bacteria in various ecosystems, optimize conditions for cellulose degradation, and explore genetic engineering strategies to enhance cellulase production and activity for sustainable biorefinery applications."

1.2 Aim

The aim of this study is to isolate and identity the cellulolytic bacteria from the gut of *psammopermes hypostoma* and to confirm their role in degradation.

1.3 Objectives

1. To isolate the cellulolytic bacteria from gut of termites.
2. To perform biochemical characterization of cellulolytic bacteria
3. To carry out molecular characterization using 16s RNA

1.4 Gap Analysis

The diversity of cellulolytic bacteria in termite gut varies in different regions of world and depends upon the nature of plantation cellolulytic bacteria associated

with the termites of fauna of Pakistan has not yet be available. Even the studies about termite fauna is also less reported. Study related to cellulolytic bacteria associated with different part of Pakistan. Limited study available. The diversity of cellulolytic bacteria still limited. There is a need to explore the cellulolytic bacterial diversity to access the need of concentration and efforts related to termite species and also to determine if there are unexplored bacterial taxa that play significant role in cellulose digestion.

Chapter 2

Review of Literature

2.1 Termites Distribution

Termites are typically found worldwide in tropical rainforests near the equator, spanning both North and South latitudes [47]. The Eastern Hemisphere boasts a higher abundance of termite species compared to Northern latitudes. There are various species of termites in the Northern Hemisphere whose biodiversity higher than Southern Hemisphere even discovered at elevations of 2000 m in Eastern Hemisphere mountains, where dispersion is more extensive than in the Western Hemisphere [48].

Termites inhabit every continent except Antarctica, showcasing a global distribution of species [50]. They are most abundant in tropical regions, subtropics, and areas with warmer climates. Termites flourish in warm, moist lowlands and coastal regions [49]. Their predominant presence is observed in environment of trophics, having the greatest diversity of species found in equatorial rainforests, and generally decreasing as one moves toward higher latitudes. There are differences in the diversity and distribution of termite species between continents and even between nations Termite species diversity and globular dispersion differ greatly. There are ten identified species in Europe, while North America hosts 50 species [49]. South America boasts a particularly high diversity, with over 400 known species. Asia, specifically in China, accommodates 435 termite species,

predominantly in environments that are mildly tropical and subtropical to south of the Yangtze River. Among the 3000 classified termite species, Africa houses 1000, showcasing a diverse ecological distribution of their mounds [50].

Seven genera were identified by the ecological distribution and species composition of termites in Ethiopia's central rift valley: *Macrotermes, Microtermes, Odontotermes, Amitermes, Angulitermes Microcerotermes* and *Trinervitermes*. *Trinervitermes* and *Angulitermes* were scarce and restricted to specific places, but the first five taxa showed a high distribution throughout the tested area [51].

With the use of maize stalk baiting and defined belt transects, termites were extracted from a variety of land-use types, including farmlands, rangelands, and protected lands. These different land-use categories were managed by humans to differing degrees; the least managed (least disturbed) land-use type was protected lands, followed by rangelands and farmlands. This distinction implies that some termite species may be declining as a result of cultivation and animal grazing. Termite colonies may starve if grass and straw consumed by livestock are eliminated, according to a theory [52].

The relative abundance of termites is influenced by the ecological characteristics of the regions under consideration. Occurrence of termite genera within specific locations is contingent upon the type of land use. Among the total 61 occurrences, *Macrotermes* was encountered 20 times (32.8%), with 10 occurrences (16.4%) in rangelands out of its total 20 occurrences [51]. All land-use types contained *Macrotermes*, *Microcerotermes*, *Amitermes* and *Microtermes*. The most prevalent habitats were rangelands for *Macrotermes*, farmlands for *Microtermes*, and protected areas for *Microcerotermes* [53].

In Manasibu district, west Ethiopia, the frequency of termites and the degree to which they harm important farm crops and rangelands were assessed. Forty percent of the 150 samples were found to be *Microtermes* species samples. Of these samples, 45 termite samples were taken from damaged maize in fifteen fields. 15 termite samples were taken from rangelands, and they were categorized into six genera: *Trinervitermes, Ancistrotermes, Macrotermes, Microtermes, Odontotermes*, and *Pseudodacanthoherms* [54].

Microtermes frequently encountered in the roots and stems of maize, as well as in the stalks of sorghum. At the base of matured teff roots with an occurrence rate of 37.5%. *Pseudodacanthoherms* and *Macrotermes* exhibited occurrence rates of 24.4% and 17.8%, respectively, in the mentioned crops. *Microtermes*, the moundless termites, exhibit a preference for seasonal dispersion, favoring the cold dry season and the arrival of rain starting to fall as the dry season draws to an end. After the first few raindrops, these termites quickly take over the earth. Heavy-duration rainstorms help to eradicate termites from the earth by washing away their roots and feeding holes [55][59].

Reticulitermes flaviceps was initially discovered and named in Taipei, Taiwan. Subsequent assessments, considering factors such as population size, lifestyle, and colony size, have identified it as the most abundant species [56]. *R. flaviceps* is found in various provinces, including Fujian, Guangdong, Hunan, Guangxi, Jiangxi, Zhejiang, Jiangsu, Anhui, Hubei, Yunnan, Sichuan, Shaanxi and Guizhou [54].

This invasive species has become an urban pest nuisance in China's north over the last ten years, having crossed the Qinling Mountain range and extended its spread from the Changjiang river basin to the Huanghe river basin, a distance of one hundred kilometers north. *Reticulitermes flaviceps* is indigenous to China and can be found in a variety of tropical, subtropical, and temperate regions of the nation [57]. *Reticulitermes*, which cause structural damage, is currently acknowledged as China's most hazardous and notorious underground termite infestation [58]. The underground termite *Reticulitermes chinensis* a significant termite species, that has been found in several Chinese sites, including Beijing, Shaanxi, Tianjin, Shanxi, Chongqing Huanggang, Changsha and the Yangtze River dripping basin [59]. This potential pest poses a threat to trees, building wood products and plant xylem [60]. Another significant Isopteran species impacting China's environment is the subterranean termite *Reticulitermes aculabialis* [61].

Earthworms receive more attention in regions like America and the UK, where ants and termites are less prevalent. The impacts of termites on soil qualities have been extensively studied in India, South America, and Africa [62]. In Africa, most

efforts examining the interaction between termites and soil focus on the fungus-growing mounds of *Macrotermes spp.*, which are not found in Australia. Australia itself is home to about 150 termite species in 25 genera [63].

2.2 Biology/ Morphology

From a phylogenetic perspective, termites can be categorized into the first and metamorphic groups based on external morphology [64]. While there have been changes in the outside appearance of soldiers, resembling scorpions in variation, their original morphology, including the thorax and head (for worker and reproductive organisms), is mostly unaltered. Termites are characterized by their small size ranging from 4 to 15 mm in length and they exhibit a variable color palette that spans from white to tan and occasionally black [65]. The alterations in morphology observed in the heads and thoraxes of soldiers serving as crucial markers for categorization and identification [66]. For instance, the translucent upper lip of *Reticulitermes chinensis* has a highly pointed shape resembling a needle while the upper lip of *R. flaviceps* is slender as in snail [**?**]. Alates (winged reproductive individuals) of *R. flaviceps* exhibit a gray-yellow structure on the front side of the thorax [67].

Among social insects, termites are unique in that their personnel can be either female or male. Some get wing buds that grow over time. Nymphs mature into whole winged adults, destined to become future kings and queens. These termites range in color from black to pale brown and their wings have an opaque grey to black tint. Swarming occurrences happen at different times depending on the species but usually follow a downpour. Termites and the massive mud formations they build are evident in tropical environments. These formations serve as natural air-conditioning systems and may house millions of individual termites [68].

Based on their ability to reproduce, termites can be divided into two groups: non-reproductives (soldiers and workers) and reproductives (queen and king). The characteristic sources of the major reproductives are used to classify them [69]. These animals, who represent actual adults in the nest, have clearly formed wings,

complex eyes and highly pigmented body. They serve as the searchers of initial nests after engaging in tandem partnerships and dispersal flights [70].

In contrast, secondary reproductives (derived from the nest of older adults) differ from primary reproductives. They play a crucial role in the colony's expansion due to their ability to reproduce and lay eggs following ecdysis (molting) [71]. On the basis of their age and form, secondary reproductives can be further divided into wing scale, micro, long, and short wing bud kinds. This classification considers the diversity within this category. Apart from reproduction, the majority of the workers are responsible for maintaining and caring for the overall health of the colony. They play essential roles in various tasks necessary for the well-being and functioning of the termite colony [55].

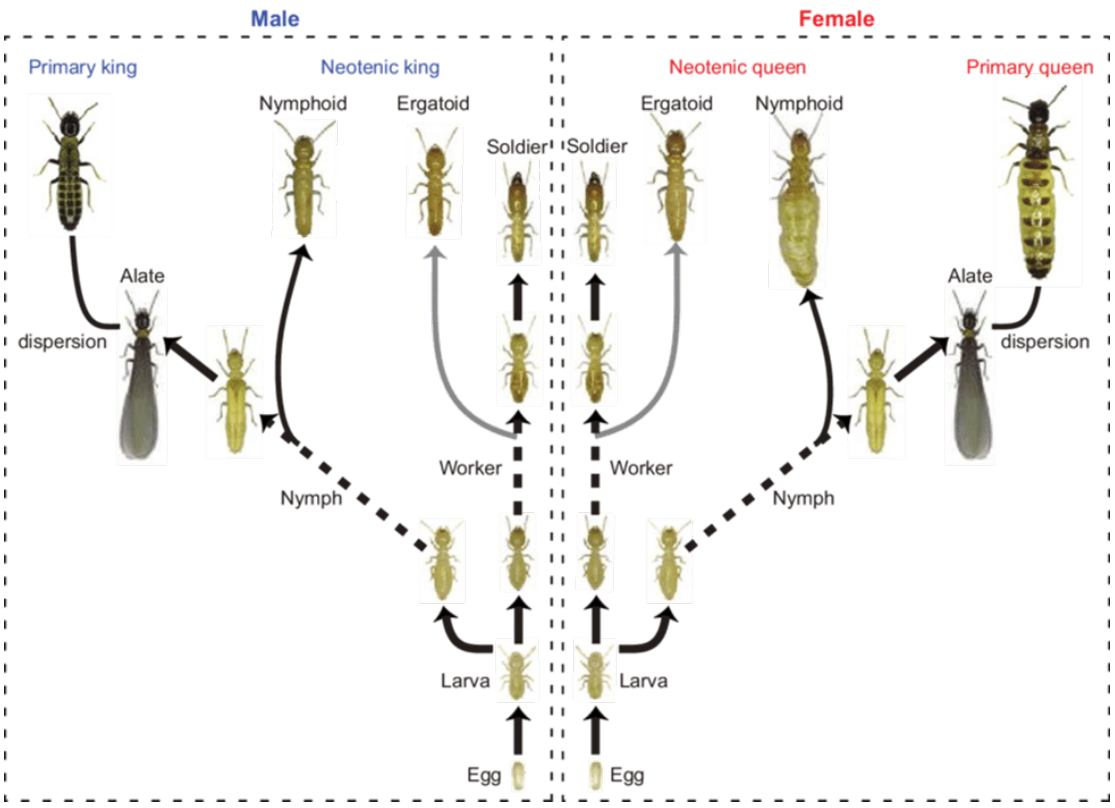


FIGURE 2.1: Cast differenciation in pathway in *Reticulitermes termites* [72]

2.3 Classification of Termites

Termites have traditionally been classified as eusocial insects within the order *Isoptera* [73]. While many scientists agree that termites are relatives of *Blattodea*

and share a common ancestry with net-winged insects, there are still debates regarding their specific relationship. Termites are considered social insects, akin to Hymenoptera, and molecular analyses of their ancestors suggest a monophyletic group, showing a close relationship with *Cryptocercus roaches* [74].

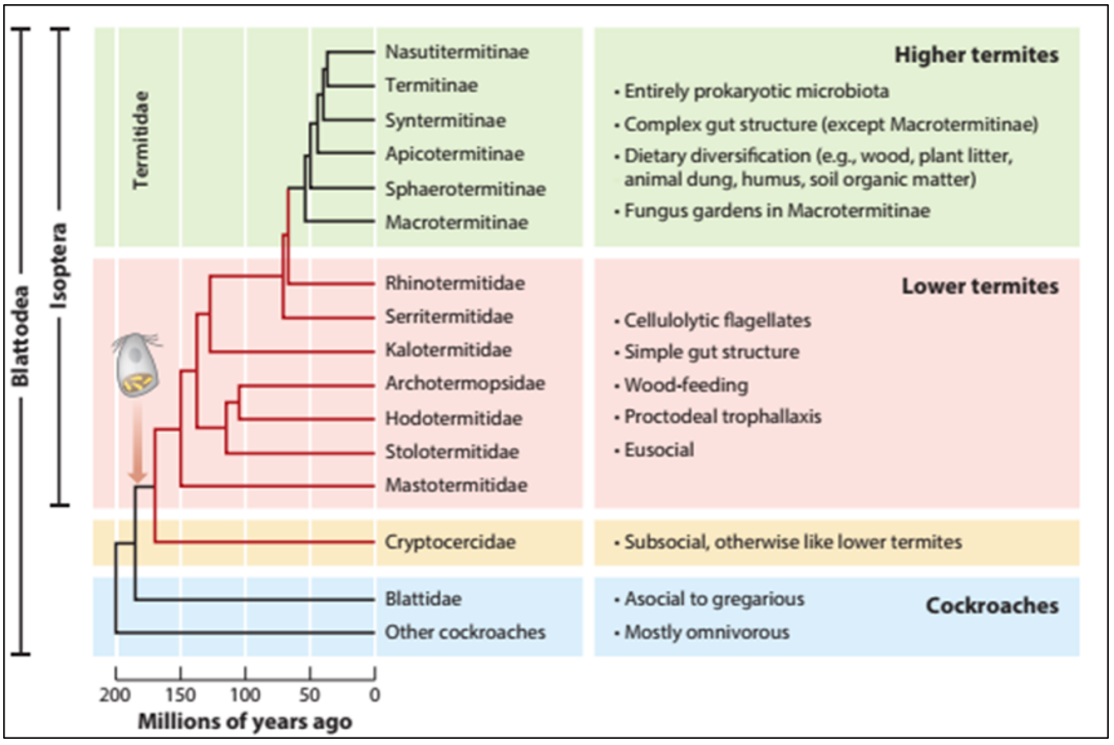


FIGURE 2.2: Termites Phylogeny showing its showing its evolutionary journey [75]

In the early part of 2009, taxonomists divided termite species into seven groups, reflecting their diverse evolutionary lineages. American researchers, Grimaldi, Engel and Krishna reclassified termites, organizing them into nine groups after 2009. The numerous families include *Hodotermitidae, Kalotermitidae, Archatermopsidae, Rhinotermitidae, Mastotermitidae, Serritermitidae, Termiteidae, Stolotermitidae,* and *Stylotermitidae* [76]. The order Isoptera, consisting of four families and four genera and divided into lower and upper termites, encompassed a total of 473 species with the majority exhibiting high destructiveness. Five of these species were discovered in Southern China [77].

2.4 Ecology of Termites

The ecology of pests including termites, focuses on understanding their behavior and interactions with their environment and other organisms. This encompasses aspects such as foraging and nesting mechanisms, as well as soil mounding as a nesting strategy and methods of searching for food. These characteristics distinguish termites from other soil insects. Termites are poorly suited to live in chilly or frigid climates because of their delicate cuticles. Termites can be generically classified into three ecological groups: underground dry wood and damp wood. Each group exhibits distinct ecological preferences and behaviors [78].

Cryptotermes brevis, a dry wood termite species that is regarded as an invasive species in Australia. Using mouth parts designed for chewing, both damp wood and dry wood termites show a predisposition for eating on dead plant materials, such as leaf litter, wood buildings, soil, crops, forests and plantations. Many different kinds of objects are at risk from termites, such as buildings, important papers, artwork, books, flooring, carpeting, and clothing. As social insects that live underground, subterranean termites build colonies and only attack dead tree parts, avoiding living trees altogether. With the largest biomass in these regions, termites are primarily restricted to tropical and subtropical environments, which are typically found between roughly 50° north and south latitudes [49]. Termites are remarkably adept at building a wide range of mound and nest sizes and configurations to house their whole colony. While arboreal termites make nests on trees, ground-dwelling termites usually create intricately sculpted mounds. By regulating condensation, these tree nests allow for water saving while acting as safe havens for the inhabitants. The colony provides care and attention to eggs and in-star larvae in the nursery chambers nestled deep within these nests. Termites are able to stay above ground because of the complex structure of their mounds, which includes a thin end that faces the sun during its hottest part. By encouraging hot air to rise within the above-ground mounds, this design helps the subterranean network's air circulation currents. For species that grow fungal gardens and those that expend a lot of energy keeping the brood within a narrow

range of temperature (±1°C's) and these currents are essential in dispersing the necessary temperature [49].

In tropical savannas, termite mounds can vary significantly in size and shape. Some species construct exceptionally large mounds, reaching an extreme height of 9 meters, especially in well-wooded areas where conical mounds are prevalent. A more typical size for these mounds in such environments ranges from 2 to 3 meters.

These mounds can also have a variety of morphologies, such as sculptured hard earth mounds, irregular domes or cones covered in grass and/or woody bushes, or combinations of these characteristics [88].

2.5 Colony Emergence and Growth

2.5.1 Life Cycle

There are three different caste kinds in the termite life cycle: workers, soldiers, and reproductive. An egg starts the cycle, which then progresses to immature termite nymphs or termite larvae, worker, pseudergate, soldier, drone, and queen stages. Their life cycle aligns with the typical social insect system, facilitating a division of labor among different castes [77].

One of the three castes soldier, worker or secondary reproductive termite could emerge from the larva. A mix of termite pheromone cues, environmental cues, and social cues influence this decision.

Until it reaches adulthood, the larva goes through a series of molts; usually, this takes three molts. Upon hatching from one of the countless thousands of termite eggs, the newly emerged individual is referred to as a termite larva or larvae.

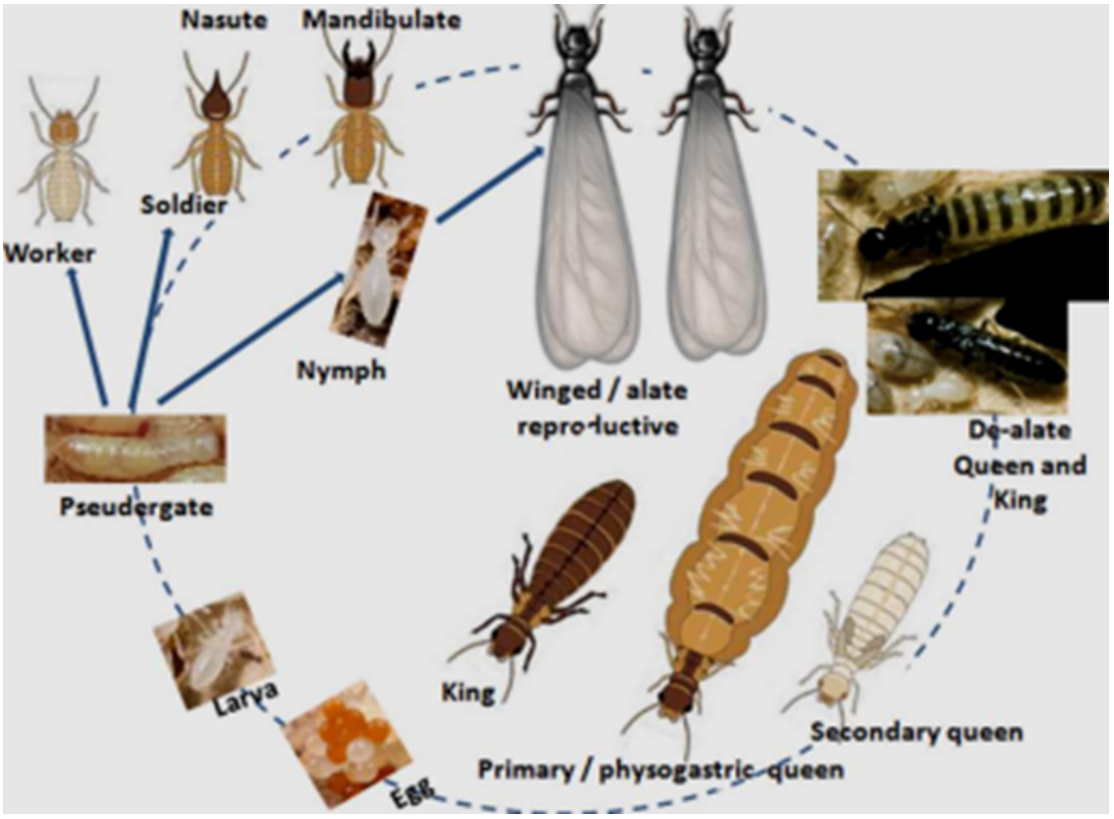


FIGURE 2.3: Lifecycle of termites [79]

The termite larva can follow one of two paths, concluding its life cycle accordingly. Its life cycle concludes with death, and it might go on to become a laborer or soldier. The larva can develop into a secondary reproductive or reproductive alate, which can then become a queen or king termite to start a new colony. The queen termite boasts the longest lifespan, averaging around 25 years. In contrast, the life spans of other termite castes range from 12 to 24 months [53].

2.5.2 Reproduction of Termites

Similar to other organisms, termites engage in reproduction to ensure the continuation of their generations. In the summer, king and queen termites congregate to create enormous groups of thousands of individuals in search of a partner [80]. After finding one other, the two partners engage in a modest wooing dance before starting their own colony. When the fertilized queen gets ready to start the process of making young termites, the male or king, helps her out. The queen capable

of laying eggs anywhere from hundreds to thousands of eggs every day during her first year of egg-laying [81].

Until there are enough young or workers in the colony, the king and queen termites share care of the colony's initial generations. The pheromones and temperatures that the eggs are exposed to determine whether the newborn termites will develop into soldiers or workers once they hatch into larvae. Workers are essential to the colony's division of labor since they are the only ones who can feed the young, developing babies, keep the peace among them, and go foraging. Because they are sterile due to their inability to procreate soldiers and workers can be either female or male [82].

For over five years, the termite colony's population grows over time. The young colony kings and queens are the first reproductive alates whom the queen will be able to give birth at this time. These reproductive alates mature and get ready to swarm, leaving the nest to establish another colony during the summer. This reproductive cycle repeats itself continuously, contributing to the ongoing expansion and establishment of new termite colonies [83].

2.5.3 Termite Mating Behavior

Termites in their adult stage engage in group flights to reproduce away from their original colony, subsequently dispersing to initiate new colonies for the purpose of laying eggs and hatching additional offspring [54]. The process of dispersion is affected by various factors such as seasonal variations, atmospheric pressure, temperature and humidity [84]. During these dispersion flights, the winged reproductive termites, known as alates, shed their wings, with the males primarily trailing the females in the process. The fundamental genetic behavior crucial to termites in the establishment of new colonies involves the formation of tandem pairings, comprised of kings and queens [84]. To initiate a new nest, the queen plays a key role in assisting the king in selecting an appropriate location [85]. Every year, hundreds of individuals are released by *R. flaviceps* from established colonies. Alates shed their wings after the dispersal flight, locate females, form

partnerships, and engage in tandem activity in order to reproduce, lay eggs, and hatch once a colony is established. The male starts the tandem behavior by keeping his hand on the lady's stomach tip [86]. The feminine assumes the lead in searching for an appropriate nesting location where both can isolate themselves and commence the formation of the initial colony. It initiates dispersal flights and undergoes maturation over an unspecified duration [87].

2.5.4 Termite Parthenogenesis

In sexual reproduction, the fusion of sperm and ovum nuclei occurs upon the completion of meiosis, triggering the activation of insect eggs [88]. In certain insect individual parthenogenesis leads to the development of eggs with in sexual reproduction, the fusion of sperm and ovum nuclei occurs upon the completion of meiosis, triggering the activation of insect eggs. In certain insect individuals, parthenogenesis leads to the development of eggs without fertilization [89]. In order to maintain regular life and activities within the colony, the survival rate of female matching is closely related to the surviving rate of male-female bonding [90]. Although parthenogenetic reproduction exhibits favorable adaptations, comparative sexual reproduction is considered advantageous, achieving comparable success despite being twice as effective [91]. The constraints on parthenogenesis stem from both genetic and developmental factors, resulting in a typically lower survival rate for its progeny compared to sexual reproduction [91].

Only a small number of Isopteran species have been shown to be parthenogenetic thus far and certain reproductive termites also employ asexual reproduction methods [92]. The mechanism of termite parthenogenesis known as asexual queen succession (AQS) is particularly unique [93]. The AQS system has been observed in various termite species including *R. verginicus, R. lucifugus, Cavitermes tuberosus,* and *Embiratermes neotenicus* [94].

In lower termites, parthenogenesis is denoted as "end fusion" while in higher termites, it is termed "central fusion" [74, 92].

The phenomenon of asexual queen succession in *R. chinensis* leads to the production of unfertilized eggs even though this species lacks the process of egg incubation [95].

Researchers investigated the early growth of fertilized and unfertilized eggs in two termite species, *R. chinensis* and *R. aculabialis*, using laser scanning and a digital microscope. They looked at exterior morphology, cleavage, and embryo development. The FF (female-female) eggs showed notable variations in size, width, volume, number of nuclei, and cleavage in 24 and 48 hours, offering insights into both modes of egg development in these two termite species [96]. Through the use of laser scanning and a digital microscope, researchers compared the embryonic development of fertile and unhatched eggs in both *R. aculabialis* and *R. chinensis*, examining external morphology, cleavage, and embryo development. Significant differences in width, size, number of nuclei, volume and cleavage in 24 to 48 hours, were observed in the FF (female-female) eggs, providing insights into both forms of egg development in these two termite species [97].

2.5.5 The hybridization of Termites

The two termite species that have genetic origins from distinct populations frequently participate in hybridization, a reproductive behavioral phenomenon [98, 99]. This process results in genetic interactions among offspring, who may inherit genes from parents belonging to different species [100]. The emergence of new economic influences in the world has coincided with this genetic variation. Some of these influences, formed through linkages between hybrids, manifest in various ways, proving ecologically compatible and successful [101].

In contrast to the parental populations, heterozygous regions generated through hybridization exhibit favorable ecological and evolutionary consequences [102]. In Southern United States an example illustrating the impact of hybridization is observed in the two invading fire ant species, *S. invicta, Solenopsis richteri* [103]. The resulting progeny from this hybridization process demonstrate dominant effects and contribute to the dispersion of these species over a wide range in certain

region [104]. In the plant kingdom in particular, the hybridization of organisms increases the likelihood of species adapting to harsh environmental conditions and surviving. Through this process, variations are produced in the offspring that provide those benefits over the parents in conditions of stress resistance, ability to grow, adaptation and viability [105]. Between the borders of adjacent populations, hybridization takes place in a variety of ecological and environmental zones, made possible by the spread of hybrid genomes and dispersal flights at common periods [106]. The resulting novel phenotypes are adapted to the native environment due to the frequent occurrence of crossbreeding in areas where territories are confined and mixed [107].

There exist gene connections among members of the local population, including parasite relationships among herbivorous arthropod [108]. This phenomenon may involve two native species or an invasive species interacting with a native species [109]. Hybridization by combining genes, increases the likelihood that parental populations will be able to adjust and adapt. It also gives rise to fresh accusers that are a mix of two ancestral generations while maintaining distinctiveness from the parental generation [110]. Reproductive isolation involves both pre- and post-zygotic barriers. Determining pre-zygotic isolation takes into account factors such as morphology, nutrition, breeding season, location and ecology [**?**]. A fair gap in several crucial criteria can prevent parents from becoming sufficiently compatible for mating and fertilization, leading to the inability to generate offspring [111]. Post-zygotic barriers can manifest in various ways, encompassing gamete isolation, developmental isolation, early embryonic death in hybrids, infertility in hybrids, and poor acclimatization and adaptation in hybrid descendants [111].

In the context of termite colonies, the contacts between soldiers and workers from established colonies were observed to be limited, with individuals displaying inter-species competition and aggression for resource access [112].

Members of alates, the winged reproductive termites, are observed to engage in competition with other species [23]. Hybridization is identified as a common outcome of cross-breeding among various species of organisms, including termites like

R. lucifugus and *Z. nevadensis* made the noteworthy observation that the dispersal flight seasons of C [113, 114].

2.5.6 Swarming

Termite colonies are commonly initiated through the dispersal of winged adults known as alates, which typically emerge from an established colony during specific times of the year. Alates stay in special chambers outside the nest for a few days or weeks after molting into winged adults. In addition to species-specific temperature, climatic conditions, and seasonal considerations, high atmospheric humidity is generally linked to the emergence and flight of alates [115]. Some species may experience only one emergence per year, while others may have multiple successive flights. Before the alates emerge, workers create escape holes, prepare surface tunnels, and occasionally construct launching platforms. During the mating flight or nuptial flight, alates (winged reproductive termites) emerge from the nest, attracted to light. Soldiers guard the exit holes to protect against adversaries and prevent alates from returning. The alates, not being strong fliers, descend near the colony, relying on wind for transportation. After landing, mating occurs, and the alates shed their wings [116]. The mated pair then travels together, with the female emitting a sex attractant. This process is crucial for the establishment of new colonies and the continuation of the termite life cycle.

After the nuptial flight, the mated pair seeks a suitable nesting location, often in soil or wood, where they construct a nuptial chamber. This chamber is sealed with a mixture of saliva and feces. Copulation occurs within this chamber, and the female stores the sperm in her spermathecal [96]. Interestingly, termite colonies typically begin with the development of worker and soldier castes from the earliest baby nymphs. This differentiation of castes is influenced by factors such as diet, environment, and the needs of the colony. It is a fascinating process that contributes to the complex social structure of termite colonies [117]. The transition from the initial care provided by reproductive to the involvement of worker and soldier castes is a crucial aspect of termite colony dynamics. The variation in colony sizes among different termite groups, from small primitive colonies to large

and highly populous colonies in more developed families, highlights the diversity within the order Isoptera [118].

2.6 Cellulose

Being the primary element of plant cell walls, cellulose is acknowledged as the most abundantly useable bio resource in the world [117]. This polysaccharide based on glucose has glycosidic linkages. Lignin and Hemicelluloses contribute 5–30% and 20–35% of the dry weight of the plant, to the composition of plant cells, with cellulose making up 35–50% of the total [119]. Together with three other cellulases, the high-capacity enzyme cellulase hydrolyzes cellulose into glucose. Three types of cellulase exist: β-D-glucosidase, endo-1,4-glucanase, and exoglucanase [119].

During their development on cellulose, bacteria create the enzyme cellulase, which aids in the digestion of cellulose. To fully break down cellulose into glucose, cellobiohydrolases, (also known as exo-glucanases) endo-glucanases and β-glucosidases must work together [120]. Random attacks by endoglucanases are made on different internal sites inside the amorphous area of cellulose fibers. Future targets for exoglucanases to attack are made visible by endoglucanases' work on cellulose. Cellobiose and oligosaccharides are formed at the reducing and non-reducing ends of the glucose chain by eliminating mono and dimers. The enzyme β-glucosidase converts cellobiose to glucose monomers. After that, glucose is moved across the membrane to take part in metabolic processes that produce energy [119]. Carboxymethyl cellulose (CMC) has been widely used for research purposes in the study of gut bacteria that produce endo-1,4-glucanase. Applications for cellulase have grown in a number of industries, making it the third most popular enzyme for industrial use worldwide [121]. It is anticipated that in the future, cellulase will be the most abundant industrial enzyme. The increasing awareness of cellulase's adaptability and efficiency in industrial processes is reflected in its rise in significance [122].

2.7 Evolution of Symbiotic Digestion

The general consensus is that termites descended from a sub social wood-feeding progenitor during the cockroach radiation [123]. According to recent molecular phylogenies, termites and the blattid cockroaches, split at least 150–170 million years ago during the Middle Jurassic [124]. The development of flagellates with cellulolysis by a common ancestor of termites (Isoptera) and its sister group, the Cryptocercidae (all assertions regarding lower termites), was a pivotal moment in termite evolution. It is thought that this acquisition has greatly improved their ability to digest lignocellulose [125]. Proctodeal trophallaxis developed to stabilize the symbiotic interaction between termites and flagellates. This characteristic makes sure that flagellates are reliably transferred between nest mates and between generations [41]. Proctodeal trophallaxis is a crucial element of the complex social behavior observed in termites. This intricate social behavior has its roots in long-lasting parental care. *Cryptocercidae* has evolved over time, culminating in an elaborate caste system and the cooperative sharing of labor among termites [125]. The flagellates make up the dual cellulolytic system of lower termites, working in tandem with the host's natural cellulolytic capabilities, which are present throughout the whole blattodean lineage [119]. When compared to other xylophagous and detritivorous cockroaches, this dual cellulolytic system is a far more effective mechanism for the lignocellulose symbiotic digestion. The synergistic action of host cellulolytic activities and flagellates enhances the termite's capacity for breaking down complex plant materials [126].

2.8 Gut Habitat of Termites

Termites' digestive tracts are tiny ecosystems made up of a variety of microhabitats that differ greatly in their biotic and abiotic surroundings. While some environmental traits are innate to the gut, others are the result of the host's physiological processes or the microbial inhabitants of particular areas. As the host has evolved, so too have the kinds of environments that are favorable for microbial colonization.

This dynamic and intricate ecosystem within the termite gut plays a crucial role in the symbiotic relationships and digestive processes crucial for termite survival.

2.9 Gut Structure

Cockroaches and termites have similar basic gut structures (Figure 2.4). Meal is transported by the foregut from the mouth to a large crop, where salivary gland secretions are used to incubate the meal. The meal enters the midgut for digestion by midgut-secreted enzymes after being further ground by the gizzard [127]. The gastric ceca and midgut epithelium then reabsorb the products of digestion. A large amount of microbes found in the hindgut (proctodaeum), which is made up of the colon (P3 and P4), rectum (P5), short ileum (P1) and enteric valve (P2). Feces are the byproducts of digestion that are produced after water and ions are removed [127]. This complicated digestive system demonstrates how the termite gut and its microbiota work together to break down complex plant sources. Termites differ from cockroaches in certain aspects of their digestive architecture. Termites have a crop that is comparable to cockroaches in size, a shorter midgut, and either a decreased or nonexistent ceca. The anterior colon (P3) of lower termites dilates significantly and forms a single, massive paunch. In cockroaches, P3 may already have some enlargement [128].

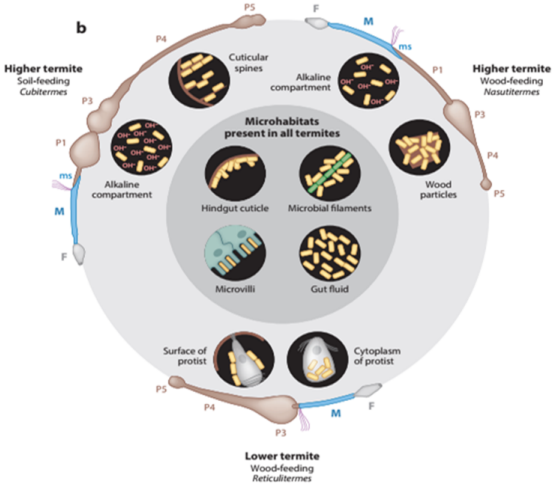


FIGURE 2.4: Structure of Termite Gut and function of different microbiome [127]

Higher termites progressively stretch and distinguish the hindgut into many proctodeal compartments, with the exception of Macrotermitinae. These adaptations in the digestive structure of termites are indicative of their specialized feeding habits and symbiotic relationships with gut microbiota [14].

2.10 Microhabitats

Microorganisms must swim quickly or attach themselves to longer-lasting intestinal particles than the watery element in order to prevent washout caused by the digesta's fast passage through the termite gut. Due of their great motility, the stomach dilations of lower termites are able to actively hold onto place within stomach. There are some flagellates that have organelles that can connect to the intestinal wall's cuticle [62]. Since flagellates make up the majority of the hindgut volume, prokaryotes in the hindgut of lesser termite's colonies the surface, cytoplasm, and even the nucleus of these protists. This arrangement within the gut showcases the intricate relationships and adaptations that have evolved to optimize lignocellulose digestion in termites [129].

The flagellate habitats last longer than a single termite because of the transmission of stomach contents between nest mates. The gut lumen itself is not a good microhabitat for bacterial cells, with the exception of large spirochetal forms that can move swiftly enough to actively retain their position. The retention duration of wood particles in higher termites that feed on wood is longer than that of the stomach fluid. Same processes observed in work of soil feeders, as tiny clay particles that are high in organic matter are held in place for a longer period of time than huge sand grains [130]. These adaptations in gut dynamics underscore the cooperative nature of termite colonies and the optimization of microhabitat conditions for efficient lignocellulose digestion.

Based on DNA content, it has been calculated that the fiber fraction is closely linked to roughly One third of the microbial biomass in the hindgut paunch (P3) of a species of Nasutitermes that feeds on wood [131]. Only the midgut of insects is endodermal, granting bacteria direct access to the peritrophic membrane-protected

epithelial surfaces. There have been reports of bacterial cell associations with the space ectoperitrophic of the mixed segment [132]. These associations highlight the complex interactions between gut microbiota and the termite digestive system.

Termites have ectodermal origins for both their foregut and hindgut, which are always covered in cuticle [131]. Acetate and other short-chain fatty acids may be more permeable through the unique pits or holes in the hindgut area's cuticle. The cuticle has numerous surfaces and is typically heavily covered with a biofilm of microorganisms [133]. Cuticle spines in the P4 compartment of specific higher termites provide more places for the microbiota to attach [134]. Insects replace all of their cuticles during ecdysis, or the molting process, which means that the hindgut must be recolonized after every molt. The termite gut microbiota's dynamic nature and ongoing adaptability to the host's digestive environment are highlighted by this cyclical process [135].

2.11 Microbiota

2.11.1 Termite Gut Fllagelates

The phylum Parabasalia is home to the bulk of termite gut flagellates [14]. Three of the six classes of parabasalids, known as hypermastigids, are restricted to the lower termites' digestive tracts. A probable adaptation to the termite diet and the microbial environment in the termite gut, these flagellates' considerable motility, supported by many flagella, prevents washout. They are large enough to phagocytize wood particles [136]. Generally smaller, the ancestral *Trichomonadea* consume dissolved nutrients or bacteria and are also seen in a variety of environments. The same evolutionary pressure most likely led to the increased size of cell seen in some lineages [137].

All species do not show this association, several lower termite species carry flagellates of the phylum *Preaxostyla*, order *Oxymonadida*. Within this group, certain lineages have evolved unique holdfasts that adhere to the cells hindgut and cuticle themselves can be so tiny that they vanish completely inside the biofilm of bacteria

[138]. The variety of symbiotic connections and the coevolution of termites and their gut bacteria are demonstrated by these adaptations.

Numerous research demonstrated that the termite gut flagellates' diversity exceeds first predictions, as demonstrated by molecular investigations [139]. Detailed phylogenetic and ultrastructural characterizations are still pending for several species. New lineages are still being uncovered by ongoing research and even morphospecies that seem to be similar at first glance have been found to include distinct phylotypes [140]. This emphasizes the idea that every species of termite has a different parasite. Using amplicon sequencing, early attempts to evaluate the diversity and community structure of termite gut flagellates have shown that universal primer sets and an enhanced phylogenetic framework are required [141]. The evolving understanding of termite gut flagellates emphasizes the complexity and richness of these symbiotic relationships [142].

2.11.2 Bacteria

Termites have a small number of dominating phyla in their bacterial gut microbiota and there is significant difference among the main host groups. libraries of Clone of 16S rRNA genes have been crucial in the last 20 years in giving a wealth of data on the diversity of bacteria found in different termite guts [143]. Recent studies have expanded this knowledge to include termite genera that were previously underrepresented [144]. A lot of the libraries were quite tiny, especially the ones from earlier investigations, leading to a severe under-sampling of gut community diversity [145].

These constraints have been overcome by the development of next-generation sequencing technologies, which have made it possible to conduct a more thorough analysis of the variations in community structure among a variety of termite species [141]. These technologies have made it easier to investigate differences between members of the same species that were collected from colonies that were geographically apart or that were fed different [146]. Researchers have explored differences

between various gut compartments or luminal fractions. The utilization of cutting-edge sequencing techniques has greatly improved our comprehension of the variety and dynamics of the termite gut microbiota's bacteria [147].

Within every termite gut community, *spirochaetes* are unique individuals [148]. They include a variety of monophyletic groupings of lineages exclusive to termites and show a high degree of phylogenetic diversity [144]. The number of distinct lineages within this group varies depending on the host group [149]. These lineages can exist as free-swimming cells or attach to the fiber fraction the surface of flagellates or both Spirochaetes could stand for many functional guilds [150]. Wood-feeding termites typically have the highest proportion of spirochetes, whereas fungus-cultivating and humus-feeding termites generally exhibit lower numbers [150].

Termites that cultivate fungus are rich in Bacteroidetes which helps explain why termites and cockroaches have similar gut microbiomes [145]. The distinct roles played by these bacterial groups in different termite species highlight the intricate relationships between the host's diet, gut physiology, and the composition of the microbial community [151].

Since they are also present in the guts of mammals, several of the dominating taxa, including Alistipes, Dysgonomonas, Paludibacter, and Parabacteroides, which are frequently recovered from termite guts show a general affinity for intestinal environments [152]. Many family-level cliques are made up only of members found in termites and cockroaches [153]. Firmicutes comprise both common gut bacteria (Lachnospiraceae and Ruminococcaceae) and very particular lineages linked to the alkaline gut compartments in higher termites" Candidatus Arthromitus" [154]. In comparison to other termite groups, macrotermitinae and cockroaches have higher abundances of protobacteria. All host groups contain Deltaproteobacteria strains linked to flagellates, as well as a deep-branching clade (the Rs-K70 cluster) and several Desulfovibrio-related lineages. The distinct distribution of these bacterial groups across termite host groups highlights the host-specific nature of certain microbial lineages in termite guts [155].

2.11.3 Archea

Methanomicrobiales, *Methanosarcinales*, *Methanobacteriales* and a deep-branching clade distantly related to the nonmethanogenic. *Thermoplasmatales* are the four main lineages of *Euryarchaeota* found in termite guts [156]. After the first isolate of the order, the latter was once known as Methanoplasmatales called by Methanomassiliicoccales. A novel mechanism of energy metabolism has been identified in all members of this lineage by comparative genomic analysis of strains from the human gut and "Candidatus Methanoplasma termitum," a highly enriched culture from the gut of a higher termite [157]. The highest variety of archaea is found in the subfamily *Termitinae* of higher termites, especially in the lineages who feeds in soil that support different archaeal communities thought to be made up of methylotrophic and hydrogenotrophic populations in each hindgut compartment [155]. The variation in archaeal communities across termite species and their specific hindgut compartments reflects the intricate relationships between the host's diet, gut physiology, and microbial community composition.

Methanobrevibacter species predominate in archaeal communities in lower termites [141]. More variety has been revealed by a survey with the help of pyrotag libraries than was previously indicated by earlier clone-based research [158]. This increased variety includes an uncultivated Thaumarchaeota lineage seen in termites that eat on soil. The use of more advanced sequencing techniques has provided a more comprehensive understanding of the archaeal communities present in lower termites highlighting the importance of continued advancements in microbial profiling methodologies [159].

2.12 Functional Amplifications of Microbiome

2.12.1 Hydrogen Metabolism

In fermentative processes, hydrogen is an essential intermediate that frequently builds up to high amounts. It is released in lower termites when the cellulolytic

flagellates oxidize polysaccharides to acetate and CO_2 [160]. We still don't know exactly which major fermenters in higher termites produce hydrogen. Rather than being the result of insufficient production, low hydrogen concentrations observed in some termite species are likely the result of closely coupled processes that make and use hydrogen [161]. During reductive acetogenesis from CO_2 a considerable amount of the hydrogen generated in the stomach of termites who feeds on wood, is transformed into extra acetate [14]. The regulation of hydrogen levels in the termite gut involves a complex interplay between microbial processes and host-microbe interactions, contributing to the unique metabolic dynamics within the termite digestive system [162].

Various isolates from both higher and lower termite species have demonstrated the capability for reductive acetogenesis. Assessments of functional genes such as fhs and coo, which participate in the Wood-Ljungdahl pathway imply that different populations of homoacetogenic spirochetes are present in both lower and higher termite species [147]. It is unclear, exactly what part the various FeFe hydrogenases (hyd) in the gut microbiota play in the synthesis and utilization of hydrogen. The ability of closely related Treponema cluster isolates to undergo reductive acetogenesis may differ [163]. It is not always the case that all distinct lineages of termite gut treponemes are homoacetogenic. The intricacies of these microbial metabolic pathways contribute to the overall dynamics of hydrogen metabolism within the termite digestive system [164].

Although in the gut microbiota of termites hydrogenotrophic methanogenesis is a distinctive mechanism, it usually has little bearing on wood-feeding species. Given that methanogens are found near the periphery of the hindgut their hydrogen limitation is most likely the cause of this [165]. It is yet unknown what causes the significantly higher methane emissions observed in termites that consume fungus and dirt. This phenomenon may be caused by the spatial organization of the methanogenic communities, which include large populations of lineages with obligately methylotrophic representatives [157]. The methanogens of lower termites that have abnormally high methane outputs are linked to flagellates found in the

gut center, which is rich in hydrogen .The complex interplay of microbial interactions and spatial organization within the termite gut contributes to variations in methane production across different termite species and dietary preferences [166].

2.12.2 Nitrogen Metabolism

The growth of termites that feed on wood is significantly hampered by the low nitrogen concentration of lignocellulose. The mechanisms of nitrogen fixation, recycling, and upgrading depend heavily on the hindgut microbiota [167]. The hindgut produces microbial fermentation products, which the host epithelia directly ingest. Digestion enzymes in the midgut can only access the biomass subsequent to proctodeal trophallaxis. The only way an insect may meet its requirements for critical vitamins and amino acids digesting microbial biomass in the midgut. This is true even for the termites who feeds on dung and humivorous species, where nitrogenous dietary ingredients are mineralized, resulting in a net production of ammonia in the process of digestion [168].This intricate process highlights the symbiotic relationship within termites and their microbiota of gut in overcoming nutritional challenges associated with their dietary habits.

A wide range of diazotrophic capacity in termite gut communities is suggested by the variety of same nifH gene, a functional marker for nitrogen fixation [169]. Symbionts of flagellates appear to be important for activities related to nitrogen fixation and/or upgrading in lower termites [170]. It is yet unknown which microorganisms in higher termites that feed on wood are causing the high rates of nitrogen fixation [168]. Fixation of nitrogen is a crucial aspect of termite gut ecology, allowing these insects to overcome the challenges posed by the low nitrogen content in their wood-based diets [162].

2.12.3 Polymer Degradation

Depolymerizing resistant plant fiber is the main job of the termite hindgut microbiota. The hindgut flagellates of lower termites generate a wide variety of glycoside

hydrolases that facilitate the effective degradation of wood [171]. This include a variety of hemicelluloses, such as xylanases, arabinosidases, and mannosidases, as well as other cellulases, such as exoglucanases and endoglucanases [172]. Recent research has identified the protist community as a significant source of chitinase activity in the hindgut of *Zootermopsis angusticollis* [173]. It's worth noting that hydrolysis occurs within the digestive vacuoles of flagellates, sequestering wood particles and potentially limiting power of luminal bacteria to the generated sugars during the depolymerization process. According to metabolomics research, lower termites' hindgut bacteria significantly contribute to the breakdown of cellodextrins [145]. Since flagellates are lacking from higher termites like Macrotermitinae, other methods of digesting fiber are used. Macrotermitinae termites metabolize wood with the aid of *Termitomyces spp.*, a fungus that breaks down lignin. In contrast to lesser termites, this fungus is grown in fungus gardens inside the nests and is not a component of the gut microbiota [144]. Older combs, which contain fungal biomass and partially digested lignocellulose, are harvested by termites. Different genera of termites that cultivate fungi have different compositions of ingested material, which influences the composition of their bacterial microbiota [122]. Since the fungal comb cannot fully breakdown fiber, further digestion takes place in the gut. Metagenomic analyses of several fungus-cultivating termite species reveal that gut bacteria, particularly those belonging to the Bacteroidetes, encode numerous glycosyl hydrolases involved in breaking down polysaccharides found in plant and fungal cell walls [174]. Different techniques have evolved in higher termites, notably in various subfamilies, to make use of partially humified lignocellulose as a nutritional resource. The breakdown of wood and plant litter, as well as the excrement left behind by herbivorous mammals, are common precursors to the humification process [162]. The amount of cellulose continuously decreases during humification, while the proportion of nitrogenous compounds and complex polysaccharides made from microbial biomass increases [144]. Termites that feed on wood and those that feed on dung differ significantly in terms of their community structures and the quantity of different glycoside hydrolase families [141]. Since they make up a sizable portion of soil organic matter are probably a key source of food for real soil feeders. This may explain the prevalence of Firmicutes

in the gut microbiota of termites that are soil-feeding [175].

2.12.4 Oxygen Consumption

Due to their small size, termites' hindgut walls allow for a large intake of oxygen [176]. Therefore, it is not surprising that purely aerobic and facultative bacteria were isolated from guts of termite. The microaerophilic bacteria that colonize the hindgut wall and oxidize acetate to the hypoxic environments seen in the peripheral areas of hindgut [162]. As demonstrated by the metabolic transition during lactate metabolism from propionate to acetate, for fermenting bacteria, oxygen acts as an electron sink [157].

Hydrogen can be used as a reductant by methanogens that invade the hindgut wall, to removal of oxygen. other useful fermentation products and acetate are produced in greater quantities thanks to this technique [177]. The microbial community in the termite's hindgut benefits from the elimination of hydrogen with oxygen. The oxidative metabolism of aromatic chemicals requires oxygen as a cosubstrate and genome sequences have shown that anaerobic spirochetes found in termite guts have latent oxygenase activity [175].

2.12.5 Environmental Factors

Both the biotic and abiotic environments have an impact on the physicochemical terms in various gut parts. Oxygen enters in the gut through the epithelia of the host on a constant basis, the gut microbiota effectively removes oxygen, leaving an anoxic environment at the core of all dilated hindgut parts [162]. It is imperative to bear in mind that, because to the small size of termite guts, diffusive transport of metabolites along steep radial concentration gradients is more significant than axial transport and convective mixing by peristalsis. Additionally, the activity of the flagellates has little effect on this process [173].

Termites' and other insects' enormous surface-to-volume ratios, when compared to the larger guts of most vertebrates, enhance the importance of aerobic processes and facilitate in the gaseous exchange and dissolved products of microbial metabolism. Different microhabitats have varying redox potentials due to changes in gut pH, oxygen status, and the generation of redox-active chemicals like hydrogen [178].

The microbiota of the various termite gut compartments is significantly influenced by the secretions of the hosts. Saliva and midgut secretions include digestive enzymes that provide sugars or amino acids as substrates for the local bacteria in the anterior gut. These enzymes do, however, also aid in the breakdown of microbial biomass [179]. The anterior gut can serve as a barrier to prevent diseases or foreign microbes from colonizing. It is uncertain how the hindgut microbiota that travels to the nest mates by proctodeal trophallaxis avoids being broken down by the gizzard, which may mechanically disrupt the flagellates [180].

The high level of alkalinity found in the anterior hindgut of termites who feed on soil is probably going to have an impact on the survival of transitory microbiota and could favor lineages that are suited for this particular environment [15].

Chapter 3

Materials and Method

3.1 Methodology Flowchart

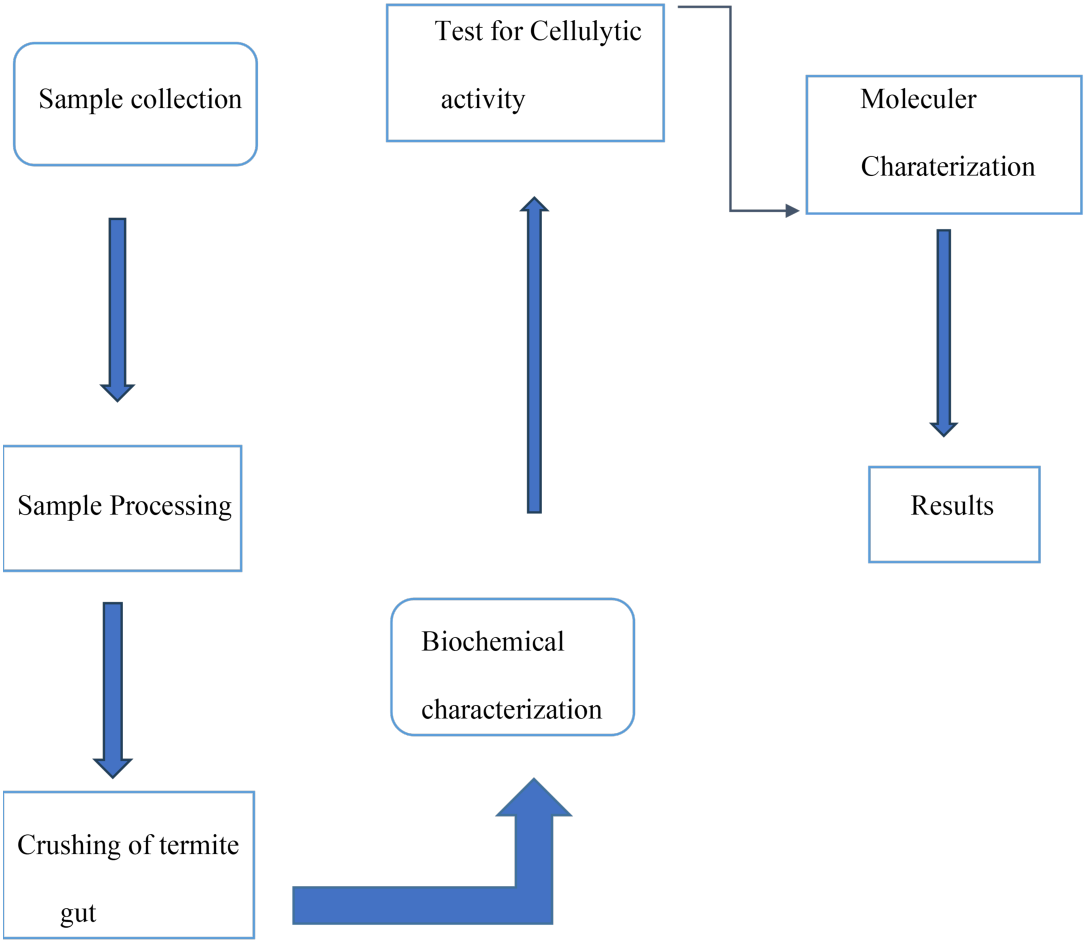


FIGURE 3.1: Proposed Methodology

3.2 List of Equipment

Laminar flow hood, weighing balance, Auto clave, Incubator, vortex, sample storage bottles, PCR thermocycler, Microscope, Grinder, Shaker, centrifuge

3.3 List of Apparatus

Petri dishes, micro-pipette, glass rods, forceps, spreader, reagent bottles, spatula, microscopic slides, spirit lamp, micro-pipette tips, filter paper, para-film tape, measuring cylinders, gloves, test tubes.

3.4 List of Chemicals

CMC (Carboxymethyl Cellulose) agar, Nutrient broth Agar, 0.1% Congo red reagent, NaCl (Sodium Chloride), 0.5% Phenol red reagent, Yeast Extract, 70% Alchohol, Ethanol, BHI (Brain Heart Infusion), Crystal violet, Gram iodide, Decolorizing solution, Safranin, Distilled water, 100% Ethanol, TBE (Tris-borate-EDTA) buffer, Alpha-naphthol, 40% KOH (Potassium Hydroxide), TSI (triple, suger, iron) agar, Ethedium Bromide.

3.5 Sample Collection

Termites were collected from different locations of Tehsil Khuiratta, District Kotli, Azad Kashmir, Pakistan. The specified location is situated at an elevation of 801.09 meters (2628.25 feet) above sea level. Termites were collected from tree trunks by scratching and from soil mounds in small glass jars. Sample were brought into the lab and stored into the refrigerator at -4^{o}C. Samples were labeled as mentioned in the table.

TABLE 3.1: Tags used for labeling of termite sample.

No.	Samples	Source	Sample ID
1	Sample 1	Tree trunk	J1
2	Sample 2	Tree trunk	J2
3	Sample 3	Tree trunk	J3
4	Sample 4	Soil	L
5	Sample 5	Tree trunk+Soil	M

3.6 Sample Processing and Culturing

3.6.1 Extraction From Termites

Termites were sterile through a process involving washing with 70% alcohol. Following the removal of each termite's head using forceps, the bodies were individually separated. The bodies were crushed, and the resulting paste from the gut of termite was utilized for isolation of bacteria.

3.6.2 Media Used for Culture

Nutrient Broth agar, consisting of Agar (15 g/l) and Nutrient Broth (9 g/l) was inoculated with the crushed termites. After the growth of bacteria in nutrient broth series of serial dilutions (10^2-10^6) were prepared to reduce the density of the bacterial culture before inoculation onto the media. Serial dilution with bacteria were immunised on Nutrient agar that was chosen for the general bacterial isolation.

3.6.3 Culturing on BHI (Brain, Heart, infusion)

A loop full of bacteria picked from the bacterial colonies on the agar and was streaked on the plates with the BHI (Brain, Heart, Infusion) media.

3.6.4 Culturing on CMC (Carboxymethyal Cellulose)

CMC media were used specifically to promote the growth cellulolytic bacteria. CMC media, composed of MgSO4. NaCl (0.23 g/100 ml), 7H2O (0.05 g/100 mL), Yeast extract (0.2 g/100 ml), Na2HPO4.2H2O (0.5 g/100 ml). CMC was mimic cellulose and the microorganisms were tested for their ability to break it down. Strains were streaked onto the CMC agar plates. Loop full of bacterial colonies were picked from agar and was streaked onto the CMC agar plates. The immunised plates were then incubated for 48 hours at 37^{o}C. This allowed the microorganisms to grow and potentially produce cellulolytic enzymes.

3.7 Screening of Cellulolytic Bacteria

Strains were once more streaked onto petri plates that had been prepared with 1% CMC agar. After that, these petri plates were incubated for at 37^{o}C 48 hours. After being incubated for a period of time, 0.1% Congo red reagent were used to flood the petri plates and allowed to stand for 20 minutes. First, 1M NaCl was used to wash the plates. The presence of cleared zones, often called halo zones, which contrasted with the Congo reagent's red color indicated favorable test results. The ratio of the colony's diameter to the total of its diameter plus the clear zone around it served as a measure of the enzyme activity. For additional analysis, only isolates exhibiting the creation of a distinct zone surrounding the colony were chosen.

3.8 Identification of Cellulolytic Isolates

A battery of routine biochemical and physical testing was used to identify each isolate. Tests for Gram and Endospore staining, motility, the methyl red (MR) test, Voges-Proskauer (VP) test, oxidase and catalase activities, TSI (triple, sugar, iron) and urease activity were among those conducted. The use of various carbon sources, including D-dextrose, D-sucrose and D-lactose, was part of the evaluation

of carbon usage. Every test was run in the proper medium at 28^{o}C. The protocols adhered to normal operating practices, and Bergey's Manual of Systematic Bacteriology was used. For every test, cultures that were between 24 and 48 hours old were used.

3.8.1 Gram Staining

To distinguish between various bacteria according to the properties of their cell walls, gram staining was used. The bacterial sample was placed on a slide and heated gently to create a heat-fixed smear. One drop of crystal violet was poured onto the smear for one minute after two drops of water were added to the slide. Following that, surplus strains were removed from the smear by washing it with distilled water. The slide was covered with gram iodine and left for a minute. The water was then rubbed off the slide once more. One or two drops of the decolorizing solution were applied to the slide to decolorize it. After applying safranin as a counterstain, the slide was left to stand for a minute. After rinsing the excess safranin with water, the slide was let to dry naturally. After then, the slides were studied under a microscope. The procedure was repeated with additional isolates.

3.8.2 Biochemical Description

3.8.2.1 Oxidase Test

The oxidase test moistened strips was used. A small amount of bacterial growth was transferred to the moistened paper. If the microbe has cytochrome c oxidase, then enzyme catalyzes the addition of electrons to the reagent. The reagent changes its color from colorless to a deep indigo blue within a short time frame, typically 10-20 seconds. The color change indicates the presence of cytochrome-c oxidase in the tested microorganism. This test is particularly useful in differentiating between oxidase-positive and oxidase-negative bacteria and is often employed in the identification of certain bacterial species.

3.8.2.2 Catalase Test

The purpose of this test was to find out if the isolated strains could break down the hydrogen peroxide. The experiment was conducted using a glass slide on which an inoculating loop was used to add a bacterial colony. After that, a 3% hydrogen peroxide drop was put to the slide, and the production of bubbles within 30 seconds was carefully monitored.

3.8.2.3 Voges-Proskauer Test

The test was used to conform the production of acetoin by bacteria. The test helped in differentiating between bacterial strains based on their ability to ferment glucose. Isolates were taken from the pure culture. After incubating aerobically for 24 hours, at 37^oC temperature, 2ml of broth was taken into the test tubes. After re-incubating remaining broth for 24 hours, two drops of 5% Alpha-Naphthol were added. Then added two drops of 40% potassium hydroxide (KOH) was added. The tubes were then gently mixed after each reagent addition. A favorable reaction was demonstrated by the development of a red color, showing the production of acetoin and suggesting a particular metabolic pathway in the bacteria.

3.8.2.4 Motility Test

This test was valuable in differentiating bacterial species based on their motility characteristics. An isolated colony was taken and inserted into the medium in test tubes using a sterile needle. The inoculated tube was then incubated for 18 hours at 35^oC until visible growth was obvious. The incubation period allowed the bacteria to grow and move away from the stab line, creating a visible pattern. After incubation, the tube was observed for bacterial growth and motility. Motile bacteria were often showed a diffuse, cloudy growth pattern radiating outward from the stab line. Non-motile bacteria were typically only grow along the stab line. The use of semi-solid agar allows for the observation of bacterial motility. Negative test is characterized by the red growth restricted to inoculation line without further extension.

3.8.2.5 Urease Test

The urease test was designed to detect microorganisms that could hydrolyze urea by using the urease enzyme. To distinguish the genus Proteus from other intestinal bacteria, this test was widely used. A discernible color shift in the test indication signifies the breakdown of urea caused by the bacterial urease activity, which raises ph. Within 15 minutes to 24 hours, a vivid magenta to brilliant pink color developed, indicating a successful outcome. In a negative test, there is no discernible color shift.

3.9 Molecular Characterization Using 16S rRNA

The 16s rRNA sequencing procedure was used for identification and classification of isolated strains at molecular level. This procedure involved the extraction of DNA ,sequencing of the 16s rRNA gene and amplification, which was conserved region found in prokaryotic bacteria.

3.10 DNA Extraction

Following protocol was used for the isolation of microbial DNA from the sample. A loop full of bacterial colony was added in the Eppendorf tube with the reagent A. After that reagent B and reagent C were added and incubated for 1 hour at 95^{o}C. Reagent D was added the tube and centrifuge for 10 minutes at 13000 rpm.

Upper aqueous layer was removed and added into the new tube. Ice chilled Reagent D was added in it and incubated for 20 minutes at room temperature and spun at 13000 rpm for 13 minutes. After discarding the supernatant, the pellet was mixed with reagent F. The resulting suspension for 5 minutes was spun at 8000 rpm. The pellet was air dried after the supernatant was discarded. Incubation was done after adding the reagent G at 600^{o}C for 30 minutes. Purified sample was stored at -20^{o}C.

3.11 Polymerase Chain Reaction (PCR) Amplification

Primers were optimized for the annealing temperature through gradient pcr method. Pre designed primers were selected.

Table 3.2: Primer Selected

Name	**Forward Primer**	**Reverse Primer**	**T(a)**	**Product size**
16s	CCTAYGGG RBG-CASCA	GGACTACNNG GGTATCTAAT	57^o	465bp

3.12 Reaction Mixture

Table 3.3: Detail of Reaction Mixture

S. No.	**Reaction Mixture**	**Volume**
01	PCR water	4 μL
02	Reverse primer	2 μL
03	Forward primer	2 μL
04	Master mix	10 μL
05	Template	2 μL
	Total volume	20 μL

Selected primers were those who had the conserved regions of 16S rRNA gene. (For example universal primer 27F and 1492R).

3.13 Gel Electrophoreses

Purified PCR products were run on Agarose gel. 30ml of TBE (Tris, Borat, EDTA) buffer added with 1% Agarose gel mixed well and boiled in microwave till gel temperature lowered to 35-40. Then Ethidium Bromide 5 μL were added for

the visibility of the bands. Ethidium was added into a comb shaped. 5 μL PCR purified samples are then loaded on the gel and after that control or 2 μL loading dye added in it.

FIGURE 3.2: Gel Electrophoresis (Bands of bacterial isolates)

3.14 16S rRNA Sequencing

The high-throughput technique employed in the earliest stages of studying microbial ecology involves utilizing the '16S rRNA sequence, which is known for its high conservation. This cost-effective approach is applied to survey bacterial communities. To identify the microbiota associated with probiotic bacteria, preserved and purified PCR strains were subjected to 16S rRNA sequencing.

3.14.1 Sequence Analysis

A tool for aligning a sequence with a reference sequence and determining the similarity index based on matches, mismatches, and gaps is the NCBI's Basic Local Alignment Search Tool (BLAST).

Chapter 4

Results

4.1 Culturing and Isolation of Strains

4.1.1 Serial Dilutions

The procedure involved crushing the bodies of the termites, and dilutions were then prepared. The resulting paste from the termite gut was utilized for bacterial isolation, lowering the initial dense culture of cells to a more manageable concentration before inoculating into the media by a series of consecutive dilutions.

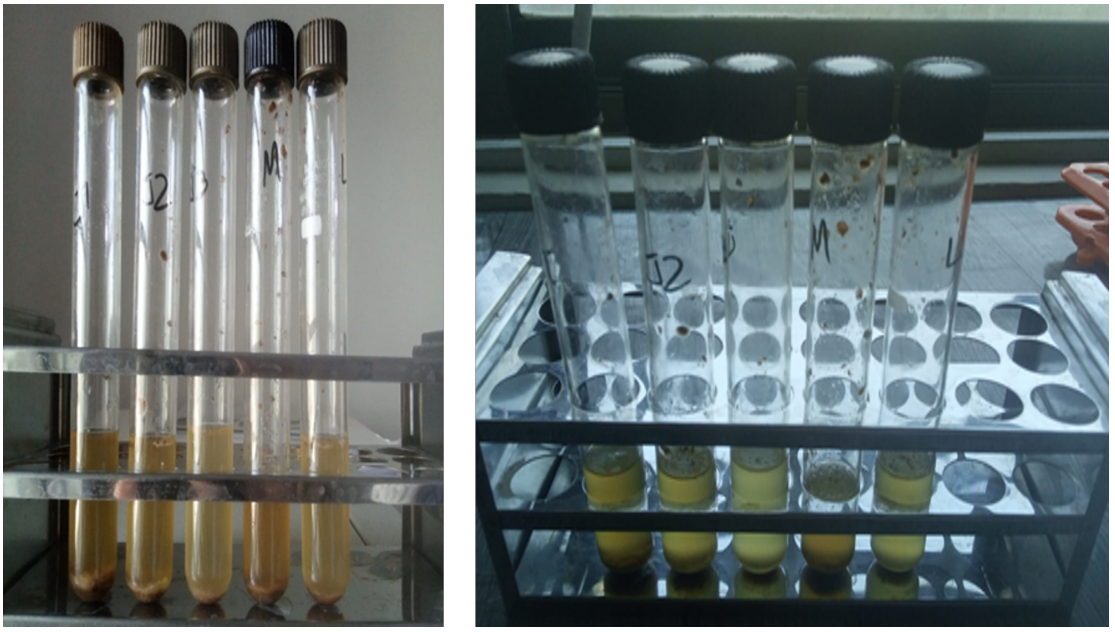
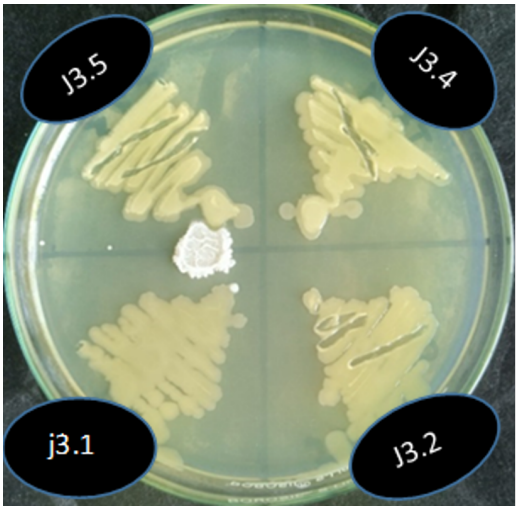


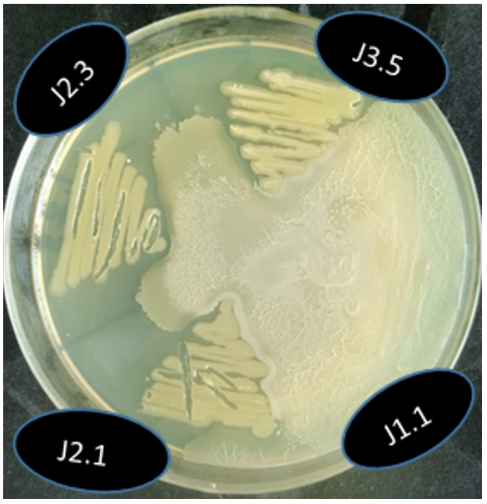
FIGURE 4.1: Serial Dilution of Termite samples

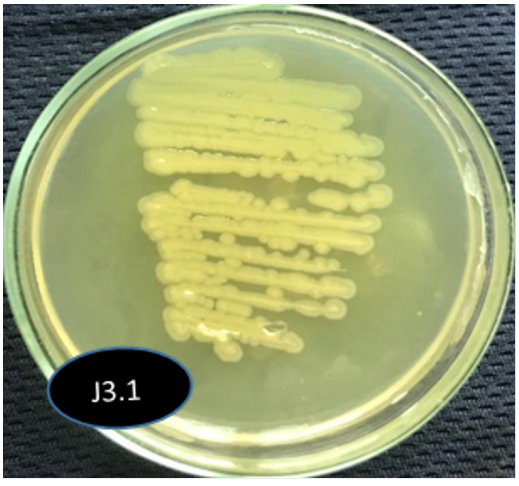
4.1.2 Isolation on Nutrient Agar Medium

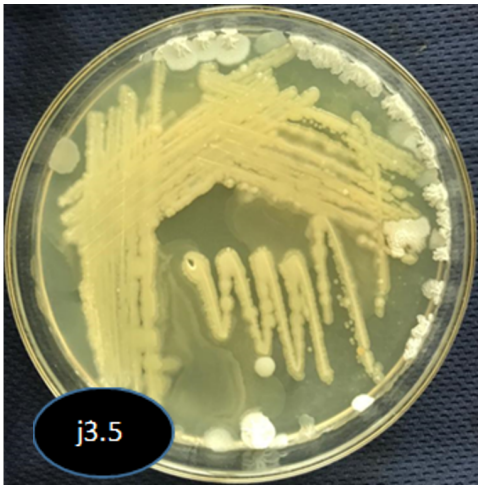
The samples collected from the termite mounds on soil or wood trees infected with termites showed the growth of different bacterial strain as shown in figure 4.2. Each sample was replicated five times.

The colonies from the two samples J2 and J3 were streaked on the nutrient agar medium. They showed growth on the medium. Two samples from J2 (j2.1 and j2.3) and four samples from the sample J3 (j3.4, j3.1, j3.3, j3.2) were showed growth.









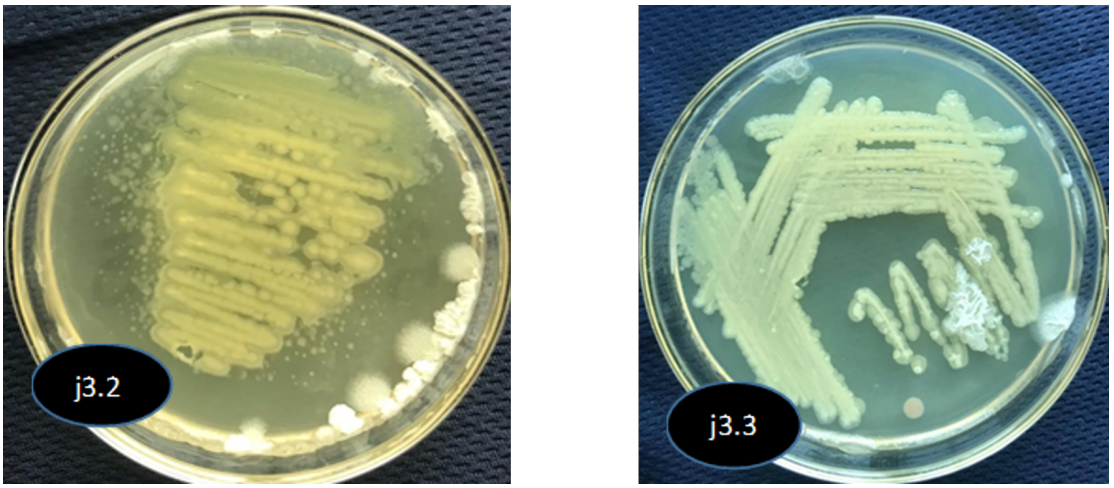


FIGURE 4.2: Microbe purification of both samples

4.1.3 Culturing on CMC Agar Media

The utilization of CMC agar enables the identification of isolates exhibiting cellulase activity on soluble cellulose. This method primarily reflects the presence of endoglucanase and beta-glucosidase activities. CMC showed growth of bacterial colonies. Out of four samples J_1, J_2, J_3 & L, two samples J_2 & J_3 showed growth. The two samples J2 and J3 were used for the further processing and remaining were not further processed.

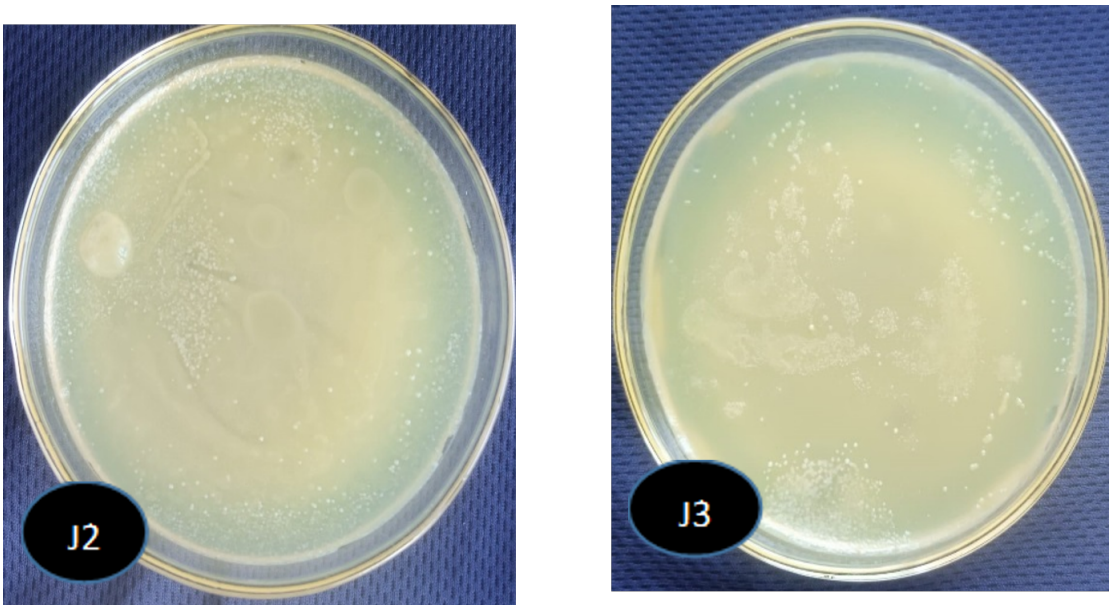


FIGURE 4.3: Growth of different bacterial strains on the nutrient agar media

4.1.4 Screening Tests for Cellulolytic Activity

After preparing carboxymethyl cellulose (CMC) agar plates, strains were streaked over them. After that, the Petri plates were put for 48 hours' incubation. The Petri plates were inoculated with Congo Red reagent after incubation and for 20 minutes they kept undisturbed. Following a 1M NaCl wash, the clearance zones also known as halo zones became visible against the crimson hue that Congo crimson had added Enzyme activity was measured for favorable outcomes by dividing the colony's diameter by the colony's diameter plus the clean zone surrounding it. For additional research, The isolates that showed a clear zone encircling the colony were the only ones selected.

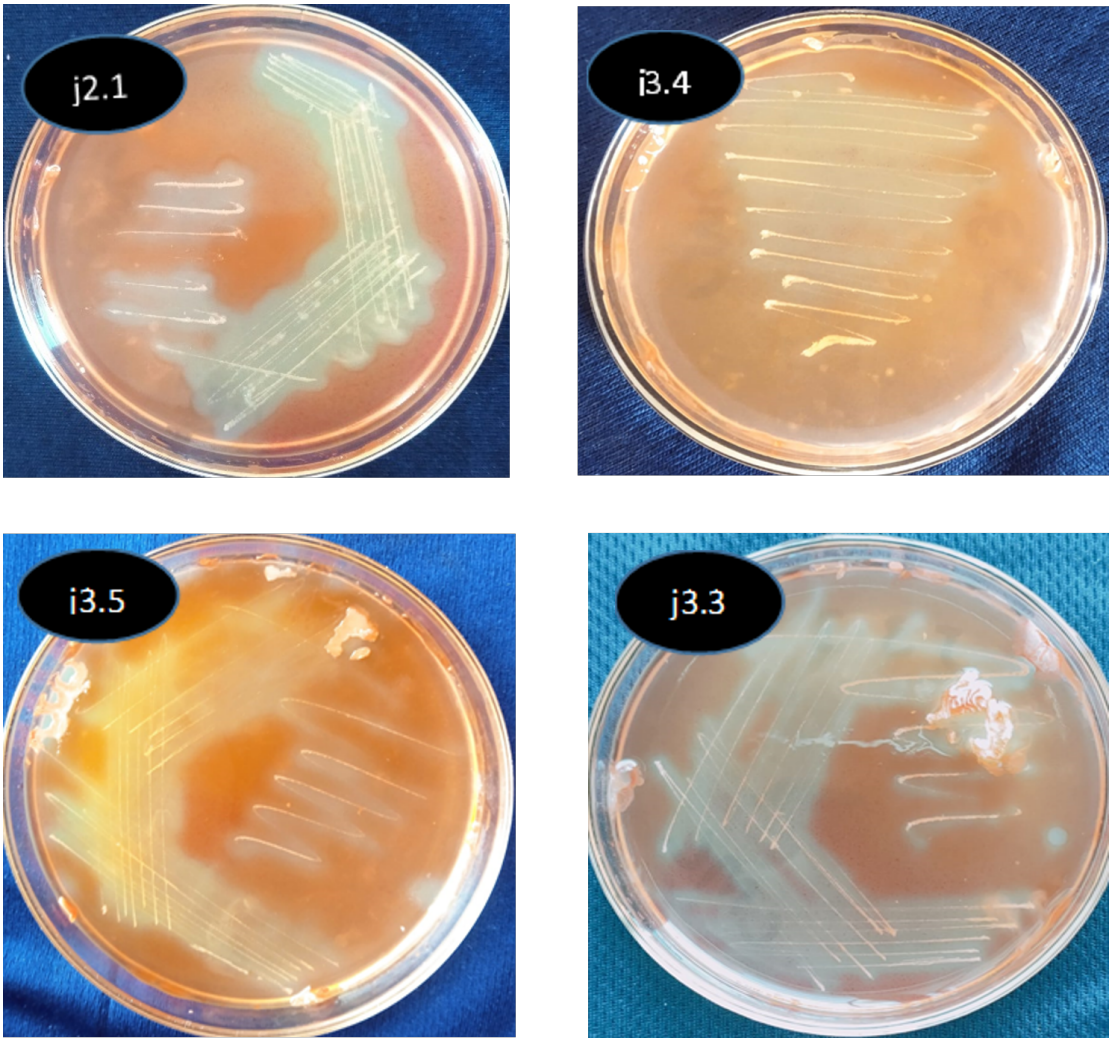


FIGURE 4.4: Isolated strains from the Bacterial sample on CMC and BHI media

4.2 Gram Staining

Gram staining performed for isolated strains indicated that out of seven stains six strains were stained positive and one strain was gram negative as shown in the table 4.1. These six strains were rod shaped and one strain was circular shaped in the staining results as shown in table 4.1. All of these strains were from tree samples.

TABLE 4.1: Morphological examination of bacterial strains isolated from termite gut

Sample	**Sample ID**	**Color**	**Margins**	**Form**
Tree	J2.1	White	Flat	Rod
Tree	J2.3	White	Flat	Rod
Tree	J3.2	White	Flat	Rod
Tree	J3.3	Off white	Flat	Rod
Tree	J3.4	Off white	Raised	Circular
Tree	J1.1	Off white	Flat	Rod

4.3 Biochemical Characterization

4.3.1 Catalase Test

The catalase test is used to determine whether the catalase enzyme is present by breaking down hydrogen peroxide and releasing oxygen and water, as seen in the reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The catalase reaction is recognizable by the swift generation of bubbles.

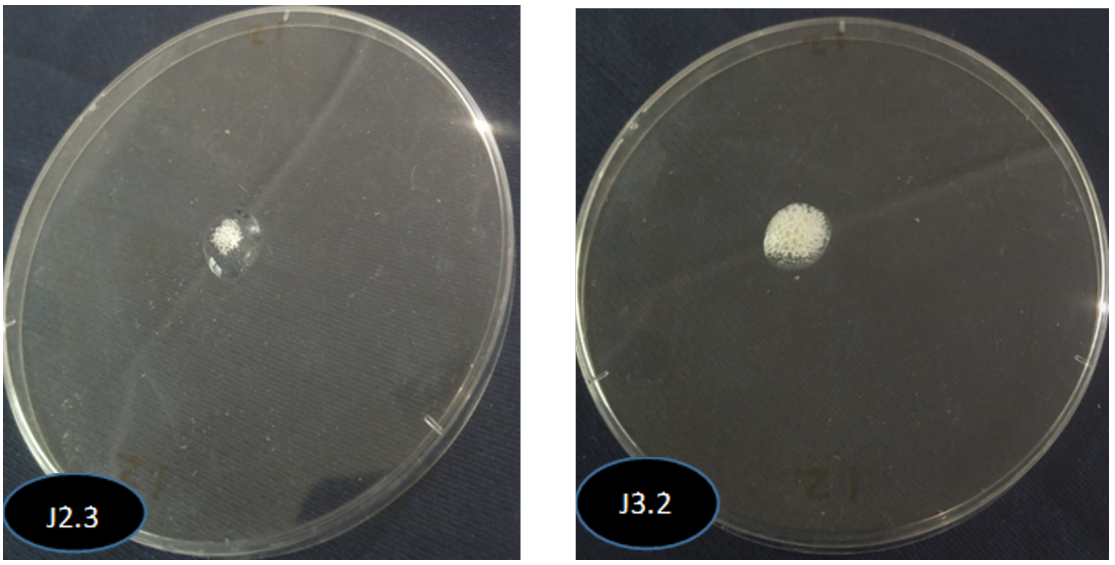


FIGURE 4.5: Catalase test results for the sample J2 & J3

All the sample as mentioned in figure 4.5 and table 4.2 were catalase positive.

TABLE 4.2: Biochemical characterization of bacterial strains isolated from termite gut sample

ID Sample	Catalase	Oxidase	Motility Ornithine	Gram Staining	Urease	VP test	TSI
J2.1	+	-	-	+	+	-	-
J2.3	+	+	-	+	+	-	-
J3.3	+	+	+	+	+	-	+
J3.2	+	+	+	+	+	-	+
J3.4	+	-	-	+	-	-	+

4.3.2 Oxidase Test

The oxidase test is used to determine which bacteria generate the essential enzyme in the bacterial electron transport chain, cytochrome c oxidase. Every bacterium that tests positive for oxidase is aerobic, meaning that it can use oxygen to receive electrons at the end of the respiratory chain. The colorless to deep indigo appearance indicated the presence of cytochrome c as a part of their respiratory chain labeled as the positive result. The reagent not oxidase, appeared colorless in the test limits, indicated the absence of cytochrome e as a part of their respiratory

chain. Out of five strains J2.3 and J3.2 showed color change and were oxidase positive. While J2.1 and J3.3 showed slight color change but J3.4 showed no change in color and were oxidase negetive.

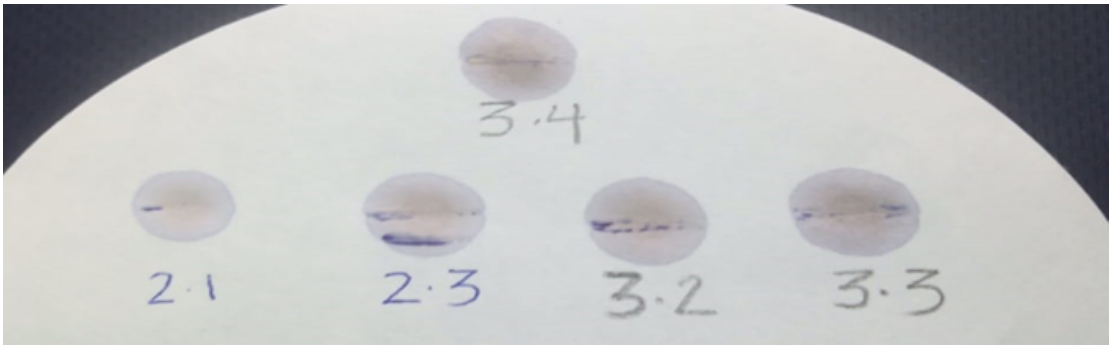


FIGURE 4.6: Oxidase test results for the sample J2 & J3

4.3.3 Voges-Proskeur Test

The Voges-Proskauer (VP) test was conducted to identify the production of acetoin, a neutral end product of glucose fermentation. The isolates were cultured in a glucose-containing broth medium and incubated aerobically for 24 hours, at 37^{o}C. Following incubation, a small portion of the culture were transferred into test tube for further examination. The Voges-Proskauer (VP) test procedure involved the sequential addition of two reagents, alpha-naphthol and potassium hydroxide (KOH). After the addition of each reagent, the tube was gently mixed. The reaction between alpha-naphthol and KOH with acetoin resulted in a red color indicating a positive result for bacterial strains that produce acetoin. In contrast, non-acetoin-producing strains either remained colorless or exhibited a light yellow color indicating a negative result for the VP test.

The test helps in distinguishing between bacteria that produce a significant amount of acetoin during glucose fermentation from those that do not. There was not any detectable change in the color after adding the reagent A and reagent B. All strains isolated were VP negative.

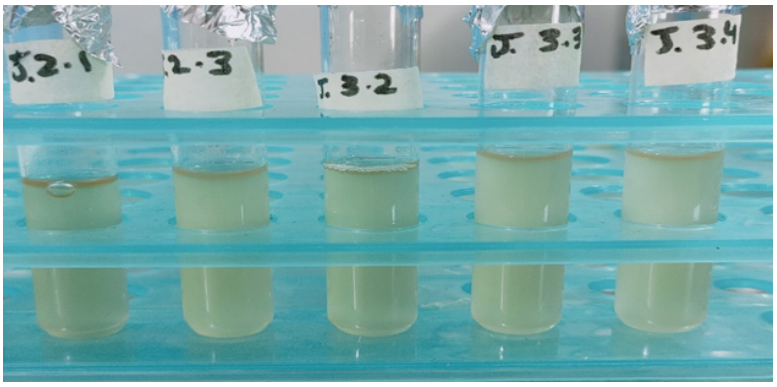


FIGURE 4.7: Results of Voges-Proskauer shows no pink color formation after adding the reagents

4.3.4 Motility-Ornithine Test

The Motility-Ornithine test was employed to assess the motility and ornithine decarboxylase activity of microorganisms. A sterile needle was utilized to pick an isolated colony and then stabbed into the medium in test tubes. The inoculated medium was incubated at 37^{o}C until visible growth was observed. The presence of a red turbid indicates positive results in region spreading beyond the line of inoculation. On the other hand, a negative test is characterized by red growth confined to the inoculation line without further extension.

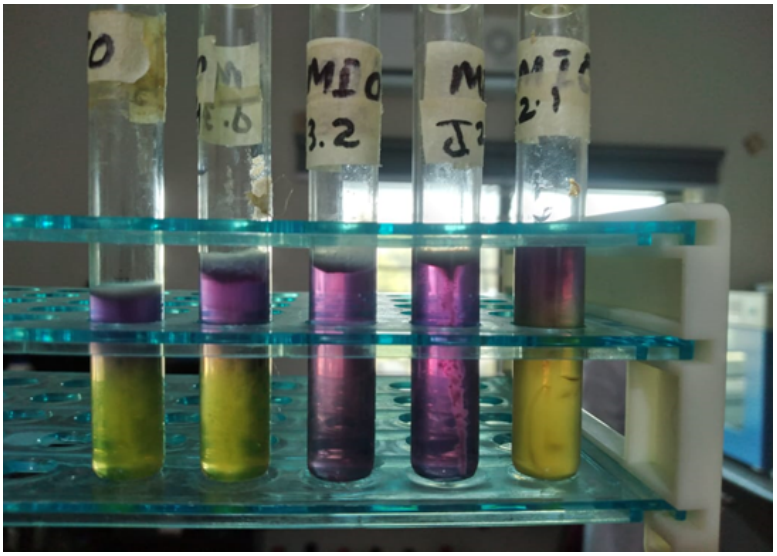


FIGURE 4.8: Motility test showing J2.1, J3.3, J3.4 Negetive results

4.3.5 TSI Test

Enterobacteriaceae are presumptively identified using the Triple Sugar Iron (TSI) Agar, which is based on the bacteria's ability to ferment glucose, lactose, and sucrose in addition to producing gas and H_2S (hydrogen sulfide).

TSI Agar is made up of peptones, yeast, and beef extract in addition to the three carbohydrates glucose, sucrose, and lactose. Included as a pH indicator is phenol red.

The tubes that hold the melted agar are slanted during the preparation process. The Triple Sugar Iron (TSI) test involves first inoculating the TSI slant by jabbing the butt all the way down to the bottom of the tube. Next, streak the surface of the slant. Make sure the closure fits loosely to allow air to pass through. After 18 to 24 hours of incubation, the results are readable at 37^oC. Red butt, red slant, and yellow butt all exhibit no signs of gas or H_2S production.

Gas production is visible in the yellow slant and butt but no H2S is present. The generation of gas and H2S is shown by yellow butt and yellow slant, yellow butt and red slant.

4.4 Molecular Characterization using 16S rRNA

From the obtained samples, two strains were identified through the sequencing of their 16S rRNA gene Sequencing.

Two bacterial strains were identified from the acquired samples by means of 16S rRNA gene sequence analysis. The multiple sequence alignment was done by using a bioinformatics tool named Clustal Omega of Sequence 2.1 and Sequence 3.2. The result of multiple sequence alignment showed the variation in sequences with Maximum Percentage Identity 99.30% and Query Coverage 97% respectively table 4.3.

The identified strains were *Lysinibacillus sphaericus* and *Lysinibacillus boronitolerans*. The sequences were submitted to the NCBI and accession no provided are mention in table 4.3 and figure.

TABLE 4.3: Molecular characterization using 16s

Sr. No.	**Sample Id**	**Scientific Name**	**Accession no.**	**Query Cover**	**Percentage Identity**
1	2.1	*Lysinibacillus boronitolerans*	PP544449	97%	98.84%
2	3.2	*Lysinibacillus sphaericus*	PP544448	97%	99.30%

Chapter 5

Discussion

The conventional classification places termites within the order Isoptera; research indicates a closer relationship to cockroaches, categorizing them as members of the *Blattodea* order [181]. Termites are categorized into two groups: lower termites including families (*Masto-, Archotermopsidae, Stolo-, Hodo-, Kalo -, Stylo-, Serri* and *Rhino -termitidae*) and higher termites (*Termitidae* family) [171]. These groups demonstrate different approaches to the digestion of lignocellulose [182]. Lower termites depend on intricate symbiotic relationships with eukaryotic flagellates and bacteria [14]. Higher termites either host a mutualistic hindgut microbiome made up only of prokaryotes or participate in an external symbiotic relation with fungus species of *Basidiomycetes* [183].

Particularly in the buccal cavity, some species of termites, both higher and lower, appear to express endogenous cellulases [184]. Contrary to the prevalent *Firmicutes* and *Bacteroidetes* in rumen and cellulolytic soil bacterial communities, the majority of particle-associated bacteria in the intestines of wood-feeding termites are *Fibrobacteres* and *Spirochaetes* [16]. This difference probably indicates different bioconversion and degradation processes. The digestive tracts of lower termite species (*Mastotermitidae*, *Hodotermitidae* and *Kalotermitidae*) are populated with numerous flagellates [185]. Symbiotic bacteria are crucial for termite digestion, and these interactions are described as mutualistic. The termite digestive system contains three parts: the foregut, midgut, and hindgut. Due to various

intestinal processes, termite digestive enzymes may require an extended period for digestion [186]. *Acetonema longum* and *Clostridium mayombei* from Macrotermes gilvus (Hagen) are among the bacteria that have been isolated and identified from termite guts [119]. In another study, four bacteria obtained from the stomach of *Odontotermes formosanus* were identified as having the capability to digest lignin and cellulose [187].

After the successful isolation of three cellulolytic bacteria from the hindgut of the underground termite *Coptotermes curvignathus*. *Enterobacter aerogenes*, *Clavibacter agropyri* and *E. cloacae* were identified as the microorganisms in question [120]. The findings offered proof of their role in cellulose degradation. From the termite gut, eight bacterial and five fungal isolates that degrade cellulose were isolated. Bacillus species, Cellulomonas species, Enterobacter species, and *Aspergillus* species were among the discovered microbes [119]. For *Aspergillus spp.*, the largest zone of hydrolysis (38 mm) during their screening for cellulase production using the Congo red assay. The isolated five bacteria from the hindgut of termites belonging to the genera *Heterotermes* and *Odontotermes* [188]. Three of these isolates were recognized as Bacillus and one each as *Staphylococcus* and *Enterobacter spp.* Three strains of Bacillus were identified from the termite *Zootermopsis angusticollis*, demonstrating their function in cellulose degradation [189]. PCR-dependent gene amplification of 16srDNA has been the primary method of species identification in environmental microbial community investigation [190]. Cellulolytic bacteria identified from *Reticulitermes lucifugus*'s intestines, demonstrating its function in the breakdown of cellulose and confirming its identity using 16S rRNA gene sequencing [191]. From the termite *Zootermopsis*'s intestines, 119 cellulolytic bacterial strains [190]. Using partial 16S rDNA sequence analysis, cellulolytic bacteria identified and isolated from the gut of *Reticulitermes lucifugus*, highlighting their function in the cellulose digestion [192].

Prokaryotes in termites' hindguts normally have a cumulative count of 107–1011 per milliliter [193]. Gram-positives, *proteobacteria*, *spirochetes* and the *Bacteroides /Flavobacterium* branch are the main bacterial taxa found in the termite stomach

according to the 16S rDNA method [194]. Variations in termite diet were associated with differences in domain-level profiles; methanogenic Archaea were found to be more common in termites that fed on soil than in those that fed on wood [195]. Numerous investigations have demonstrated that spirochetes are the predominant bacterial community in the contents of the hindgut [196]. In pure culture, five species have been successfully isolated [197]. These gut bacteria are essential for the breakdown of cellulose, hemicellulose, oligosaccharide processing, degradation of aromatic compounds, and nitrogen fixation. The redox status in the termite gut is shaped in part by these microbes [191]. Bacteria and archaea do not have a random distribution; rather, they inhabit particular microhabitats [198]. A detailed examination of *Nasutitermes spp.*, in different intestinal sections *Spirochetes, Firmicutes, Fibrobacteres, Bacteroidetes, Proteobacteria* and *Actinobacteria* to be compartmentalized [191]. Contentious debates have centered on the problem of termites' bacteria's digestion of cellulose. From termites, a variety of cellobiose-using bacteria and cellulolytic have been effectively identified [199]. The termite stomach's mostly facultatively anaerobic or microaerophilic cellulolytic bacteria contrast with the rumen's entirely anaerobic cellulolytic bacteria, which include Bacteroides, Butyrivibrio, and Ruminococcus with titers of up to 107 per millilitre of gut. Termite guts are dominated by Bacillus species [191].

Numerous bacterial strains have been identified, mostly from Proteobacteria and Gram-positive bacteria. These strains have the capacity to break down mono- and oligosaccharides that are produced when cellulose and hemicellulose are hydrolyzed. The isolates' oligosaccharide-hydrolyzing enzyme activity have been evaluated [200]. *Homotocetogenic spirochetes* are assumed to be engaged in polysaccharide degradation which is the third step. while spirochetes are implicated in the breakdown of oligosaccharides [201]. Numerous other bacteria that use oligosaccharides and monosaccharides have been discovered [202]. Sixteen cellulolytic bacterial strains, including those of the Dyella, Chryseobacterium, and Bacillus species, were isolated from the stomach of R. speratus. The inadequate endoglucanase (EG) activity of all isolates in an artificial growth medium led the scientists to hypothesise that the bacteria may not create cellulases in the gastrointestinal tract. Termites and protozoa's cellulolytic activity may be the cause of this [203].

A wide range of genes linked to bacterial activities that are cellulolytic and xylanolytic were discovered through metagenomic analysis of the bacterial microbiota in hindgut higher termite that feeds on wood, *Nasutitermes spp. Microcerotermes spp.*, is a higher termite found in Thailand. Bacillus subtilis strains with remarkable hydrolytic capabilities, such as endoglucanase, F-pase, and β-glucosidase, were obtained from it [204].

Lysinibacillus sphaericus (previously known as *Bacillus sphaericus*) [1] is a Gram-positive, mesophilic, rod-shaped bacterium commonly found on soil. It can form resistant endospores that are tolerant to high temperatures, chemicals and ultraviolet light and can remain viable for long periods of time. It is of particular interest to the World Health Organization due to the larvicide effect of some strains against two mosquito genera (Culex and Anopheles), [2] more effective than Bacillus thuringiensis, frequently used as a biological pest control. *L. sphaericus* cells in a vegetative state are also effective against Aedes aegypti larvae, [3] an important vector of yellow fever and dengue viruses.

Lysinibacillus sphaericus comprises a group of motile Gram-positive spore-forming bacilli. Members of this group are characterized by their terminal endospore, the capability to utilize acetate as the sole carbon source, and the presence of lysine and aspartic acid in their cell-wall peptidoglycan. Some *L. sphaericus* strains with larvicidal activity have been successfully used in vector-control programs against malaria, filariasis, yellow fever, dengue fever, and West Nile virus. Commercially available formulations containing *L. sphaericus* spores include JianBao ®, VectoLex ®, VectoMax ®, Spherimos®, and Spicbiomoss®. *L. sphaericus* has other environmentally relevant capabilities, including degradation of organic contaminants such as hydrocarbons and dyes; promotion of plant growth via nitrogen fixation; production of indole acetic acid; and solubilization of silicates and phosphates – as measured by the enhanced germination of tomato, cucumber, and rice seeds – and the adsorption of toxic metals (cadmium, lead, arsenic, mercury, chromium) and precious metals (gold).

Chapter 6

Conclusion and Future Work

Termites belonging to the order Isoptera, are cellulose-eating insects. Termites are the highest abundance in both numbers and species observed in tropical rainforests. Termites, social insects forming colonies and able of ranging from a few hundred to over a million individuals, play a vital role in the degradation of diverse materials. Termites contributes to biodiversity by creating conducive conditions for plants and other biota. Besides offering ecosystem services, various termite species also pose economic significance as pests affecting agricultural crops, forest plantations, and the structural wood components. Dry-wood termites, such as those belonging to the Cryptotermes genus, thrive in small colonies within wood and can endure extended periods of dry conditions. Termites play a vital ecological function by aiding in conversion of plant cellulose into substances that can be recycled within the ecosystem, supporting new growth. The first objective was to isolate the cellulolytic bacteria from gut of termites. For this purpose, crushed gut of termites cultured on nutrient agar and isolation was performed on CMC revealed the cellulolytic bacteria. Second objective was meant to perform biochemical characterization of cellulolytic bacteria. Gram staining performed for isolated strains indicated that out of seven stains six strains were stained positive and one strain was gram negative. These six strains were rod shaped and one strain was circular shaped in the staining results. All of these strains were from tree trunk samples labelled as J2.1, J1.1, J3.2, J2.3, J3.3 and J3.4.

Morphological inspection involved examination of six bacterial colonies. The colonies were white to off white with powdery to glossy appearance with flat and raised margins observed with naked eye. By breaking down hydrogen peroxide and releasing oxygen and water, the catalase test is used to determine whether the catalase enzyme is present. The quick formation of bubbles is indicative of the catalase reaction. Every isolated strain tested positive for catalase. An important enzyme in the bacterial electron transport chain, cytochrome c oxidase, is produced by certain bacteria, and these bacteria can be identified using the oxidase test. Out of five strains J2.3 and J3.2 showed color change and were oxidase positive.

While J2.1 and J3.3 showed slight color change but J3.4 showed no change in color and were oxidase negative. The Voges-Proskauer (VP) test was conducted to identify the production of acetoin, a neutral end product of glucose fermentation. All strains isolated were VP negative. The Motility-Ornithine test was employed to assess the motility and ornithine decarboxylase activity of microorganisms.

Motility test showing J2.1, J2.3, J3.4 negative results and J3.2 and J3.3 shows positive results. The Triple Sugar Iron (TSI) Enterobacteriaceae are presumed to be identified on agar media based on their ferment ability of glucose, sucrose and lactose as well as their ability to create gas and hydrogen sulfide (H2S). J2.1 and J2.3 stains show negative results J3.2, J3.3 and J3.4 show positive results.

6.1 Future Recommendations

In this investigation, we were able to separate and identify two strains of aerobic, symbiotic cellulolytic bacteria from the termite's digestive system. They help in the breakdown of cellulose in the termite's stomach together with the termite's own cellulases enzymes and cellulolytic flagellates.

1. It is crucial to conduct genomic characterization to identify bacterial strains and enzymes involved in cellulose digestion.

2. Exploring anaerobic cellulolytic bacteria specific to the gut of termite species is essential, considering the efficiency of these insects in decomposing lignocellulose.

3. Better control strategies for these insect pests may result from interfering with the mutual interactions between symbionts and termite hosts. The eradication of aerobic cellulolytic bacteria can modify the anaerobic conditions required (flagellates), which may lead to their eventual demise. This disruption affects cellulose digestion and eliminates a significant portion of the symbiotic organisms crucial for the survival of termites.

4. The cellulolytic potential of these bacteria can be further explored for applications in fermentation and ethanol production.

5. The application of these bacteria can be evaluated for bio-inoculation to enhance soil fertility by decomposing organic material, contributing to a reduction in environmental pollution.

6. The electron flow involved in the breakdown of biopolymers by anaerobic microbial communities can be investigated and understood through the use of cellulolytic bacteria.

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