Evaluation of Endogenous and Symbiotic Cellulase Activities in Subterranean Termites: Their Role in Biomass Conversion and Termite Control Strategies

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Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University

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Evaluation of Endogenous and Symbiotic Cellulase Activities in Subterranean Termites: Their Role in Biomass Conversion and Termite Control Strategies

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By

Muhammad Afzal

Presented to

Department of Zoology

Faculty of Biological Sciences

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Islamabad

2021

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Dedicated

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My Beloved Late

Parents

,

Acknowledgments

I am highly grateful to ***Allah Almighty*** *for His countless blessings throughout my life and for helping me to achieve my goals and for giving me the strength in the completion of this project. May Allah guide me on the right path (Ameen). Peace and blessings are upon the* ***Holy Prophet Hazrat Muhammad (PBUH)****, the most perfect among all human beings ever born and Who guided his followers to seek knowledge from cradle to grave.*

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Contents

List of Abbreviations

ANOVA	Analysis of variance	Ml	Milli litter
AWPA	American wood protection associations	MOE	Molecular operating environment
BBD	Box Behnken design	MS	Mass spectrophotometry
BX	Beechwood xylan	NIH	National institute of health
β-glu	β-glucosidase gene	N	Number of samples/individuals
CB	Cellobiose	OD	Optical density
CBE	Conduritol b epoxide	ODP	Observed dead population
CBH	Cellobiohydrolase	PCR	Polymer chain reaction
CBI	Cellobiomidazole	PDB	Protein data bank
CS	Corn stovers	pNPC	p-Nitrophenyl pyranocellobioside
CCA	Chromated copper arsenate	pNPG	p-Nitrophenyl pyranoglucoside
CI	Confidence interval	PE	*Populus eumericana*
CMC	Cello methyl cellulose	PMFS	Phenylmethylsulfonyl floride
df	Degree of freedom	pNPM	p-Nitrophenyl mannoside
dH_2O	Distilled water	pNPX	p-Nitrophenyl xylobioside
DMSO	Dimethyl sulfoxide	pNPA	p-Nitrophenyl arabinoside
DNA	Deoxyribonucleic acid	PR	*Pinus roxburghii*

DPPH	1,1-diphenyl-2-picryl-hydrazy	PTGS	Post-transcriptional gene silencing
EDTA	Ethylenediaminetetraacetic acid	*R. flavipes*	*Reticulitermes flavipes*
endo	Endoglucanase gene	RH	Rice husk
exo	Exoglucanase gene	RH	Relative Humidity
FMCB	Fluoromethyl cellobiose	RMSD	Root mean square deviated
FMG	Fluoromethyl glucose	RNAi	RNA interference
FP	Filter paper	RNA	Ribonucleic acid
GHF	Glucohydrolase family	SC	Sugarcane bagasse
H. indicola	*Heterotermes indicola*	SD	Standard deviation
HPLC	High performance liquid chromatography	SDS	Sodium dodecyl sulphate
IC_{50}	Inhibition concentration 50%	SDS-PAGE	SDS polyacrylamide gel electrophoresis
kDa	Kilo dalton	SE	Standard error
KPa	Kilo pascal	SDF	Standard database format
LC	Liquid chromatography	TP	Total population
LC_{50}	Lethal concentration 50%	UV-Vis	Ultraviolet visible
MeOH	Methanol	WA	Wheat arabinoside
Mg	Milligram	$\overline{X}$	Mean

List of Tables

List of Figures

	intermediate. While other carboxylic group acts as an acid/base catalyst and perform glycosylation and de-glycosylation via protonating	
1.6	Soldiers of (a) *H. indicola* Wasmann and (b) *R. flavipes* Kollar	**10**
2.0	Gel electrophoresis confirming the results of partial cellulase genes amplification by qPCR using genomic DNA (G) and cDNA (C) across the different body sections of termites	**28**
2.1	Cellulase genes expression fold of β-glucosidase (a), exoglucanase (b), endoglucanase (c), and (d) indicating symbiotic cellulases in thorough out gut region of *R. flavipes* and *H. indicola.* Alphabets indicating significant difference ($p < 0.001$) between different cellulase genes' expression in different gut regions using Tukey's LSD test	**37**
2.2	Distribution of different molecular weight proteins in different gut regions of *R. flavipes* (a) Head, SG: salivary gland, FG: Foregut, MG: midgut, HG: hindgut and carcass. Whereas, figure (b) indicating protein distribution and purification in *H. indicola*, 1: whole gut, 2: gel purification of thorax region, 3: gel purification of abdomen region, 4: crude of thorax along the head region, 5: crude abdomen region, 6: precipitated protein from the head region. Red arrow indicating β 1-4, endoglucanases, while yellow indicating β-glucosidase, blue indicating exoglucanase	**38**
2.3	Total cellulase activity of different gut regions of termites on Whatman No. 1 filter paper disks.	**39**
2.4	Total cellulase activity among different gut tissues *H. indicola* and *R. flavipes.* Alphabets indicating significant difference ($p < 0.001$) between different cellulase genes' expression in different gut regions using Tukey's LSD test	**39**

	corn stove, d) sugarcane bagasse, e) rice husk, and f) filter paper (control) in a humidity control acrylic chamber under 26°C and 75±5% relative humidity condition.	
3.3	Host fraction indicating endogenous enzyme source in *R. flavipes*	**62**
3.4	Symbiotic fraction indicated by hindgut of *H. indicola* along their flagellates.	**63**
3.5	Termite feeding assay results indicating percent weight loss of feedstocks and change in termite for *R. flavipes* a) and *H. indicola* b) after 14 days of continuously feeding.	**67**
3.6	The percent survival rate of *H. indicola* and *R. flavipes* by feeding on lignocellulose diets a) *Populus* (cottonwood), b) pine wood, c) corn stovers, d) sugarcane residue, e) rice husk concerning f) control (filter paper).	**68**
3.7	*In vitro* saccharification assay optimization by *R. flavipes* whole gut extract at the different time interval of incubation against feeding stocks a) *Populus* wood (cottonwood), b) Pinewood, c) corn stovers, d) rice husk, e) sugarcane residue, f) filter paper (control).	**69**
3.8a	Monosaccharide released after 12 hours in vitro saccharification assay from the feeding stocks *of Populus eumericana wood* (PE), *Pinus roxburghii* (PR), Corn stovers (CS), rice husk (RH) and Filter paper (FP) as control by the host and symbiotic fractions of *R. flavipes*.	**70**
3.8b	Monosaccharide released after 12 hours in vitro saccharification assay from the feeding stocks *of Populus eumericana wood* (PE), *Pinus roxburghii* (PR), Corn stovers (CS), rice husk (RH), and Filter paper (FP) as a control by the host and symbiotic fractions of *H. indicola*.	**71**

SUMMARY

Lower subterranean termites primarily feed on wood and wood products due to their dual cellulolytic systems comprising endogenous and symbiotic origin. The distribution of total cellulase, endoglucanase, exoglucanase, β-glucosidase, and xylanase activities were mapped out throughout the gut of termite species *Heterotermes indicola* and *Reticulitermes flavipes* using specific substrates. Native SDS-PAGE confirmed the cellulase activity distribution patterns by exhibiting the relative protein bands distribution of specific size throughout the termite gut. Seven partial genes encoding the two endogenous and five symbiotic cellulases belonging to different glucohydrolase families GHFs 1, 7, 9, 42, 45, 11 and 7 in *H. indicola* and *R. flavipes*. These genes encode proteins that significantly share similarity with previously reported cellulase genes including endoglucanase, β-glucosidase, and exoglucanases belonging to different GHFs. Quantitative (qRT-PCR) revealed that β-glucosidase and endoglucanase genes which encode GHF1 and GHF9 cellulases significantly expressed higher in salivary glands as compared to other gut parts respectively, whereas exoglucanases (GHF7) and other cellulases (GHF 42, 45, 11 and 7-5) expressed only in hindgut region where cellulolytic protozoa reside. Cellulase genes' expression positively correlated with respective specific types of cellulase activities and t-*test* insignificant differences between *H. indicola* and *R. flavipes*.

In second objective, protein-level *in vitro* and *in vivo* saccharification potential to biofuel producing feedstocks sugarcane bagasse, pinewood, cottonwood (*Populus* spp.), corn stover, rice husk by the guts of *H. indicola* and *R. flavipes.* The focus of this objective was (i) to measure the saccharification potential of feedstocks with respect to control diet (filter paper) by termites' gut, and to determine impacts of feedstock on foraging activities and survival behavior. Sugarcane bagasse, pinewood, cottonwood, and corn stovers were saccharified at significantly high levels as compared to the control diet and rice husk. The gut protein extract from both termite species saccharified all lignocellulose material regardless of feedstock loading. However, *R. flavipes* showed significantly ($p<0.05$) high saccharification potential than *H. indicola*. Consumption of the sugarcane bagasse, pinewood, and corn stover was maximum by *R. flavipes*, whereas *H. indicola* exposed cottonwood and sugarcane feedstocks as the most palatable food source with 100% survivability. Results supported the "diet adaptation" hypothesis (Karl and Scharf, 2015) by indicating β-glucosidase, exoglucanase, and xylanase activities significantly highest for sugarcane

bagasse, pinewood, and cottonwood as compared to rice husk and control diets in both termite species. These results are important from the perspective of physiological changes in termites that lead to gut microbial environments changes and 100% survivability on exclusive sugarcane bagasse, pinewood, and cottonwood feedstocks.

In the third objective, a cellulolytic bacterial strain was isolated from the gut of building infesting termite species *H. indicola* and *R. flavipes* for the production of ionic-solvent tolerant cellulase. After screening by congo red, strain HI-08 was identified as *Bacillus licheniformis* using 16S rDNA sequence analysis. It was accounted for maximum cellulase production on 2% sugarcane bagasse (1156 U/mL). The crude cellulase was purified by $(NH_4)_2SO_4$ precipitation, affinity and size exclusion chromatography, with 12.02 purification fold and 32.33% overall recovery. Approximately 55 kDa molecular weight of gel-purified cellulase was revealed by SDS-PAGE analysis. The optimum temperature and pH for maximum cellulase activity were determined as 45°C and 7.0, respectively. Kinetic analysis of purified cellulase showed the K_m and V_{max} be 2.24 mg/mL and 454.05 µg/mL/min, respectively. Statistical optimization using RSM indicated that sugarcane bagasse was the most useful agricultural waste for cellulase production, which proved its candidature in industrial bio ventures.

In the fourth objective of this project, dry lab and wet lab approaches were conducted to cut off the lignocellulose activities of termites by targeting termite cellulases including endoglucanase, exoglucanase, and β-glucosidase using ecofriendly carbohydrate-based cellulase inhibitors. Before conducting wet laboratory bioassays, in dry lab studies 3D structure of three main cellulases endoglucanase, exoglucanase, and β-glucosidase from *R. flavipes* were modeled using Modeler 9.24v and validated via Structure Analysis and Verification Server (SEVES) comprising on the package of different programs such as PROCHECK, Verify3D, ERRAT, and Ramachandran plot. Interactive binding of carbohydrate-based cellulase inhibitors (cellobiomidazole, fluoromethyl cellobiose, fluoromethyl glucose, glucose, and cellobiose) with enzymes was assessed by GOLD scoring. The results of *in silco* studies revealed cellobiomidazole, cellobiose, and fluoromethyl cellobiose showed higher promising binding affinities with exoglucanase and β-glucosidase as compared to endoglucanase based on GOLD docking scores. These results were supported by wet-lab studies, in which the efficacy of these inhibitors was investigated by conducting biochemical and feeding bioassays. Cellobiomidazole, cellobiose, and

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In the last objective of the present study, silver oak (*Grevillea robusta* Cunn.) (Proteaceae) leaf extract was investigated for its termiticidal properties against the termite *H. indicola* in the laboratory tests. An ethanol-solvent system was used to remove the extract from dry leaf powder via the Soxhlet apparatus. A dose-dependent foraging response and mortality were observed after continuous exposure to a dose range of 1 to 20 mg/mL of extract based on dry extract weight. Results indicated that maximum (> 90%) termite mortality occurred at 20 mg/mL along with a significant reduction in termite tunneling activity. The LC_{50} was calculated at 5.47 mg/mL using the concentration series data. In filter paper antifeeding and repellency bioassays, a significant reduction in the total population of gut protozoa compared to untreated and negative controls (solvent) was observed. After 2 weeks of no-choice continuous feeding on filter paper treated at 20 mg/mL, the hindgut protozoan population was reduced by 62.90%. The extract induced resistance against termite feeding activities to the non-durable cottonwood and pinewood after vacuum pressure treatment. At the highest concentration tested, 100% mortality was recorded after exposing termites to cottonwood and pinewood treated with silver oak leaf extract. HPLC-DAD-ESI-MS results of the extract showed a high level of phenolic compound, alkyl-resorcinol. Results indicated that silver oak leaf extract has termiticidal properties and could be of value in the development of botanical insecticides for termite control in wood products.

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General introduction

Termites are eusocial insects, and they are efficient lignocellulose degraders that are found globally in tropical, subtropical, & temperate areas (Potter, 2011; Hassan, 2017). Termites thrive on living and dead plant materials that show their critical role in nitrogen and carbon mineralization in the ecosystem (Kudo, 2009; Brune, 2014). Agriculture waste is the highly profuse carbohydrate-based energy source in the natural ecosystem and termites perform the significant role in lignocellulose decomposition. The bioconversion of cellulose-containing raw material by termite cellulases is a considerable attention to develop procedures for the operative utilization and treatment of the cellulosic material as an inexpensive source of carbon for energy production (Lynd, 2002; Ballesteros *et al*., 2004). To understand the lignocellulose degradation system in termites, the cellulolytic enzymes and their activities should be elucidated in termites. Such studies may subsidize to the plant biomass optimization, the process of degradation and uncover the novel targets to termite control strategies. However, termites are highly wood destructive pests and a serious menace to the wood in services such as building furnishers, goods stores, bridges, poles, railway sleepers, and forest in addition to fallen and standing valuable fruit and ornamental trees (Su *et al*., 2012). Global termite damage has been estimated at the US $ 22-45 billion annually [1–3]. In Southeast East Asia, damage attributed to subterranean termites is estimated at almost 400 million USD/year [4]. Therefore, worldwide constant endeavors are directed to termite control.

1.1. Termite classification

Recently, termites are scientifically grouped to an epifamily (Isoptera or Termitoidae) of the Blattodea due to their close relevance with cockroaches (Beccaloni and Eggleton, 2013; Krishna, 2013). Approximately seven families and 280 termite genera comprising on 3105 species of termites have been described, however, damage to wooden material posed by 80 species [5]. Generally, the classification of termites carried out into seven families: Rhinotermitidae, Termopsidae, Kalotermitidae, Serritermitidae, Termitidae, Hodotermitidae, and Mastotermitid (Figure. 1.1) (Lo and Eggleton, 2011). The Mastotermitidae is native to Australia with only one wood-feeding species and most primitive family in termites. Rhinotermitidae family consumes wooden structures vigorously in temperate zones and Serritermitidae termites are much like family Rhinotermitidae that confined to South America with few species. The Termposidae nest inside

the wood and thrive on wet dead logs. Likewise, Kalotermitidae is dry wood termites and Hodotermitidae is litter decomposer and grass-harvesters. The largest group in termites is Termitidae, consisting of more than 2000 species and accounting 75% of total known termite species. Termitidae family members have diverse feeding preferences and further divided into seven subfamilies which are Nasutitermitinae, Macrotermitinae, Sphaerotermitinae, Foraminitermitinae, Apicotermitinae, Syntermitinae, and Termitinae (Figure 1.1).

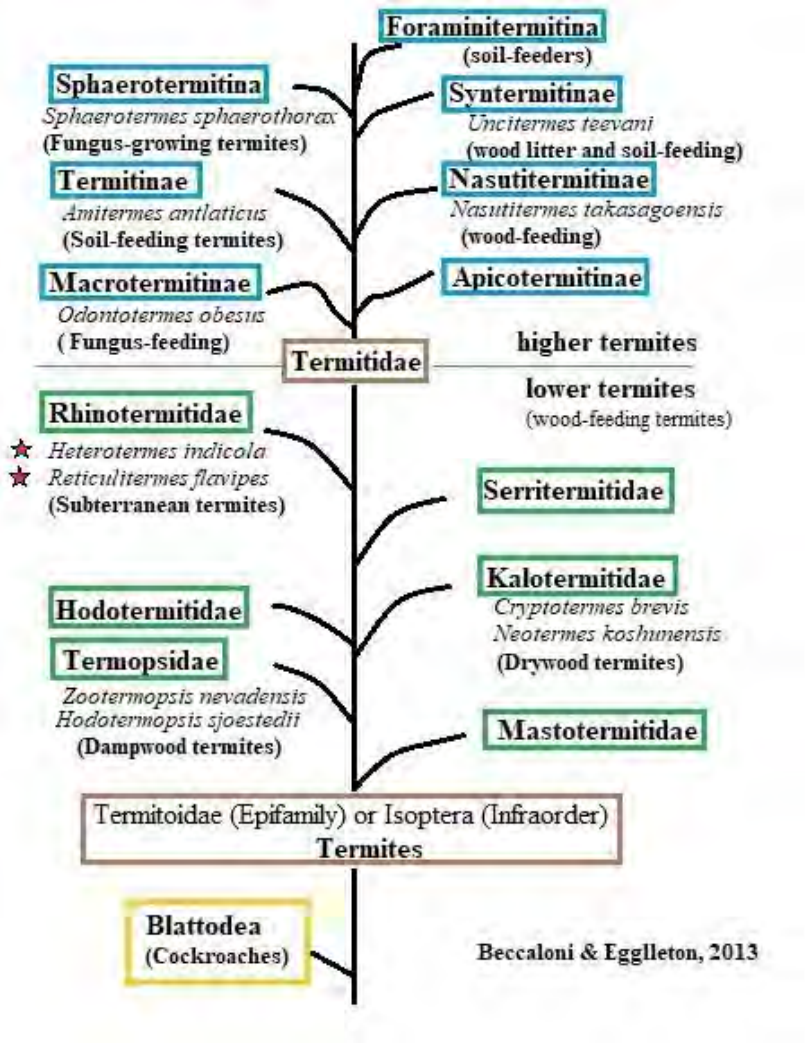


Figure 1.1. Termites' phylogeny concerning family and subfamilies of Termitidae. Asterisks indicating the termite species used in this study. Both subterranean termites are lower wood-feeding species in the urban environment of Pakistan. The cellulolytic potential of these termites species is still unexposed.

Conventionally, termites have been classified into higher and lower ones on the basis of symbiotic flagellated protozoa presence or absence in termites' hindgut (Brune, 1998; König *et al*., 2013). The termites in the family of Termitidae do not have the protozoan symbionts in their hindgut and referred to as, "Higher termites", whereas other families harbor a large number of symbiotic protozoa in their hindgut and named as, "Lower termites" (Brune, 1998).

On the basis of foraging behavior, termite are further classified into 03 major types such as dampwood termites, dry wood termites and the subterranean termites. Each species has unique biology and behavior that impacts their habitat, nest building strategy, and their likelihood to damage structures. Drywood termites as by name, typically live inside dry wood, such as dead tree logs, structural dry woods, hardwood flooring and they belong to the family Kalotermitidae. No moisture or soil contact is required to drywood termites as required by subterranean termites. However, dry wood termites typically damage the wood at slow than subterranean termites, since their colonies are smaller than subterranean termites. Dampwood termites as names suggest, they infest to wood with high moisture content and belong to family Hodotermitidae. Dampwood termites are rarely infested in the home or other wood structures in an urban environment since these wood structures typically do not carry enough moisture content. Usually, damp wood termites are larger than subterranean termites and most species don't require contact with soil for infestation.

Among total termites' species, subterranean termites are accounted for 38 species and important wood and wood products infesting pests due to their cryptobiotic nature (Eggleton, 2000; Brune, 2014; Hassan, 2017). Frequently, subterranean termites belong to the family Rhinotermitidae and their live-in soil and attack the wood structures by building trails made up of mud for the movement from the soil in the direction of wood, which is attached or above the earth surface. Its fallouts in greater termite infestation by increasing the moisture content transferred from soil to the wooden materials (Kirker *et al*., 2013). Subterranean termites, which can habitat in each continent, except Antarctica, are responsible for the major termite damage globally.

1.2. The digestive tract of subterranean termites

The digestive tract of termites is laterally structured micro hotspots with variances in digestion events and endosymbiotic community assemblies (Kohler *et al*., 2012; Ni and Takuda, 2013). The

termite gut entails three major parts foregut, midgut, and hindgut (Figure 1.2), with an ectodermal origin, the foregut of subterranean termite is an esophageal duct that has an engorged anterior section known as, “the crop” and a subsequent section, “the gizzard” that performs the grinding of ingested wood particles mechanically (Ni and Tokuda, 2013). Generally, the mid-gut is later to the foregut with an endodermal origin in insects is known as a discharge spot for gastric enzymes and the absorption of the nutrients is uniform and columnar. The excretory structures, “Malpighian tubules” are typically present at the termination of midgut to excrete the nitrogenous wastes in the gut lumen (Ni and Tokuda, 2013). The hindgut of subterranean lower termite is the largest organ that is further divided into dilated compartments P1, P2, P3, P4, and P5 (Watanable and Tokuda, 2010).

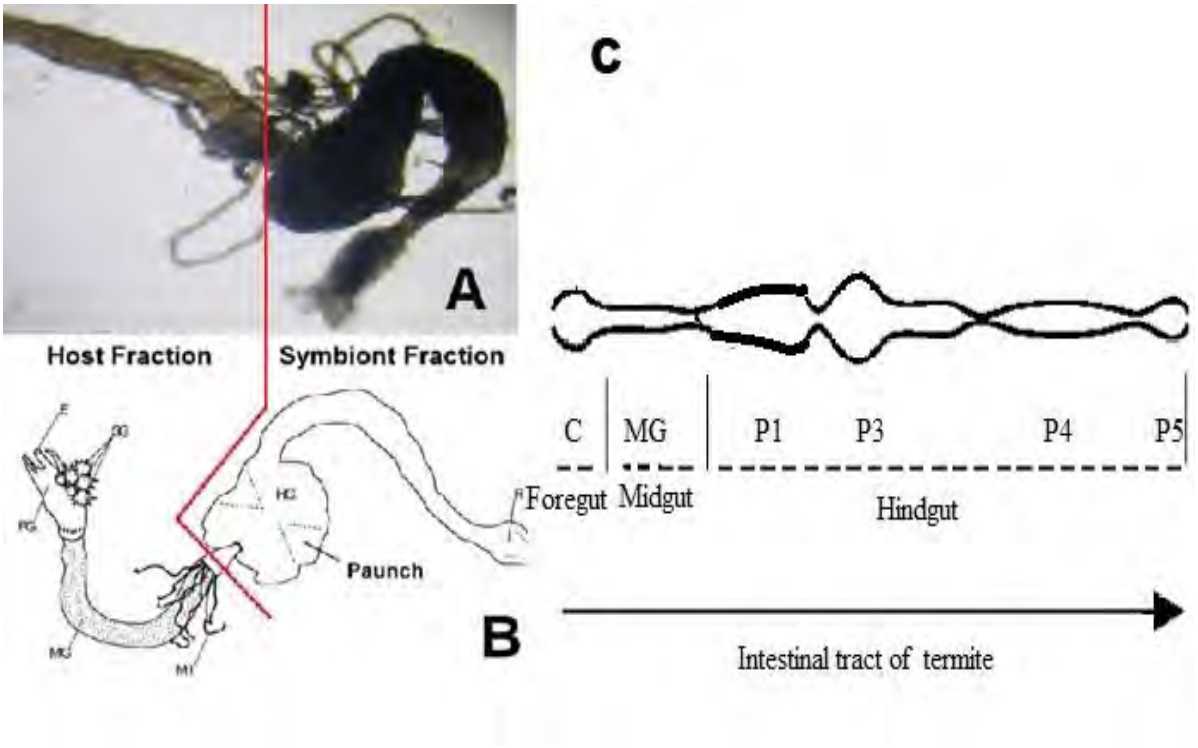


Figure 1.2. (A) The intestinal tract of subterranean termite *Heterotermes indicola* representing the collaboration of symbiont fraction and host fraction. (B) The sketch was described by Scharf *et al*. (2011) by indicating its parts; (E) esophagus, (SG) Salivary glands, (FG) Foregut, (MG) Midgut, (MT) Malpighian tubules, (HG) Hindgut and (R) Rectum. (C) sketch indicated different segments P1, P2, P3, P4, and P5 of the hindgut of subterranean termite.

Among these sections, P3 has usually enlarged to harbor the bulk of facultative, obligate, and anaerobic microbiota in an anoxic environment. It is a hotspot in subterranean termite’ gut due to the high rate of microbial activities and known as, “Paunch” (Breznak, 2000; Brune, 2005). The

microbial community residing in lower termites' paunch termites encompasses three major organisms' domains; bacteria, archaea, and eukaryotic (Hongoh, 2011; Ni and Takuda, 2013). In addition to the digestive gut of the subterranean termites, salivary glands similarly subsidize expressively to the metabolic makeup of the termite by releasing endogenous enzymes.

1.3. Lignocellulosic biomass

Lignocellulose from agriculture contains lignin (18-35%), hemicellulose (15-35%), and cellulose (20-50%). Cellulose a linear natural polysaccharide, serene of the D-glucose subunit that accompanying with β-(1,4)-glycosidic linkages, is the major part of plants (20-50%) and the utmost copious renewable biomass source for bioethanol production on earth (Tomme *et al*., 1995; Uchima *et al*., 2009). The microfibrils of cellulose are interconnected by hydrogen bonding with the addition of Van der Waal forces that generate a crystalline structure of cellulose and it is one of the factors that decrease the efficacy of enzymatic hydrolysis.

Besides, the intimate connotation of cellulose with hemicellulose and lignin that create an intricate network, decrease the cellulose hydrolysis and availability of cellulose degrading enzymes to cellulose fibers (Uchima, 2012). The subsequent most ample part of lignocellulose is the hemicellulose, and it gives rigidity to cellulose and lignin via hydrogen and covalent bonding respectively. Hemicellulose is composed of heterogenous groups of linear and branched sugar polymers at different sizes such as pentoses (D -xylose and L-arabinose), hexoses (D-glucose, D-mannose, and D-galactose) and acids (D-glucuronic, D-galacturonic and 4-O-methyl-glucuronic acid) (Uchima, 2012). Lignin is also a component of lignocellulose biomass and it also provides the rigidity to the lignocellulosic complex by forming different kinds of linkages. Lignin is a heteropolymer amorphous component and it entails three different types of phenylpropane units such as p-coumaroyl, sinapyl and coniferyl alcohols that are connected by various kind of forces (Uchima, 2012).

1.4. Cellulases and hemicellulases as glycoside hydrolases (ghs)

Glycoside hydrolases (GHs) perform biological hydrolysis of cellulose and hemicellulose polymers in different glycosylation steps based on the enzyme and substrate categories (Figure 1.3 and 1.4). Based on the specific activity, sequence of amino acids, and 3D structures, different families of the glycoside hydrolases (GHs) have been classified. GHs entails a copious enzymes

class with multiple ranges of substrate and structures specificities that decompose the polysaccharides into smaller carbohydrate monomers such as glucose, mono, and disaccharides (Uchima, 2012). In nature, the synergistic effect of three main types of cellulases mediates the complete and efficient hydrolysis of cellulose: (i) endoglucanases (endo-1,4-β-glucanases, EC 3.2.1.4); (ii) exoglucanases, including cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) and exoglucohydrolase (1,4-β-D-glucan glucohydrolase EC 3.2.1.74); (iii) β-glycosidase (EC 3.2.1.21).

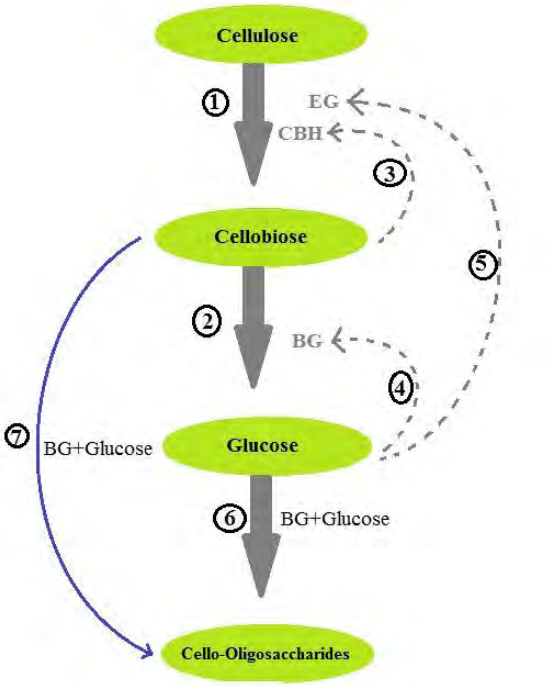


Figure 1.3. Schematic pathways of cellulose hydrolysis, Dark grey arrows heads (1, 2) show the main hydrolysis reaction, dotted grey arrows (3, 4, and 5) indicate reverse reaction due to end-product inhibition. Blueline arrow (7) exhibiting the trans glycosylation reactions. EG, endoglucanase; BG, β-glycosidase and CBH, cellobiohydrolases

However, for the complete hydrolases of hemicellulose entails the broad range action of the hemicellulases because of the flexible association of the substrate (Uchima, 2012). Hemicellulases could be glycoside hydrolases or carbohydrate esterases that are including endo-β-1,4-xylanases (EC 3.2.1.55), xylan 1,4-β-xylosidases (EC 3.2.1.37), α-glucuronidases (EC 3.2.1.139), α-ł-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterases (EC 3.1.1.72), β-1,4-

mannosidases (EC 3.2.1.25), arabinan endo-1,5-α-ł-arabinosidases (EC 3.2.1.99) and mannan endo-1,4-mannanases (EC 3.2.1.78) (Uchima, 2012).

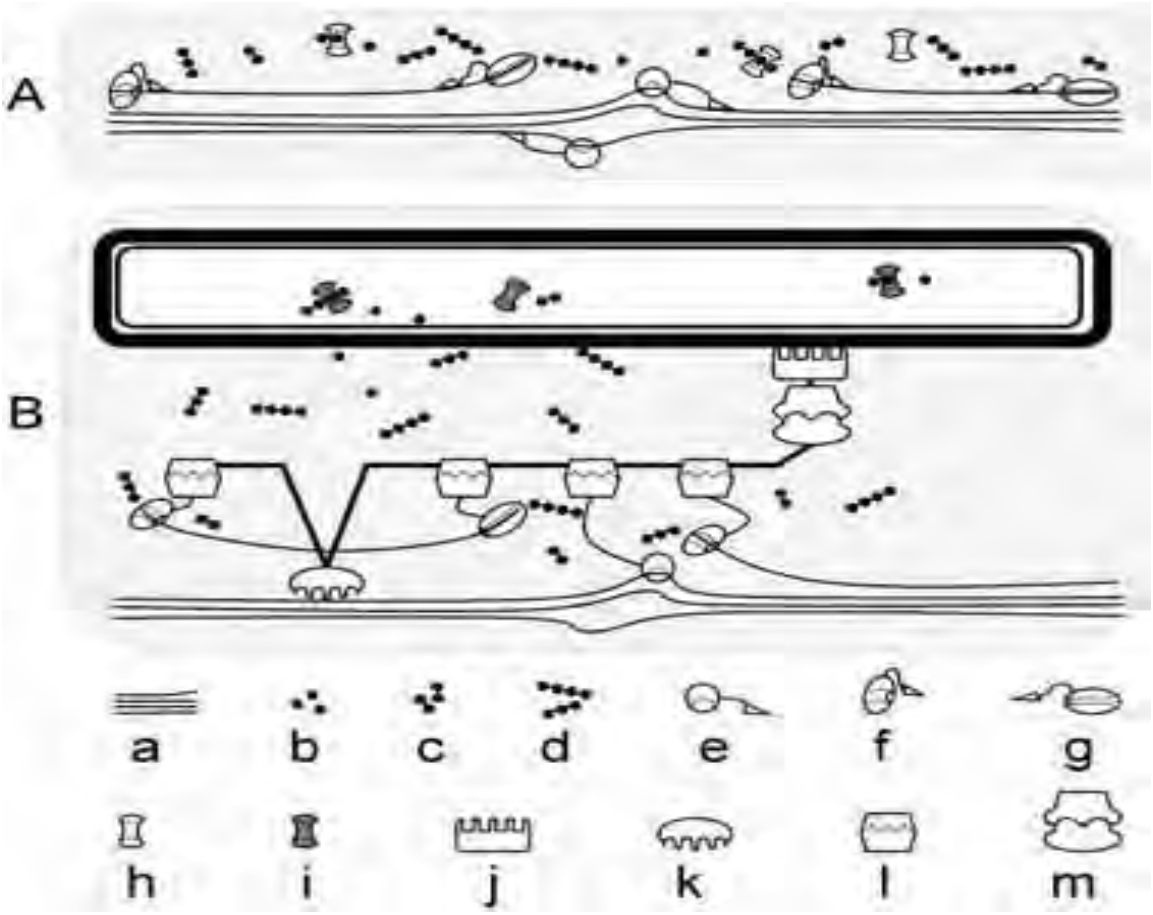


Figure 1.4. Schematic description of the decomposition of cellulosic polymers by non-complexed (A) and complexed (B) different cellulases mechanism (Zhang and Zhang, 2013). (a) Cellulose; (b) Glucose (c) Cellobiose (d) Oligosaccharides; (e) Endoglucanase with carbohydrate-binding modules; (f) Exoglucanase with CBM acting on reducing ends; (g) Exoglucanase with CBM acting on non-reducing ends; (h) β-glucosidase; (i) Cellodextrin phosphorylase (j) S-layer homology module (k) CBM (l) Type-1 dockerin cohesin pair; (m) Type-II dockerin cohesin pair.

1.4.1. Endoglucanase (EGs)

Endoglucanase (EG) generates new ends of polysaccharides chain by random cleaving of internal glycosidic β-1,4-linkages in the cellulose fiber (Lynd *et al*., 2002). They act on the amorphous region of cellulose and produce reducing and non-reducing ends known as nicks of cellulose fibers (Figure 1.4). Different catalytic modules of endoglucanase belong to GHFs are produced by

different organism sources such as bacteria, fungi, protists, animals, and plants (Li and Wilson, 2008). The crystalline structure of most endoglucanases depicts the catalytic modules that have active site of cleft-shape, which helps to bind it with cellulose chain and cleave the polysaccharides to generate the glucose, soluble or insoluble cellodextrins, and cellulose fragments. Some EGs act processively depends on its ability to hydrolyze the crystalline cellulose into major products if cellodextrins or cellobiose (Cohen *et al*., 2005; Li and Wilson, 2008; Yoon *et al*., 2008; Zhang and Zhang, 2013).

1.4.2. Cellobiohydrolase (CBH) or exoglucanases (EGs)

Cellobiohydrolases (CBHs) degrade the fibers further by acting on reducing and non-reducing ends of polysaccharides chains in a processive manner liberating the cellobiose and glucose units as major products (Figure 1.4) (Beguin and Aubert, 1994; Uchima, 2012). It effectively works on microcrystalline cellulose, apparently like peeling cellobiose chains from microcrystalline cellulose (Teeri, 1997; Cohen *et al*., 2005). CBHs are important cellulases produced by bacteria, fungi, and wood degrading termites with catalytic modules belonging to glycoside hydrolases families 7, 48, and 74 (Zhang and Zhang, 2013). The tunnel shape active site enables the CBHs to attach with reducing and non-reducing ends of cellulose and hydrolyze in a unique processive manner (Vocadlo and Davies, 2008). The product of CBHs is cellobiose and glucose that acts as directly and indirectly inhibitors of EGs and CBHs and causes the reduction in hydrolysis rate as well as the product yields (Andric *et al*., 2010).

1.4.3. β-glucosidases

β-glucosidases (BGs) are exotype cellulases, which breakdown the glycosidic bonds and hydrolysis of the soluble products of endo and exoglucanases (cellodextrins and cellobiose) from the non-reducing end (Figure 1.5) (Uchima, 2012). BGs are the institute in entirely the realms of living organisms such as bacteria, eukaryote, and archaea. it does not show a significant activity to hydrolyze the insoluble cellulose (Zhang and Zhang, 2013) however, it hydrolyzes the cellobiose and cellodextrins which are inhibitors for CBHs and EGs. Aerobic microorganisms like fungi and bacteria produce extracellular BGs whereas, anaerobic microbes produce BGs in the cytoplasm. Only termite gut symbionts like protozoa and bacteria produce both extra and intracellular BGs. Different BGs have a different source of production, like bacteria, fungi, plants, and wood-feeding insects with catalytic modules without carbohydrate-binding module (CBM) belonging to GHs families 1, 3, and 9.

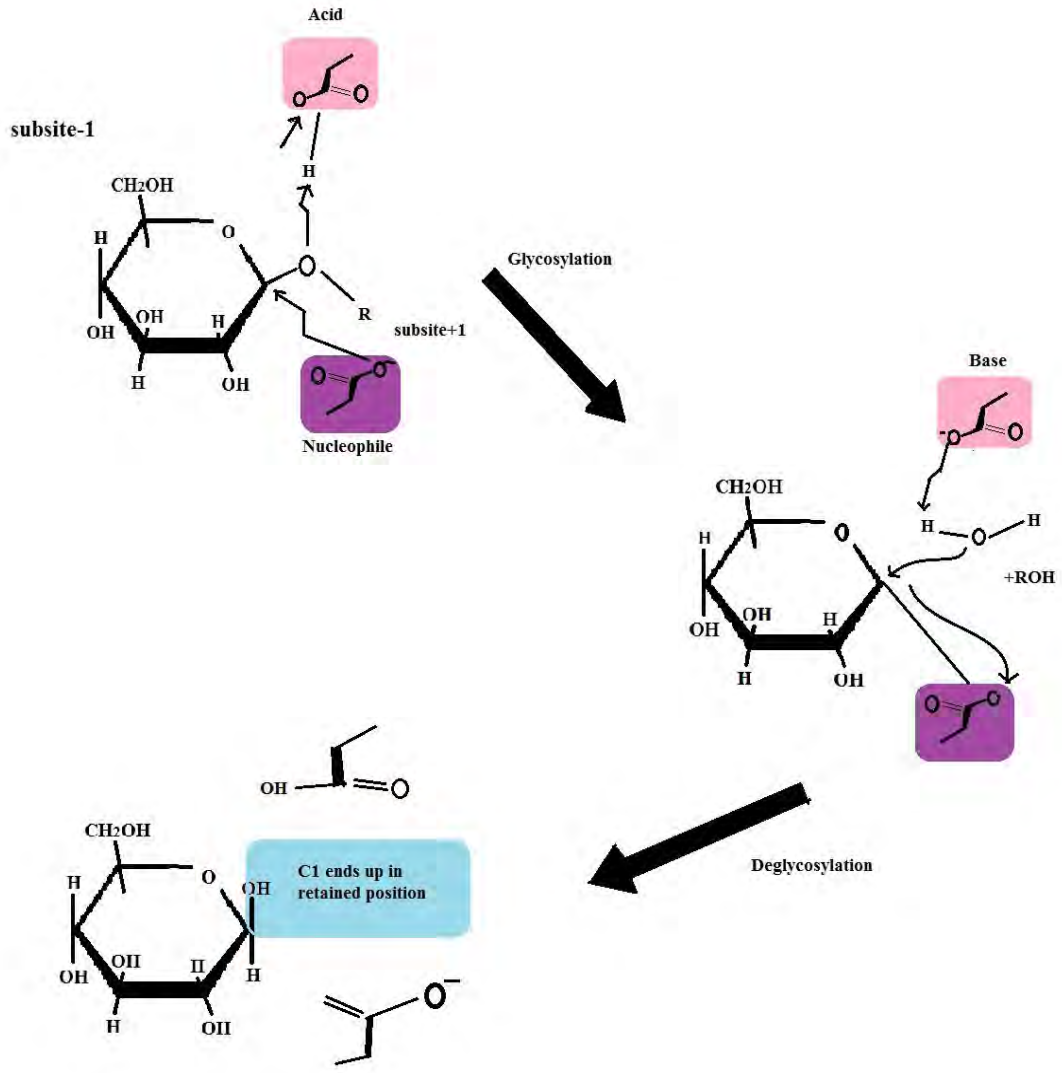


Figure 1.5. β-glucosidases retention via glycosidase mechanism in which one carboxylic group attacks the substrate as a nucleophile to form an intermediate. While other carboxylic group acts as an acid/base catalyst and perform glycosylation and de-glycosylation via protonating (Uchima, 2012).

1.5. Lower termite's cellulolytic systems and its role in biomass conversion: *Heterotermes indicola* and *Reticulitermes flavipes*

Heterotermes indicola Wasmann (Figure 1.6a) and *Reticulitermes flavipes* Kollar (Figure 1.6b) (Blattodea; Rhinotermitidae) are the most devasting subterranean and wood-destroying lower termites occur predominantly in tropical and subtropical regions of the world (Maiti, 2006; Buczkowski and Bertelsmeier, 2017). Besides, infesting wooden structures, *H. indicola* can damage other cellulosic materials. *H. indicola* colonies attack wood in service where their colonies

are cryptically located. Termites construct trails made up of mud for the movement from the soil in the direction of wood, which is attached or above the soil. Its fallouts in greater termite infestation by increasing the moisture content transferred from soil to structures (Kirker *et al*., 2013; Afzal *et al*., 2018).

Reticulitermes flavipes is also known as eastern subterranean lower termite and most commonly exist in North America. Eastern subterranean termites are the most economically wood devastating organism in the urban environment of the United States and characterized as pests. Termites' infestation is mostly invisible, because of their cryptic nature, they destroy the wood like a silent runner and most people become aware of their infestation when the annual swarming of reproductive caste (alates) occurs in structures.

Figure 1.6. Soldiers of (a) *Heterotermes indicola* Wasmann and (b) *Reticulitermes flavipes* Kollar.

Both species play a critical role in the degradation and recycling of wood or another lignocellulosic biomass. They are also considered as important pests because they eat our wood structure and cause significant economic loss by their cellulolytic mechanisms in their guts. However, it could be beneficial if these natural termite mechanisms and role serve as an ideal biomass conversion model of lignocellulose to valuable energy products. Recently, global warming and escalating costs of fossil fuel creating an alarming situation to find eco-friendly energy sources such as bioethanol from agricultural and forestry waste. According to an estimate, in the USA alone >1 billion tons/year of lignocellulosic biomass could be harvested that can replace approximately 30% of the total gasoline consumption in the US. In the process of bioethanol production, a crucial step is the hydrolysis efficiency of cellulases against the cellulose and hemicellulose.

Wood-feeding termites are one of the most important and effective sources of efficient cellulase producing invertebrates on the earth and their digestive system act as the most efficient

natural bioreactor in the world that could be valuable in exploiting the cellulose-based biofuel. Wood-feeding lower termites can degrade 74-99% cellulose and 65-87% hemicellulose from lignocellulosic materials (Prins and Kreuen, 1991; Tokuda, 2007; Uchima, 2012; Brune, 2014). They show multiple strategies for the metabolism of lignocellulose and count on intricate triple-stage cooperative communications with the unicellular zoo-flagellates and prokaryotic bacteria. Additionally, many lower termite species also secrete endogenous cellulases from the salivary glands and wall of the midgut. The prokaryotic biome in lower termite gut mostly consists of Spirochetes and Fibrobacteres that reflect distinct and novel biomass conversion pathways in the lower termite hindgut. To understand the utilization of lignocellulose by termite, distribution patterns of cellulolytic enzymes in the intestinal tract of termites are required, since it varies by termite species, caste, and developmental phases (Shimada and Maekawa, 2010; Ni and Tokuda, 2013). Generally, wood-feeding termites are belonging to lower termite groups that harbor the symbiotic flagellated protists in their hindgut. The symbiotic hindgut protozoa removal from the wood-feeding termite *Reticulitermes flavipes* caused in the forfeiture of wood-feeding capability of termite, that is indicating the significant role of this microbiota in wood digestion by releasing different cellulolytic enzymes (Cleveland, 1924; Uchima, 2012). In lower termites, a dual cellulolytic system has been purposed complete and efficient digestion of cellulose through the gut passage. They digest the cellulose by two types of cellulases.

i. **Endogenous cellulases**
ii. **Symbiotic cellulases**

Endogenous cellulases are secreted from the midgut wall and salivary glands, contain endoglucanases (endo-type cellulase) (Tokuda *et al*., 2004). These cellulases are coded by termites' genome whereas, symbiotic cellulases refer to enzymes produced by hindgut symbionts. Lignocellulose, particularly amorphous cellulose is partially digested by endogenous cellulase, and then partially digested cellulose travels to the hindgut, where symbiotic cellulase further digests crystalline region of cellulose by the gut microbiota through the endocytosing process. In the endogenous system, lower termites secrete endo-β-glucanases and β-glucosidases in the salivary glands, and these enzymes mixed with ingested wood materials that degrade the amorphous cellulose (Ni and Tokuda, 2013). The chewed wood particle partially digested in the midgut region. The endogenous enzyme and gut microbiota work synergistically to digest the cellulose efficiently,

but do not localize in the same compartment of gut (Tokuda *et al*., 2007). Generally, endogenous cellulases show strong hydrolytic activity against carboxymethyl cellulose (CMC) in the salivary glands, which is 45-85% of total termite gut cellulolytic activity (Tokuda *et al*., 2004). Endogenous β-glucosidase activity in wood-feeding lower termites is higher in the salivary gland than higher termites (Tokuda, 2002). The pertinent genes encoding of these carbohydrate degrading enzymes have been identified and divided into > 100 families and subfamilies on the basis of the similarity in the sequences of their amino acids (Watanabe and Tokuda, 2010; Lo *et al*., 2011; http://www.cazy.org). Frequently, adherents of the same glycoside hydrolase (GH) family posses' structural similarities but show different substrate specificities and it indicates their evolutionary origin.

To understand the **symbiotic cellulase** system, we must evaluate their role in biomass conversion by *in vitro* cellulolytic assays. Besides that, morphology, and genomic (cDNA library) properties of gut microbiota are also important to recognize their cellulolytic system. The cDNA library of the mixed population of gut flagellates and prokaryotes from different lower termite species has been constructed and examined in previous studies (Brune, 2014; Scharf, 2015b; Watanabe *et al*., 1998). The functionally important gene in the microbiota of termite gut has been screened out and analyzed comparison through expressed sequence tags (EST) [9]. Several endo and exo-type cellulases belonging to glycosyl hydrolase families (GHFs) 7, 9, 45, 5 cellobiose, and as well as β-glucosidases, hemicellulases coding genes have been identified in symbiotic microbiota of termites (Brune, 2014). The presence of a diverse gene pool in each cellulase gene family predicts that a variety of cellulases operate in concert to attain efficient digestion of highly polymerized cellulose material (Ohkuma, 2008). It is important to confirm the correlation between cellulase genes and activities expressed in microbial symbionts of termites' gut to determine the extent of enzymatic synergism because flagellated protists endocytose the cellulose and degrade within their body (Inoue, 2005).

Prokaryotes like bacteria, spirochetes, and nematodes also play a significant role to fix the product of cellulose. In lower termites the contribution of prokaryotes is probably low quantity but, it is not negligible. In higher termites, the roles of prokaryotes even fungi present in termite gut also help to digest the cellulose material. In lower termites, the digestion of cellulose in their

hindgut in highly efficient way is only possible due to the presence of cellulolytic bacteria and endo-flagellates.

1.6. Saccharification response of termites to lignocellulosic biomass

Lignocellulose biomass is a promising sustainable source for biofuel production because of its availability and richness on earth. However, it is critical to degrade the hemicellulose and lignin polymer compressing the cellulose polymer that is the major source of many renewable products. Therefore, the breakdown of lignin is important to access the core hemicellulose and cellulose. Functional lignocellulose dilapidation has been conversed mostly by hemicellulase and cellulases fit in to glucohydrolase superfamily (GHs) and other associative enzymes (Lynd *et al.*, 2008; Horn *et al.*, 2012; Jönsson *et al*., 2013; Levasseur *et al.*, 2013; Rajarapu and Scharf, 2017). As earlier mentioned, the complete depolymerization of cellulose required the synergistic action of endoglucanase, exoglucanase, and β-glucosidase, whereas, the breakdown of hemicellulose not only assisted by endo and exo-xylanase of GH5 but also depolymerized by GH9 endoglucanase in the wood-feeding termites of the current study that target the core fiber of cellulose from the center (Scharf *et al*., 2011a,b). In the last few decades, wood-feeding termites have gained the interest of scientists in the production of biofuel because of their efficient innate potential to digest the refractory agricultural biomass by enzymatically and have been studied to decipher lignocellulose mechanisms (Breznak and Brune, 1994; Geib *et al*., 2008; Sun and Scharf, 2010; Sethi *et al*., 2013a; Ni and Tokuda, 2013; Scharf, 2015; Janusz *et al*., 2017).

In the developed countries, such as in Japan, Germany, and USA, *Nasutitermes takasagoensis* a higher termite and *Coptotermes formosanus, C. gestroi*, and *R. flavipes* the lower termite have been studied broadly from genomic towards functions to elucidate their degradation termite physiology towards lignocellulosic biomass (Zhang, 2008; Franco Cairo *et al*., 2011; Scharf, 2015; Rajarapu and Scharf, 2017). Several enzymes belonging to diverse GH families have been recognized, isolated and checked their activity against different substrates (Tokuda *et al*., 1997; Watanabe *et al*., 1998; Tokuda *et al.,* 2007; Lo *et al*., 2011; Scharf *et al*., 2011). However, there is little known about the lignocellulose breakdown mechanism in wood-feeding insects belonging to different geographical regions which is a limiting factor in biomass saccharification. Termites species *Reticulitermes flavipes* belong to United States and *Heterotermes indicola* belong to South

Asia countries and considered as model organisms for lignocellulose saccharification [6,27]. In addition to endogenous secretory enzymes, these lower termites also harbor symbiotic protozoa and bacteria that work as fermentation analogs against lignocellulose biomass (Breznak and Brune, 1994; Morrison *et al*., 2009; Watanabe and Tokuda, 2010a; Scharf, 2015a). The recombinant and crude enzymes consistent with termite hemicellulases categorically showed their significant role in lignocellulose saccharification of a complex lignocellulose source pine dust, corn stove and soybean residue under *in vivo* and *in vitro* bioassay conditions [13,21].

1.7. Termite control background

a) Via Prevention

Termite infestation is like a menace, once the colony has settled, control of Rhinotermitidae family is very difficult due to their living habits, behavior, and adaptability characteristics. Consequently, in zones where termite infestation is possible, preventive measures are at a premium, since prevention is better than treatment [31].

b) Wood/tree species selection

Wood resistance or durability against termite infestation is always reliant on the species of wood/tree and termite (Rasib *et al*., 2017). Before using the wood in service, investigations about termite wood-feeding preference in laboratory and field condition is a key point. If a wood/tree species are considered termite susceptible and inadequately durable for a particular use, then precautionary actions should be adopted, such as satisfactory preservative treatment. Natural wood resistance against termites depends on the wood hardness and natural chemical repellent to termite present in wood (Su *et al*., 1984; Gautam and Henderson, 2011; Rasib and Ashraf, 2014). The discovery of natural toxin and hideous extracts has led to wider research for the toxin in plants. These toxins in resistant wood against termites may cause repellency, digestive toxicity, neurotoxicity, protozoacidal (antimicrobial agents) (Ahmed *et al.,* 2007; Ahmed *et al.*, 2011; Pandey *et al*., 2012; Abbas *et al*., 2013). Extraction of these toxins from wood/tree and their activities against termites have been reported in the last few decades (Ohmura *et al*., 2000; Bläske and Hertel, 2001; ; Bläske and Hertel, 2009; Elango *et al*., 2012; Hassan *et al*., 2017 Ahmed *et al*., 2018). Wood modification is also a relatively novel development in wood protection, but not a substitute for wood preservation. Inwood

modifications, a chemical reaction is applied with cell-wall components of woods that alter the wood properties including durability, hardness, dimension stability, and moisture resistance (Miltz *et al*., 1997; Jones *et al*., 2003). So, the selection of durable wood/tree species is significantly important to prevent termite attack.

c) Construction practices

Two major factors in the urban wood structure environment significantly affecting the risk of termite attack are moisture and the presence of deadwood wreckages. Moisture contents stimulate the subterranean termites strongly to establish their colonies in a building, although subterranean termites can transport the moisture by themselves by creating humidity in the tunneling tubes [31]. However, proper ventilation of subfloor areas, prevention of leaks in the plumbing drainage areas, control of seepage from attached water sources such as water tanks are essential for termite attack prevention. Modern building regime, in which cleanliness of wood debris in soil, avoiding direct contact of soil and wood, keeping wood structures furnished or painted are also important practices to prevent termite infestation [31].

d) Cutoff termite access to the structure

To keep away wood structure from termite access in the urban environment, different physical and chemical barriers have been developed (French, 1994; Lenz, 2002; Su, 2002; Nobre and Nunes, 2007). In physical barriers, civil engineers prefer to dig out buffers containing mineral particles of different grades, stainless steel meshes, and concrete with plastic sheets along the building foundation (Su *et al*., 1991; Ewart, 2000). Insecticides are also used in chemical barriers that are used along with or beneath the building foundation, and these pesticides should be active for 5-10 years and should not be harmful to human health and the ecosystem. Besides, that the blend of chemical and physical barrier, such as polymer sheets impregnated with insecticides has shown significant reduction in termite breaching and it is commercially practicing [31]. However, the risk of leaching and underground water contamination with pesticides always causes serious environmental threats.

1.7.1. Control of active termite infestation

Once a termite sets its colony size in a building or wood in service, it will not be easy to

eradicate termite menace. There are few methods available to reduce the infestation, however, the success of all these methods depends on field and laboratory trial efficacy.

i. Via synthetic pesticides

The most common methods to control the existing termite infestation in wood structures are either fumigation or to apply chemicals extensively on wood in service and soil openly or using bait matrix system or induction of chemicals via pressure treatment to preserve timber (Su and Scheffrahn, 1998). Soil treatments approach by chemical insecticides have back draws in the form of emerging termite resistance and environmental concerns. Major pesticide classes pyrethroid, organophosphate, organochlorine, carbamates, and dicarboximides are commercially available and have been used on largescale after world war II.

Besides of it, the use of synthetic pesticides has been reduced the termites' attack significantly, however, the persistence, stability in soil and UV and pesticides' residues have created many environmental issues including bioaccumulation in the food web of the natural ecosystem [31]. Major insecticides are neurotoxic to invertebrate and vertebrates and inhibitors of mitochondrial respiration [2]. In brief, growing environmental awareness and new regulations for urban pest management have significantly cut off the amount of chemical use and enhanced their target specificity. This reduction in pesticide usage against termites has led towards a more integrated approach such as biological controls, and new generation chemicals (insect growth regulators) (Zhou *et al.*, 2008).

ii. Physical treatments

Non-chemical wood protection methods exist, however, a few documented and difficult at largescale applications [31,51]. In these approaches, only the woo log termites that are present in it will be demolished but the colony foundation will remain integral. These approaches are removal of queen and mound, flooding, suffocating by toxic repellents, electrocution, dielectric heating by microwave or radiofrequency energies, heat treatment and submit the infested materials to a low temperature such as liquid nitrogen (Su *et al*., 1991; French, 1994; Lewis and Haverty, 1996; Su, 2002). All these methods are localized and just for infested spot treatment and its efficacy strongly dependent on accurate delimitations of termite infestation. In the case of subterranean termites, the tunneling galleries have a diffused

interconnected network and temperature treatments cannot eradicate whole termite colony and with time, structures can be reinfested.

iii. Biological control

In biological control, natural enemies of termites such as predators (ants, lizards, frogs, and mammals), parasites (nematodes), and pathogens (entomopathogenic fungi) are involved in termite control (Grace, 1997; Rath, 2000; Chouvenc *et al*., 2011). These methods are expensive and have concerns about outdoor applications and to maintain the quality of wood in service after application [55]. Entomopathogens such as nematodes and fungi are under manipulation and suggested as a possible non-synthetic pesticide alternative for termite control (Grace and Zoberi, 1992; Culliney and Grace, 2000; Wang *et al*., 2002; Wu *et al*., 2014; Damme *et al*., 2016; Cuthbertson and Audsley, 2016). In backgrounds of nematodes are considered as a rapid host-seeking and aggressive penetrating microbial agent [61,62]. In some reports, nematodes reduced their infection due to termite resistance such as grooming behavior of termites and to culture and maintenance of nematodes are difficult, which increases the failure of nematode treatments as termite control agents [63].

In cases, of fungi, several strains have been studied and evaluated as termite control pathogens, but results are still not conclusive at commercial or field level. Two virulent strains, *Metarhizium anisopilae,* and *Beauveria bassiana* have been most widely documented and studied for termites control pathogens (Yendol and Paschke, 1965; Bao and Yendol, 1971; Lai *et al.,* 1982; Rath, 2000; Wright *et al.,* 2002; Chouvenc *et al.,* 2011). Entomopathogenic fungi invade the insect via integument and lead the host to death through depleting their metabolic activities by releasing toxic in their body. The spores of fungi are likely to be transferred to another nestmate by grooming activities and if fungal pathogens become dormant and unable to achieve their virulence in termites, then defense mechanism of colony decreased the infection [59,65,67,69]. Field application of fungal conidia showed variable results against different termite species in a different environment. Since environmental factor always affects the virulence and inhibit the germination of conidia in field colony that cause the ineffectiveness of field treatment. These pathogens could be

effective at short-term to eliminate active infestation through local treatment, but as long-term pathogenicity decreases by environmental and termite resistance mechanism.

iv. Baiting system

In the last 20 years, the baiting system is found most successful management tool in developed countries to eliminate termite infestation. In baiting method, a very low amount of the active ingredient is used by mixing with an attractive substrate to enhance the foraging activities, since the effectiveness of bait depends on the willingness of termite to consume the bait (Rojas and Morales-Ramos, 2001; Potter and Hillery, 2002; Rojas and Morales-Ramos, 2009; Verma *et al*., 2009; Rust and Su, 2012; Evans and Iqbal, 2015). If a toxicant bait is presented with an optimal food source, it increases the high foraging activity and resulted in a high intake of toxicant too [70]. In temperate areas, bait technology showed significant results to reduce the termite's infestation in temperate regions, however in tropical areas, the baiting system showed variable results, maybe due to diverse and high richness of termites. Another back draw of the baiting method is that the population of termite colonies doesn't suppress so immediately.

v. RNAi as biopesticide

RNA interference (RNAi) signifies a revolutionary technology for performing functional genomic research for highly targeted pests. RNAi technique also known as post-transcriptional gene silencing (PTGS) and it is a normal mechanism of gene regulation and a defense system against viruses in a eukaryotic cell by depletion of the mRNA and significant reduction in targeted gene expression (Hannon, 2002; Baum *et al*., 2007; Zhu *et al*., 2008b; Kupferschmidt, 2013). The significant advance has been made in this technique in the field of pest management because of elucidation of gene silencing mechanism in eukaryotic organisms (Price and Gatehouse, 2008; Zhang *et al.,* 2013; Zotti *et al.*, 2018). The delivery of double standard RNA (dsRNA) by RNA interference transformative approach (transgenic food) has shown a promising method to induce gene silencing in a specific pest, however, it is still not still practical to every target under field conditions. Another major limitation of this technique is to maintain transgenic food stability for termites and regulatory approval, which is a costly and time-consuming process.

1.7.2. Termite control strategies used in this study

a) Cellulase inhibitors as termite control agents

The field applicability of all above mentioned termites control methods have limiting factors such as environmental, cost effective and robustness concerns. So, there is a need of specific-targeted ecofriendly approach in termite control strategies. As mentioned earlier, the wood digestion mechanism in termites carried out by the collaborative action of three types of cellulases endoglucanase, exoglucanases, and β-glucosidases and convert the cellulose into monomers such as glucose and energy with approximately 100% efficiency (Breznak and Brune, 1994; Inoue *et al*., 1997; Watanabe *et al*., 1998; Watanabe and Tokuda, 2010b). Substantial research has been performed toward the discovery and characterization cellulases in termites, however, disproportionally a few types of research have been carried out towards exploring enzyme activities as a novel target in the control strategies of termites. In past few years, only a few beta-glucosidase inhibitors including palladium, gluconolactone, *p*-coumaric, sinapinic and ferulic acid, conduritol B epoxide (CBE), and 1-deoxynojirimysin have been used in different study of cellulase inhibitions (Peciarová and Biely, 1982; Dale *et al*., 1985; Fan and Conn, 1985; Gross *et al*., 1986; Legler and Bieberich, 1988; Legler, 1990; Lassig at al., 1995; Zhang *et al*., 2012; Zhu *et al*., 2005). In this sense, a few studies have been conducted and observed minor inhibition of the cellulase in *Coptotermes formosanus* under *in vivo* conditions using numerous carbohydrate-based and non-carbohydrate-based inhibitors in the United States (Zhu *et al.,* 2005; Zhou *et al*., 2008). However, they just used selective no-choice assay conditions and measured limited parameters. Besides, no such studies or projects carried out in developing countries, where the tremendous use of synthetic pesticides creating serious environmental issues such as in Pakistan.

Keeping in view the importance of cellulase as target agent in termite control strategies, it was also an aim of present study to target the cellulolytic system of lower termites by inducing non-toxic carbohydrate-based inhibitors.

b) Toxic plant extract as termite control agents

Up to the present time, a myriad of laboratory and field studies have been conducted to test the efficiency and safety of plant-based insecticides to develop an innovative method to control subterranean termites. Nearby interest has been increased for naturally occurring botanical

compounds for use as insecticides and wood preservatives (Fatima and Morello, 2015; Gonzalez-Laredo *et al*., 2015; Hassan *et al*., 2017). Most plant-based pesticides hinder the foraging activities, growth, reproduction, and mating processes.

The secondary metabolites reside in the plants are considered as primary and common source of biopesticides and these compounds are not directly involved in the physiology and biochemical processes of the plants [93]. Several studies have examined the extracts of *Tectona grandis* L. (Teak), *Azadirachta indica* A. Juss. (Neem), *Eucalyptus camaldulensis* Dehnh (River red gum), *Pinus roxburghii* Sarg. (Chir pine), *Dalbergia sissoo* Roxb. (Indian rosewood), *Chamaecyparis nootkatensis* D. Don (Alaskan yellow cedar), *Thuja plicata* D. Don (Western red cedar), *Sequoia sempervirens* D. Don (Red wood), *Juniperus occidentalis* (Western juniper), *Morus alba* L. (White mulberry), *Targetes erecta* L. (Marigold), *Jatropha curacus* L. and neotropical *Solanum* sp. as possible alternative biocides (Carter *et al*., 1983; Grace and Yamamoto, 1994; Maistrello *et al*., 2003; Acda, 2009; Kirker *et al*., 2013; Hassan *et al*., 2017). Botanical biocides occur as naturally sequestered compounds in leaves, bark, and heartwood of plants and trees, where they often confer resistance to xylophagous insects (Adedeji *et al*., 2017). These bioactive compounds can induce contact repellency and reduce feeding in termites. Hassan *et al*. (2017) reported squalene, 2-methyl-9, 10-anthracenedione and 1-methyl-3, 4-dihydroisoquinoline as termiticidal agents isolated from Teak heartwood (*Tectona grandis* L.) extract.

In a similar study, Hassan *et al*. (2018) found high level of resorcinol in white mulberry that was toxic to *Reticulitermes flavipes* (Kollar). Rasib *et al*. (2017) showed that coniferyl alcohol from *Saraca asoca* Roxb. (Ashoka) and carboxylic acid from *Magnolia grandiflora* L. (Southern Magnolia) were feeding deterrents to *Odontotermes obesus*. The compounds chamaecynone from *Chamaecyparis pisfera* (Swara cypress), limonoids from *Phellodendron amurense* (Amur cork tree), Azidiractin from *Azidirachta indica* (Neem), loganin from *Guettarda speciosa* (Zebra wood), 7-methyljuglone from *Diospyros virginiana* (Eastern persimmon), ferruginol and nezukol isolated from *Taxodium distichum* (Bald cypress) were found toxic to termites (Saeki *et al*., 1973; Carter *et al*., 1978; Yaga and Kinjo, 1985; Scheffrahn *et al*., 1988; Kawaguchi *et al*., 1989; Ishida *et al*., 1992). Different solvents and aqueous extractives of *Eucalyptus camaldulensis* Dehnh, *Dalbergia sissoo* Roxb, *Melia azedarach* Linn, *Pinus roxburghii* Sarag, *Morus alba* L., *Diospyros sylvatica* Roxb and *Azadirachata indica* Juss. have been exhibited the antitermitic activities

(Qureshi *et al*., 2015; Hassan *et al*., 2016; Mankowski *et al*., 2016; Afzal *et al*., 2017). More plant species such as the *Lantana camara* leaves extract, the rhizome extract of *Alpinia galangal*, the seed and leaves extract of *Azadirachta indica*, *Jatropha curcas*, *Maesa laceolata*, *Chenapodium ambrosoids* and *Vernonia hymenolepis* have been previously testified as promising candidates in termite control studies [42,94–96].

In addition, naturally occurring biopesticides particularly plant-based toxic compounds and minerals usually degraded in a short period of time and not persistent in the environment, hence these type of pesticides are more safer to the environment [94,97]. So, keeping in view the importance of naturally occurring plant-based biopesticides, the leaves extract of Silver oak (*Grevillea robusta*) commonly found in Pakistan was trialed against the building infesting subterranean termite species.

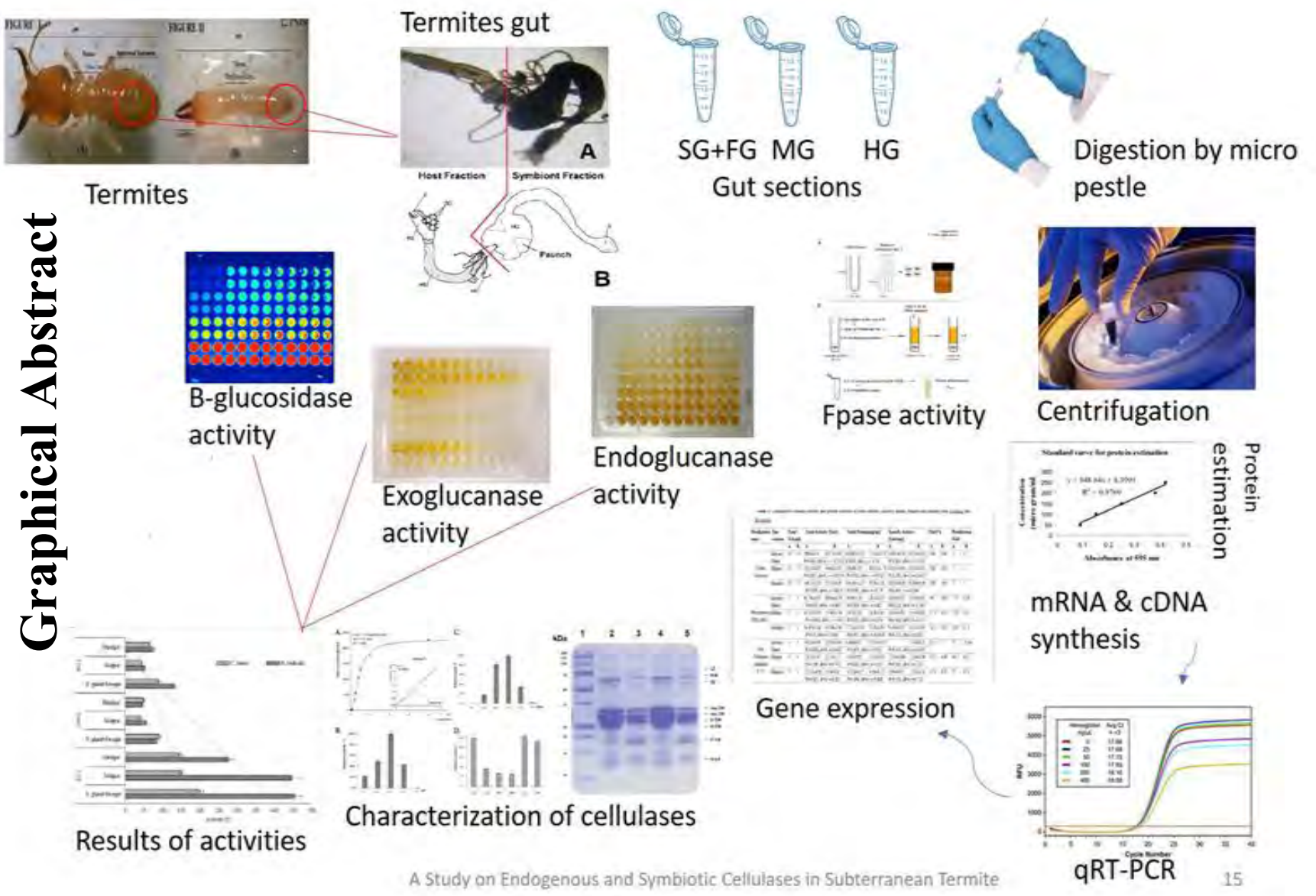
Objectives

Keeping in view, the high concerns of the urban environment regarding energy sources and environmental contamination by using chemical insecticides in termite control measures, the following main objectives have been carried out in this study.

- To explore the distribution of endogenous and symbiotic cellulase activities and their correlation with cellulase gene expression in the gut of two subterranean termites *H. indicola* and *R. flavipes*.
- To isolate and evaluate the potential of endogenous and symbiotic cellulase fractions for the saccharification of agriculture feedstocks by the lower termites *H. indicola* and *R. flavipes*.
- To isolate potential cellulolytic bacterial strains from the hindgut of termites (*H. indicola* and *R. flavipes*) and explore their saccharification potential and characterization.

Termite Control

- Carbohydrate-based inhibition of the cellulolytic activities in *H. indicola* and *R. flavipes* infestation using carbohydrate-based inhibitors under *in vitro* and *in vivo* conditions: Evidence from dry and wet laboratory approaches.
- To examine biocidal effects of toxic plant in termite control strategies.


Graphical Abstract
Termites
Termites gut
A
Host Fraction
Symbiont Fraction
Paunch
B
SG+FG
MG
HG
Gut sections
Digestion by micro pestle
B-glucosidase activity
Exoglucanase activity
Endoglucanase activity
Fpase activity
Centrifugation
Protein estimation
mRNA & cDNA synthesis
Gene expression
Results of activities
Characterization of cellulases
qRT-PCR
A Study on Endogenous and Symbiotic Cellulases in Subterranean Termite
15

Distribution of cellulolytic activities and their correlation with cellulase gene expression throughout the gut of *Heterotermes indicola* and *Reticulitermes flavipes*

Abstract

Lower subterranean termites primarily feed on wood and wood products due to their dual cellulolytic systems comprising endogenous and symbiotic origin. This study explored the distribution of cellulase activities and their correlation with cellulase gene expressions throughout the gut of two lower termites belonging to different geographic regions. Primarily, the present study mapped out the distribution of total cellulase, endoglucanase, exoglucanase, β-glucosidase, and xylanase activities throughout the body regions (head, salivary glands, foregut, midgut, hindgut, and whole gut) of termite species *H. indicola* and *R. flavipes* using specific substrates. Native SDS-PAGE confirmed the cellulase activity distribution patterns by exhibiting the relative protein bands distribution of specific size throughout the termite body regions. This study also recognized seven partial genes encoding the two endogenous and five symbiotic cellulases belonging to different glucohydrolase families (GHFs), which are GHF 1, 7, 9, 42, 45, 11 and 7-5 in *H. indicola* and *R. flavipes*. The quantitative real-time PCR (qRT-PCR) exposed that β-glucosidase and endoglucanase genes which encode GHF1 and GHF9 cellulases significantly expressed high in salivary glands as compared to other gut parts respectively, whereas exoglucanases (GHF7) and other cellulases (GHF 42, 45, 11 and 7-5) expressed only in hindgut region (where cellulolytic protozoa reside). Cellulase genes' expression positively correlated with respective specific types of cellulase activities measured in each body part of termite. *T-test* results suggest that there is no significant difference between *H. indicola* and *R. flavipes* regarding cellulolytic activities and gene expression outcomes.

2.1. Introduction

Cellulose is the major component (45-54%) of plant biomass and the most abundant carbohydrate-based energy source in the natural ecosystem. Cellulose a linear natural polysaccharide composed of D-glucose subunits linked with β-(1,4)-glycosidic bonding to form long chains of cellulose microfibrils (Tomme *et al*., 1995; Uchima *et al*., 2009). Besides, the intimate association of cellulose with hemicellulose and lignin that create a complex network, decrease the cellulose hydrolysis and accessibility of cellulolytic enzymes to cellulose fibers (Uchima, 2012).

The cellulose biodegradation is mainly achieved by all cellulase producing organisms. However, the efficient hydrolysis of cellulose is performed by the combinatorial feat of three main cellulases identified as endoglucanases (EGs, EC 3.2.1.4), cellobiohydrolases (CBHs, EC 3.2.1.91), and β-glucosidases (BGs, EC 3.2.1.21) (Beguin and Aubert, 1994; Samejima and Igarashi, 2004; Sugio *et al*., 2006). While endoglucanases generate new ends of polysaccharides chain by arbitrary cleaving of β-1,4-bonds of amorphous cellulose, cellobiohydrolase also known as exoglucanase digest the reducing and non-reducing ends of polysaccharides by releasing cellobiose and cello-oligosaccharides that inhibit the further formation of glucose. Thus, β-glucosidase plays a crucial role by hydrolyzing cellobiose into glucose or oligomers and reduces such inhibition in the catalytic activity of exoglucanase or cellobiohydrolase (Lynd *et al*., 2002). Likewise, partially degraded crystal cellulose, which is not as firm as cellulose is readily decomposed by xylanases in termite (EC 3.2.1.8).

By considering the importance of cellulases (EGs, CBHs, and BGs) in biomass conversion, numerous studies have been reported on the cellulase isolation, production and characterization of cellulases from microbes such as bacteria and fungi for biomass utilization (McBride *et al*., 2005; Kotaka *et al*., 2008; Liu *et al*. 2009; Nascimento *et al*., 2010). Since it had been believed that only microbes could degrade the cellulose profoundly and that animals digest the cellulose by the symbiotic action of intestinal microbes. Although, Termites' digestive mechanisms are capable of metabolizing different biopolymers found in wood, soil components, and crops. Mechanisms such as grinding, decomposition, humification, and mineralization of cellulose resources contribute to nutrient recycling (de Albuquerque Lima *et al*., 2014). Subterranean termites are well known as worldwide pests that destroy the wood in service, crops, and tree plantation viciously. However, they also play a keystone role in the carbon cycle by owing a profoundly efficient ability to digest 74-99% of the cellulose present in the plants' biomass (Sugio *et al*., 2006; Strassert, 2010; Sharma *et al.*, 2015). Termites have been classified into lower and higher ones based on the presence or absence of symbiotic flagellated protozoa in their hindgut (Brune, 1998; Konig *et al*., 2013). Lower termites are aggressive wood-feeders, and the dual cellulolytic system is present in their gut passage for the complete cellulose hydrolysis known as endogenous and symbiotic cellulases (Nakashima *et al*., 2002; Ni and Tokudu, 2013; Konig *et al*., 2013). In earlier, it was thought that the digestion of cellulose in lower termites solely depends on gut endosymbionts (Cleveland, 1923). However, the discovery of symbiotic cellulase genes in the termite genome supported the hypothesis, that "gut symbionts are also related to other biological functions such as

acetogenesis, nitrogen fixation, and nitrogen fixation in termites" (Bignell, 2000; Scharf *et al*., 2015).

In the last decade of studies, it has been detected that both endogenous and symbiotic enzymes are found in lower termites (Scharf *et al*., 2003; Zhou *et al*., 2007; Sethi *et al*., 2013) and it supports the hypothesis that both endogenous and symbiotic cellulases help the termites in wood digestion. Both types of cellulases are postulated to be complementary for each other in the hindgut of termite for converting cellulose termites ingest (Nakashima *et al*., 2002; Brune, 2014). Although several endogenous and symbiotic cellulases and their coding genes have been identified from termite in the last decade, however, their relative role and expression in termites remain mostly uncharacterized. Previously, Zhou *et al*., (2007) discovered one endogenous origin cellulase gene Cell-1 belongs to GHF 9, and three symbiotic origin genes Cell-2, -3, and -4 belong to GHF 7 in *Reticulitermes flavipes*. Specifically, they have observed the GHF 9 related cellulases exclusively expressed in the salivary gland and foregut, whereas GHF 7 cellulases are highly expressed in the hindgut. Likewise, Nakashima *et al*. (2002) proposed two independent cellulolytic systems in *Coptotermes formosanus*, one is in the midgut called endogenous and the others in the hindgut which is from symbiotic flagellates. Another hypothesis developed after symbiotic communities' roles is that lower termites especially wood digesting termites have developed a collaborative cellulolytic system to get energy and carbon available in cellulose biomass (Zhang and Lynd, 2004; Tokuda, 2007; Zhou *et al*., 2007; Brune, 2014; Bujang *et al*., 2014a, b).

As a step forward in defining the wood digestion in lower termite, the main objective of this chapter was to expose the cellulolytic activities corresponding to cellulase gene expressions throughout the digestive tract in two subterranean termites *H. indicola* Wasmann and *R. flavipes* Kollar (Blattodea; Rhinotermitidae) belonging from different zoogeographic areas. Both species are the most devasting wood subterranean lower termites and *H. indicola* occurs predominantly in tropical and subtropical regions of Pakistan, India, Afghanistan, and China (Maiti, 2006), whereas *R. flavipes* is distributed in all states of USA except Alaska.

Specific objectives of this study were as follows; (1) to evaluate different cellulolytic activities in different gut regions of termite workers, (2) to determine the endogenous and symbiotic cellulase gene expression among different gut regions of termite workers, (3) to determine the correlation between cellulase gene expression and its activities and to compare all above objects between *H. indicola* and *R. flavipes*. By these study objectives, this study

displays durable contract among relative cellulase activities and gene expression distribution outlines for cellulase including endoglucanases, β-glucosidases, exoglucanases, and xylanases. The distribution patterns of cellulolytic potential in subterranean can be used in biomass conversion as well as novel targets in termite control strategies.

2.2. Materials and Methods

2.2.1. Termites

The colony of *H. indicola* was collected from the infested basement stores at the Department of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan in monsoon 2018, and identified as *H. indicola* colony by soldier morphology key (Akhtar, 1984). Baits using plastic bottles containing wet toilet tissue rolls and molasses were used to attract and collect termites. Bottles were buried to half-length in the bookshelves of a dark storeroom followed by regular inspection every 20 days. After every inspection, baits were brought to the laboratory termites were separated in specific dimensions (L×H×W= 15×20×15 cm) plastic box and kept at 26 ± 1°C and 60-70% RH (Watanabe *et al*., 2002). Whereas *R. flavipes* from the field colony was collected at Purdue University campus West Lafayette IN, the USA in summer 2019 using cardboard baits. After each collection survey, termites were brought to the laboratory and established termite colonies in a dark room within the sealed plastic box (30×24×10 cm) at 26 ± 1°C and 65±2% RH using pine wood steaks and paper towel beds to clear termite gut from wood debris. Experiments on *R. flavipes* were conducted in USA, while experiments on *H. indicola* in Pakistan.

2.2.2. Termite DNA, RNA isolation and cDNA synthesis

Termite genomic DNA was mined from termite head by following the protocol of DNeasy blood and tissue kit (QAIGEN®, USA) and used standard PCR procedure to amplify ten cellulase genes (both endogenous and symbiotic origin). Cellulase gene amplification by PCR were conducted by synthetic oligonucleotides on the basis of each gene ORF (Figure 2.0), through the templates of cDNA and DNA genome. Total RNA was extracted from hindgut, midgut, foregut, salivary glands, head, symbionts, and caracas of termite using SV total RNA isolation kit (Promeg©, Wisconsin USA) to investigate the distribution and expression of cellulase genes. cDNA was synthesized from 4 µg of total RNA extracted from each part of the termite's gut using the SensiFast™ cDNA synthesis kit (BioLine, USA).

The total RNA, cDNA and genomic DNA quality and quantity were assessed with nanodrop (NANODROP 2000C, Thermo Scientific) and agarose gel electrophoresis (Sambrook *et al*., 1989).

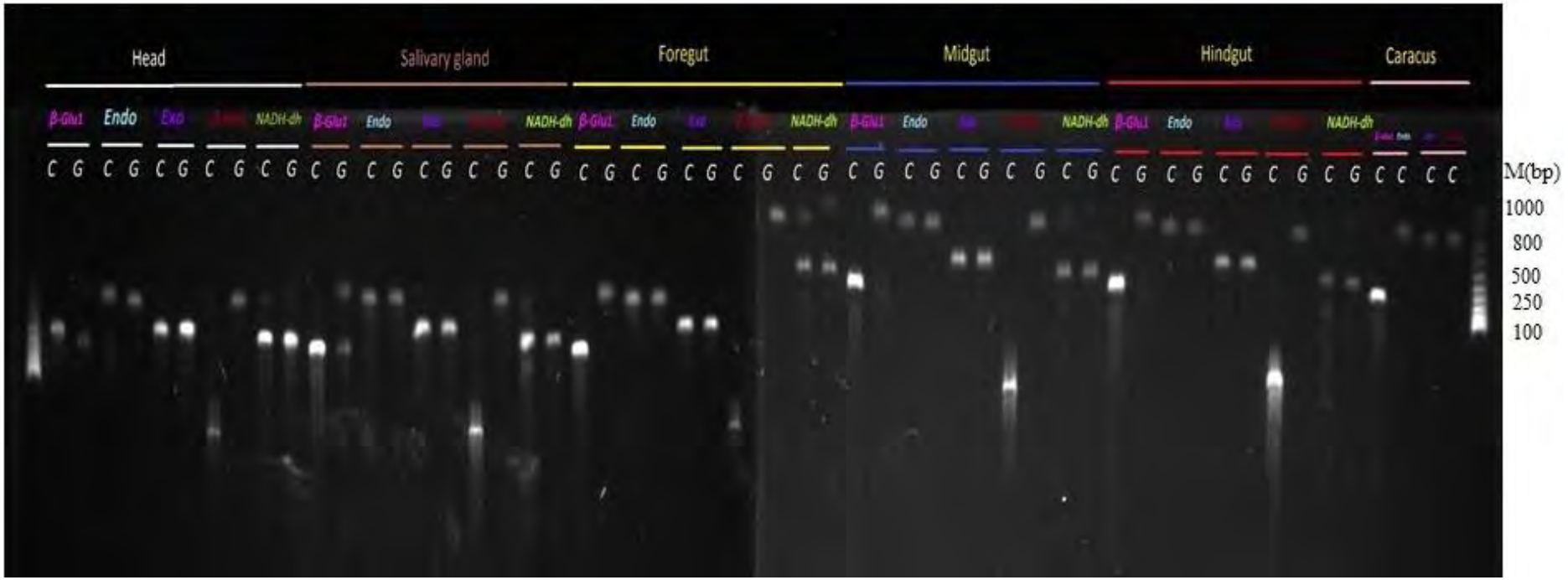


Figure 2.0. Gel electrophoresis confirming the results of partial cellulase genes expressions by qPCR using genomic DNA (G) and cDNA (C) across the different body sections of termites.

2.2.3. Phylogenetic analysis

To find the regional similarities among cellulase sequences of termites, parallel quests with resulting the nucleotide sequences were carried out using both BLASTx and BLASTn tools in GenBank NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) under default settings and E-value for the

implication of similarity were 2×10^{-10} and smaller (Eric *et al*., 2014). Amino acid sequences were aligned with other close termites and symbionts cellulase genes using Mega X software.

To determine the origin of partial amplified cellulase genes of *R. flavipes* and *H. indicola* and their partial amino acid sequences were used for alignments and phylogenetic analysis using MEGA X software. The neighbor-joining method was used to infer the evolutionary history during tree construction of cellulase genes and evolutionary distance was used to infer the tree. A p-distance method was used to compute the evolutionary distances, which are in units of several amino acid differences per site. Tree analysis involved 67 partial and complete amino acid sequences and ambiguous positions were removed using pairwise deletion option in MEGA X.

2.2.4. Expression profiling of cellulase genes

Expression of 07 cellulase genes (Table 2.1) belonging to different glucohydrolase families in the hindgut, midgut, foregut, salivary glands, head, symbionts, and caracas of termites were exposed by the quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using the CFX™ Real-Time PCR detection system with SensiFAST™ SYBR® No-ROX Mix (BIOLINE, USA). Primers for qRT-PCR were designed from Expressed Sequence Tag (EST) data of termite species *R. flavipes* (Scharf *et al*., 2003) using Primer 3 online software (http://bioinfo.ut.ee/primer3-0.4.0/).

Primer sequences with temperature and references are provided in Table 2.1. qRT-PCR was performed as described previously (Scharf *et al*., 2005; Zhou *et al*., 2007) using *NADH-dh* as a reference or control gene. However, SensiFAST™ SYBR® No-ROX Mix protocol amplification conditions; initial denaturation 95°C for 2 min, denaturation at 94°C for 10 sec, annealing at 60°C for 20 sec, extension at 72°C and melting curve from 50 to 90°C with increment 0.5°C were used to amplify the target and control genes. The quality of primers and qRT-PCR amplification efficiency for both control and target genes were verified by the presence of a single gene-specific product by agarose electrophoresis, melting curve, and sequencing of products. Simultaneously, relative expression levels for specific target genes in different parts of termites were normalized to reference or control gene *NADH-dh* and determined using $2^{-\Delta\Delta CT}$ method reported by Livak and Schmittgen (2001). ANOVA test followed by LSD *t*-test for mean separation was performed using statistical software SPSS IBM 20 to determine significant relative gene expression.

Table. 2.1: Primers details that were used in the study of cellulase gene expression change using qRT-PCR.

Target	Primers Sequence (5-3)'	Annealing(°C)	Source
Exoglucanase	*Exo* F- GGCCTTGCAAATTGTGAGGG	60.00	This study
GH7	*Exo* R- CCAAAACCACCCCAGGATCA	59.90	This study
Endoglucanase	*Endo* F- TCACAAGCAAGCAGGCATAC	60.02	[99]
GH 9	*Endo* R- CATACGCGCGATAAGTAGCA	60.02	[99]
β-glucosidase	*Bglu* F- TGAAAATGGCTTCTCCGACT	59.81	This study
GH 1	*Bglu* R- TTGCTGACTCCTTGGGAACT	59.84	This study
GHF 45	GHF 45F- AAAGCTGATGTCTCCCAGCC	50	This study
	GHF 45R- CGGCGGCAAAACCATATGAG	50	This study
GHF 42	GHF 42F- GCTATGGGCAGACAAAGGGT	50	(Peterson *et al*., 2015)
	GHF 42R- TTGTCACTGCGAAGTCCTGG	50	(Peterson *et al*., 2015)
GHF 11	GHF 11F- CTTCAGGGTGCCCGAAGAAT	50	This study
	GHF 11R- AAACCCGAGGGAATTCCACT	50	This study
GHF 7-5	GHF 7-5F- TCATGACAGCGGGACGACTAAT	50	This study
	GHF 7-5R- CCGAATAATCTGCTCCCTCAAT	50	This Study
NADH-dh	F- CCTCAGCAACGCTACTATTC	50	[101]
Reference	R- CCCAATGGCTGATAACAC	50	[101]

2.2.5. Termite tissue preparation

Group of 40 termite workers collected by aspiration, weighed and immobilized on ice 5-10 minutes before dissections and manipulation. Overall, six groups of 40 termite workers were dissevered, 03 were dissected and stored in 100 mM sodium acetate buffer (SAB, pH 5.6) and the other three in 100 mM sodium phosphate buffer (SPB, pH 6.5). The former buffer used total cellulase activity, endoglucanase, β-glucosidase, and xylanase activity, whereas the latter was used for cellobiohydrolase (exoglucanase) assays. Individually termite was truncated, and last abdominal segment was pulled to remove whole gut by using sterilized forceps. The whole termite body was divided into head, salivary glands, foregut, midgut, hindgut, and symbionts. Tissue extracts were prepared according to Zhou *et al*., (2007) with minor modifications. Each tissue was placed in a microcentrifuge tube and homogenized with a micro pestle attached to a homogenizer motor in an ice-cooled 1 ml buffer. The resulting homogenates were centrifuge at 14000 rpm and 4°C for 20 minutes. The supernatant was collected and referred to as crude cellulases.

2.2.6. Protein estimation and SDS-PAGE

Soluble protein contents of the crude cellulases were determined by the Bradford assay. Enzyme extract (160 µl) was combined with 360 µl of Bradford reagent and add 1050 µl water to bring the final volume to 1.5 ml in test tubes. Absorbance was read at 595 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad USA). The standard curve was produced by combining BSA standard with Bradford reagent in the same amount as that of enzyme extracts. The molecular weight of the cellulolytic enzymes in crude and purified form was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli *et al.* (1976) using 5% staking and 10% resolving gel. The gel was stained using 250G Coomassie brilliant blue and de-stained in de-ionized methanol acetic acid-water (Sambrook and Russel, 2001). The approximate molecular weight of cellulases enzymes was projected by running illustrations in SDS-Page and compared standard PageRuler^{TM} pre-stained protein ladder, 10-180 kDa (Thermo-Scientific Pvt Ltd., USA).

2.2.7. Cellulase activity assays

i. Total cellulase activity

The total cellulase activity of the crude extract was determined under the protocol pronounced by the International Union of Pure and Applied Chemistry (IUPAC) using the Whatmann No. 1 paper as a substrate. Filter Paper Unit (FPU) was determined and defined as the micromole of glucose equivalent liberated per mint of reaction. The strips of Whatman filter paper No. 1 (1×4 cm) were dipped 1 ml of 0.1 M sodium acetate of pH of 5.5 as substrate and for blank reagent, the filter paper was incubated just with 1.5 ml of buffer. Before adding a 0.5 ml enzyme, the reaction tube was warmed up to 50 °C and after enzymes addition, the tube was incubated at 50 °C for 1 hour. The reaction was stopped by adding 3 ml of dinitro salicylic acid (DNSA) reagent and boiled for 5 min followed by cooling at ice for 5 min. Absorbance was taken at A_{540} through spectrophotometer (SmartSpec 3000, Bio-Rad USA) and determined total cellulase activity by comparing OD with glucose standard using a linear equation.

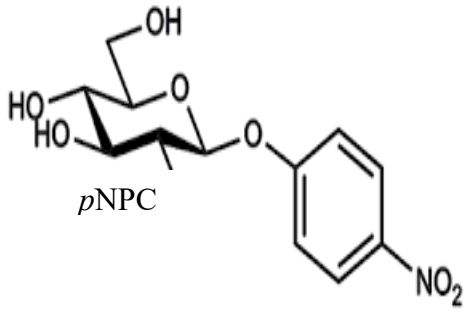
ii. Endoglucanase activity

Endoglucanase activity was determined using 0.5% w/v carboxymethyl cellulose (CMC; Sigma, St. Louis, USA) diluted with 0.1 M sodium acetate buffer (SAB) of pH 5.5 as substrate. 100 µl crude enzyme was mixed with 400 µl of the substrate and 500

µl of acetate buffer was used as the reagent blank. The reaction was incubated at 40 °C for 1 hour and stopped by the addition of 1 ml of tetrazolium blue reagent. The reaction was boiled for 5 min and cold on ice to measure the absorbance of reducing sugar at A_{660} using a spectrophotometer (SmartSpec 3000, Bio-Rad USA). The resultant absorbance was analyzed by linear equation using glucose as standard. The unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg glucose per minute/ml.

iii. Exoglucanase activity

The substrate *p*-nitrophenyl-β-D-cellobioside (pNPC; Sigma, St. Louis, USA) at 10 mM was used to determine the exoglucanase activity of crude extract of subterranean lower termites *H. indicola* and *R. falvipes*. The substrate was diluted with 0.1 M sodium acetate buffer of pH 5.5. The reaction was initiated by mixing 100 µl of crude with 400 µl of the substrate and 500 µl of 0.1 M acetate buffer of pH 5.5 was used as the reagent blank. The reaction was incubated for 30 min at 45 °C and stopped by adding 1 ml of 0.6 M Na_2CO_3. The hydrolysis product of pNPC is *p*-nitrophenyl (pNP) that is appeared in the form of yellow color. The absorbance of the reaction was read at 540 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad USA).



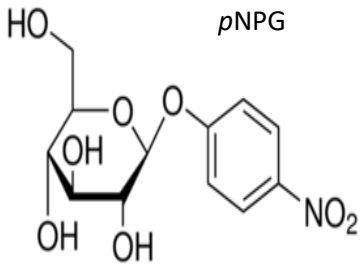
*p*NPC

One unit of exoglucanase (U) was defined as the amount of exoglucanase that releases 1 µmol of pNP per minute under standard assay conditions.

iv. β-glucosidase activity

The *p*-nitrophenyl-β-D-glucopyranoside (pNPG Sigma, St. Louis, USA) at 10 mM was used as a substrate to determine the β-glucosidase activity of *H. indicola* and *R. falvipes.* 100 µl of the crude enzyme was mixed with 400 µl of the substrate and 500 µl of 0.1 M acetate buffer of pH 5.5 was used as the reagent blank. The reaction was incubated at 45 °C for 30 min and stopped by adding 1 ml of 0.5 M Na_2CO_3. The indication of

yellow color defines the release of *p*-nitrophenyl (pNP) that was measured by taking absorbance at 410 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad USA).



The result was counted by comparing absorbance with a standard curve of *p*-nitrophenyl that was created using five different concentrations (0.0625, 0.125, 0.250, 0.500, and 1 mM). One unit of β-glucosidase (U) was defined as the amount of β-glucosidase that releases 1 µmol of pNP per minute under standard assay conditions

v. Xylanase activity

Xylanase activity was performed as described by Zhou *et al*., (2007) with a few modifications. The beechwood xylan as a hemicellulose substrate (>90% of xylose residues) was used by preparing a 0.5% stock solution in 0.1 sodium acetate buffer (pH 5.6). A stock solution was prepared by boiling the prepared solution for 2 hours until xylan fragments disappeared, then centrifuged at 4000 rpm, 22-25°C for 10 min, and collected supernatant as a stock solution. For the assay, the reaction mixture consisted of 10 µl of crude enzyme and 90 µl of substrate solution. Two parallel reactions were run at 25°C, one for 10 minutes to establish baseline activity and the other was run for 2 hours. The reactions were stopped by adding 100 µl of 1% DNSA solution and microplate immediately incubated at a boiling temperature in the water bath for 10 minutes before placing it on ice for 15 minutes. The absorbance was taken at 546 nm and the absorbance difference between 10 minutes to 2 hours was used to determine the specific activity.

2.2.8. Characterization of cellulase activities

The characterization of cellulase activities recovered through from ultra-centrifugation of crude enzymes was carried out by using different variables. The variables used to optimize the activity of cellulases were temperature, pH, metal ions, organic solvents, and inhibitors.

a) Effect of temperature

To evaluate the effect of temperature on the activity of cellulases (EGs and β-glucosidase activities), the reaction mixtures were incubated at different temperatures (35, 45, 55, 65, 75, and 85°C) for 30 min. The 3,5-Dinitrosalicylic acid method was used to determine the activity of EGs and xylanase, whereas β-glucosidase activity was used standard pNPG activity method as discussed in the enzyme assay section.

b) Effect of pH

The effect of pH on cellulase activities (EGs and beta-glucosidase activities) was studied by measuring the activity at varying pH values ranging from 4-10 using a buffer at concentration 0.1M sodium acetate. The reactions were incubated at 40°C for 30 min and all activities were assayed under standard conditions as mentioned in the enzyme assay section.

c) Effect of metal ions

Different metal ions were used [102] to determine their effect on EGs and beta-glucosidase activities. Metal ions $MgSO_4$, $CuSO_4$, $FeSO_4$, NaCl, NH_4Cl, and $BaSO_4$ were incubated with enzyme reaction mixture at 40°C for 30 min. All residual activities were estimated against the control (no metal ion) under standard assay conditions.

d) Effect of surfactants

The effect of surfactants (tween-20, tween-60, tween-80, triton X-100, SDS, commercial detergent like Surf) and oxidizing agents (sodium chlorite and hydrogen peroxide (H_2O_2) of concentration (1.0%, w/v) on EGs and beta-glucosidase activities was assessed. Reactions were incubated at 40°C for 30 min.

e) Effect of organic solvent on enzyme activity

All activities were incubated with 30% (v/v) of different organic solvents such as xylene, toluene, isopropyl, n-hexane, ethanol, n-Butanol, benzene, and chloroform at 40°C for 30 min. All residual activities were estimated against the control, in which the solvent was not present under standard assay conditions.

2.2.9. Kinetic analysis

The influence of substrates CMC, pNPG, and pNPC, on the reaction velocity of the cellulase activities, was determined according to Robyt *et al*. (1990). The crude cellulases were incubated with various concentration of CMC (0.5-4%), pNPG (0.125-15 mM), pNPC (0.5-25 mM), and xylan (0.5-2%). All enzymatic activities were assayed under the standard condition

as mentioned in the previous enzyme assay section The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Line weaver-Burk plots of Michaelis-Menten equation (Lineweaver *et al*., 1934).

2.2.10. Statistical analysis

Statistical analysis of specific activities among different termite gut tissues was determined using the One-way ANOVA LSD Tukey test. A paired *t-test* used to compare activities among termite species in SPSS software V 20.0. A Pearson correlation was determined among cellulase gene expression and cellulase activities in different gut regions using R. language.

2.3. RESULTS

2.3.1. Relative cellulase gene expression through the gut of *R. flavipes* and *H. indicola*

Two endogenous (*endo* and *β-glu*) and five symbiotic origins (*Exo*, *GHF 7-5, 45, 42,* and *11*) cellulase genes were expressed across the gut region of *R. flavipes* and *H. indicola* (Figure. 2.1). In these experiments, carcass (dead tissue without gut) was used as a control to normalize the expression profile of cellulase genes in different termites' gut regions. The expression levels of endogenous and symbiotic cellulases indicated different results in both termite species. Two endogenous (*endo* and *β-glu*) and one reference (*NADH-dh*) gene indicated similar reaction efficiencies, with r^2 value for CT values across the gut regions of both subterranean termites' ranges from 0.965 and higher. Endogenous *β-glucosidase* showed over 2500-fold higher expression level in the salivary gland relative to the other gut regions (F = 463.36 (*df:5*); P< 0. 001) in *R. flavipes*, whereas, in *H. indicola* < 2000-fold expression change in the salivary gland which was not significantly different (T-Value = 0.32; P-Value = 0.763) from *R. flavipes*, however significant different (F = 97.70 (*df:5*); P< 0.001) to their other gut regions.

Conversely, the second endogenous gene *Endo* exhibited approximately 2000-fold expression change in the hindgut of *R. flavipes* that were not sufficiently different only to other gut regions (F = 298.99 (*df:5*); P< 0.001). But it is also significantly different (T-Value = 1.54; P-Value = 0.0183) from *H. indicola*, which was approximately 1000-fold expression change in hindgut and highly different (F = 78.39 (*df:5*); P< 0. 001) to other gut regions. In case of symbiotic cellulases, *Exo, GHF 7-5, 45, 42,* and *11* were predominantly expressed in the hindgut relative to carcass tissues and another gut region of *R. flavipes* (F=843.19 (*df:5*); P< 0.001), but the expression fold is low than endogenous origin cellulase genes. *Exo* showed same distribution in the gut regions of *H. indicola* (F= 29.09 (df:5); P< 0. 001), however, there is not significantly different (T-Value = 0.67; P-Value = 0.530) among both termite species regarding symbiotic gene expression levels. Among all symbiotic cellulase, the cellulase gene belongs to glucohydrolase family 11 showed significantly higher (F=155.90 (*df:5*); P< 0.001) expression fold as compared to other symbiotic cellulase genes *Exo, GHF 7-5, 45* and *42* in the hindgut region of both subterranean termites. However, there was no significant difference in symbiotic gene expression among *R. flavipes* and *H. indicola* (T-Value = 0.16; P-Value = 0.879). All symbiotic cellulase genes and reference gene β-actin_5C1 reaction efficiencies showed little difference with r^2 value (0.875) for CT values.

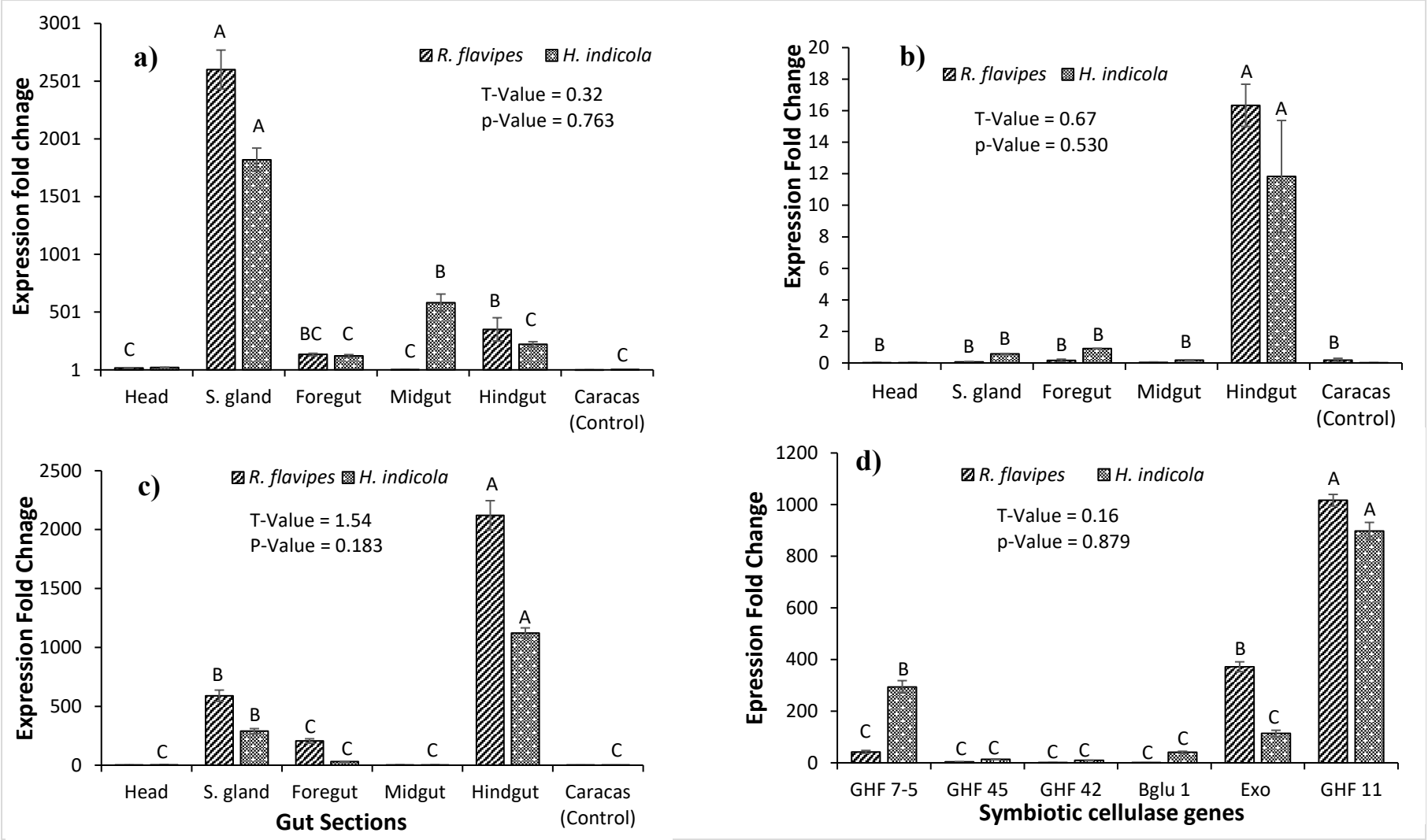


Figure 2.1. Cellulase gene expression fold of β-glucosidase (a), exoglucanase (b), endoglucanase (c), and (d) indicating symbiotic cellulases in thorough out gut region of *R. flavipes* and *H. indicola.* Alphabets indicating significant difference ($p < 0.001$) between different cellulase genes' expression in different gut regions using Tukey's LSD test.

2.3.2. SDS-PAGE

A distinct band of MW 60kDa was observed in a crude sample of the head region, a 48kDa in salivary gland and hindgut (Hg) region indicating the presence of β 1-4, endoglucanases (Figure. 2.2a). Lighter bands were observed in *R. falvipes* purified cellulases as compared to *H. indicola*. A prominent band of molecular weight of 68 KDa was observed in the whole gut, thorax, and abdomen region of *H. indicola*. which indicates the presence of β-glucosidase (Figure. 2.2b). There is no significant difference was observed between the distribution and size of β-glucosidase and endoglucanase in both species, while exoglucanase was observed only in *R. flvipes*.

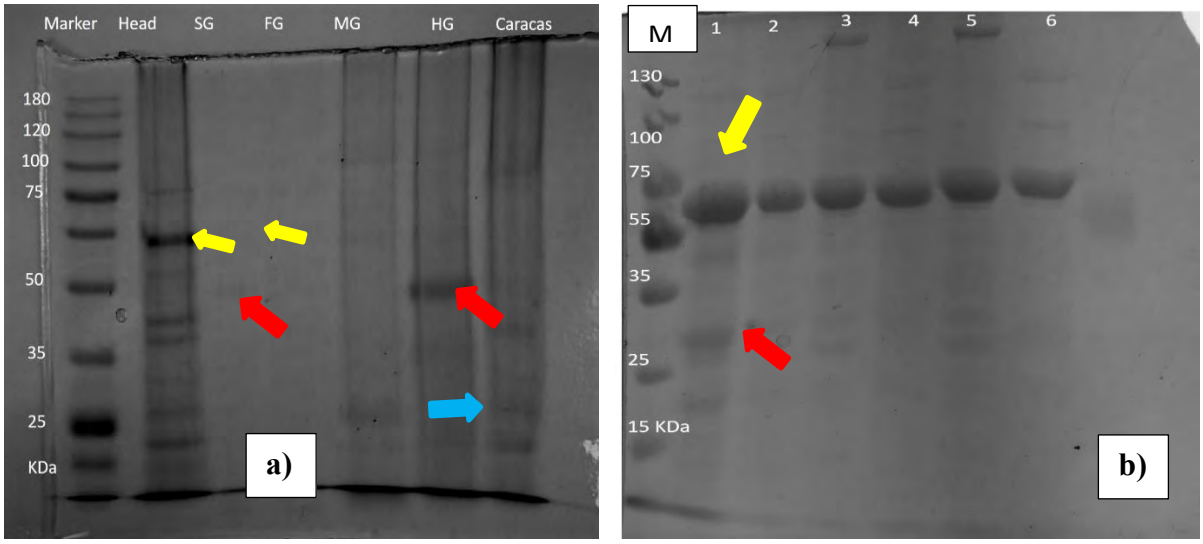


Figure. 2.2. Distribution of different molecular weight proteins in different gut regions of *R. flavipes* (a) Head, SG: salivary gland, FG: Foregut, MG: midgut, HG: hindgut and carcass. Whereas, figure (b) indicating protein distribution and purification in *H. indicola*, 1: whole gut, 2: gel purification of thorax region, 3: gel purification of abdomen region, 4: crude of thorax along the head region, 5: crude abdomen region, 6: precipitated protein from the head region. Red arrow indicating β 1-4, endoglucanases, while yellow indicating β-glucosidase, blue indicating exoglucanase**.**

2.3.3. Total Cellulase activity (FPase assay)

A significant difference (T-Value = 21.04; P-Value = 0.000) was observed among the distribution of FPase activity across the gut regions of *R. flavipes* and *H. indicola* (Figure. 2.3 and 2.4). In *R. flavipes*, the salivary gland showed significantly high (F= 76.60 *(df=6)*; P= 0.000) FPase activity (>2500 µmol/min/ml) by releasing maximum of glucose as compared to other gut regions. Whereas the minimum total cellulase activity was observed in the midgut of termite.

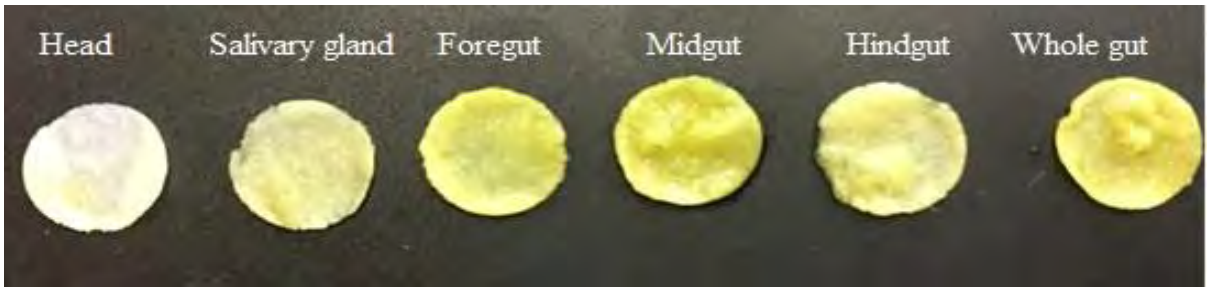


Figure. 2.3. Total cellulase activity of different gut regions of termites on Whatman No. 1 filter paper disks.

Likewise, the distribution patterns of total cellulase activity in *H. indicola* were the same as in *R. flavipes*, but the amount of activity was significantly low in *H. indicola.* In *H. indicola* maximum total cellulase activity was observed in salivary glands followed by protozoa extract > hindgut > head and midgut with significant difference (F= 71.18 (*df= 6)*; p = 0.000).

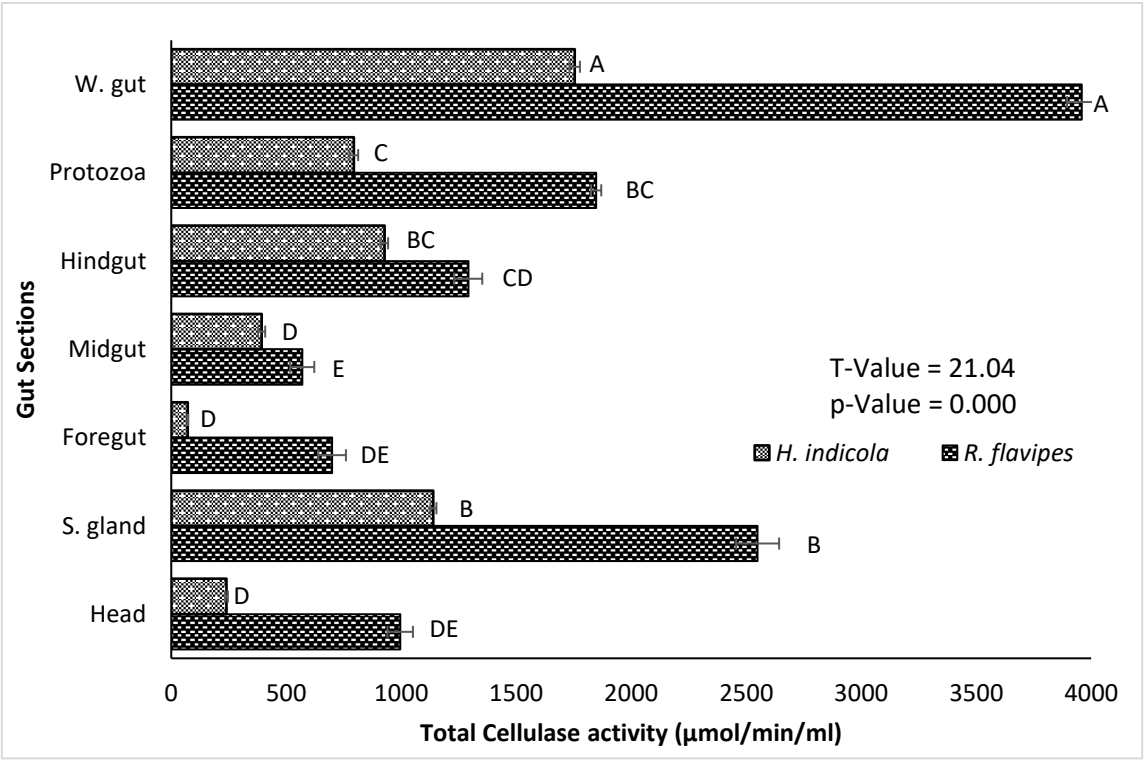


Figure. 2.4. Total cellulase activity among different gut tissues *H. indicola* and *R. flavipes.* Alphabets indicating significant difference ($p < 0.001$) between different cellulase genes' expression in different gut regions using Tukey's LSD test.

2.3.4. Endoglucanase activity

Endoglucanases are the important cellulases to start the mechanism of cellulose breakdown. Table 2.2. indicating total endoglucanase activity, total protein, and its specific activity per termite for *R. flavipes* and *H. indicola*. In *R. flavipes* the distribution of total endoglucanase activity was maximum (2312.75±34.82 µM/min) from the endogenous cellulase system that is

found in the salivary gland and minimum endoglucanase activity was observed in the head (157.26±10.67 µM/min) and hindgut (396.90±21.95 µM/min) region. whereas the total protein contents were maximum (103.01±5.12) in the head region followed by foregut (52.00±0.54) and hindgut (33.80±1.17 mg/ml). The specific activity of endoglucanase in *R. flavipes* was maximum in the salivary gland and foregut regions which are significantly different as compared to other gut regions ($F = 26.90$ *(df: 5);* $P = 0.001$).

Likewise, in the case of *H. indicola* endoglucanase total and specific activity was observed significantly different throughout gut regions, however, it was not significantly different from *R. flavipes* expect in the salivary gland and whole gut as shown by paired *t-test* in Table 2.2. Conversely, the protein concentration in *H. indicola* was observed maximum in the head region (124.47±0.12 mg/mL) and slightly more than *R. flavipes* (103.01±5.12 mg/mL).

Table 2.2: Comparative endoglucanase activity and protein contents of the crude enzyme from different gut regions of *H. indicola* and *R. flavipes*.

Gut section	Total Activity (IU=µM/min)		Total Protein (mg/ml)		Specific Activity (IU/mg/termite)	
	A	B	A	B	A	B
Head	157.26±10.67^{d}	240.6±8.76^{cd}	103.01±5.12^{b}	124.47±0.12^{b}	0.095±0.01^{d}	0.096±0.01^{d}
	T Value = -2.27; P-Value = 0.075-		T-Value = -2.74; P-Value = 0.056		T-Value = -0.06; P-Value = 0.480	
Salivary gland	2312.75±34.82^{b}	840.7±22.92^{b}	26.00±0.35^{d}	24.46±1.75^{d}	4.45±0.010^{a}	1.71±0.01^{a}
	T-Value = 10.5; P-Value = 0.006		T-Value = 0.68; P-Value = 0.717		T-Value = 5.52; P-Value = 0.984	
Foregut	425.54±7.95^{cd}	55.80±7.16^{d}	52.00±0.54^{c}	22.00±1.25^{d}	0.41±0.055^{d}	0.12±0.01^{cd}
	T-Value = 13.75; P-Value = 0.997		T-Value = 19.77; P-Value = 0.999		T-Value = 9.19; P-Value =0.994	
Midgut	659.44±11.30^{c}	446.1±11.33^{bc}	31.00±1.051^{d}	80.32±1.73^{c}	2.11±0.01^{bc}	0.27±0.08^{c}
	T-Value = 3.15; P-Value = 0.956		T-Value = -64.79; P-Value = 0.000		T-Value = 14.52; P-Value = 0.998	
Hindgut	396.90±21.95^{cd}	277.3±9.95^{cd}	33.84±1.17^{d}	97.8±1.185^{c}	0.60±0.01^{cd}	0.18±0.002^{cd}
	T-Value = 1.35; P-Value = 0.845		T-Value = -9.29; P-Value = 0.006		T-Value = 5.68; P-Value = 0.985	
Whole gut	2969.70±65.95^{a}	2021±12.33^{a}	164.00+2.50^{a}	173.40±10.2^{a}	3.42±0.15^{ab}	0.58±0.01^{b}
	T-Value = 2.55; P-Value = 0.037		T-Value = -1.77; P-Value = 0.110		T-Value = 4.64; P-Value = 0.978	

Mean ± SD with different alphabets indicating significant difference at p<0.001 using Tukey's test. Each mean is based on 3 replicates: A = *Reticulitermes flavipes;* B = *Heterotermes indicola*

2.3.5. β-glucosidase activity

The specific activity of β-glucosidase was evaluated based on V_{max} against the release of nitrophenol as shown in Figure. 2.5. β-glucosidase activity was most active in whole gut homogenate followed by salivary gland and hindgut in both termite species *H. indicola* and *R. flavipes.*

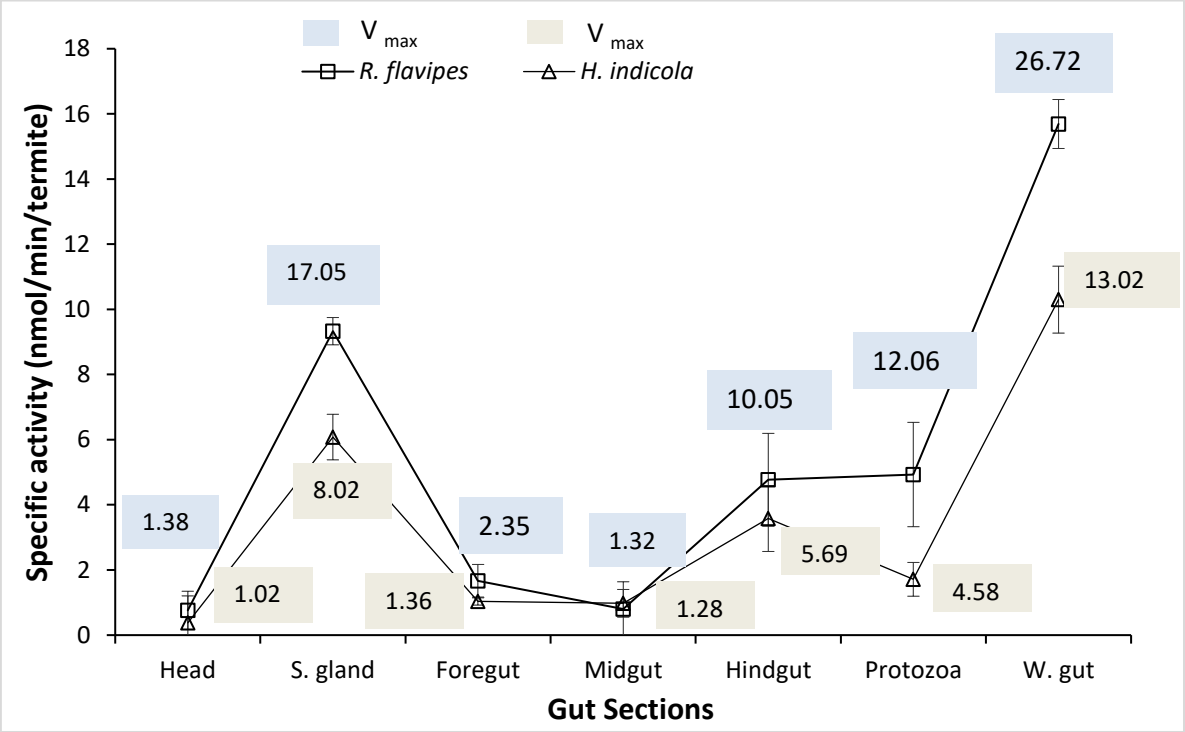


Figure. 2.5. Distribution of β-glucosidase with V_{max} in *R. flavipes* and *H. indicola* indicating the velocities of reactions under kinetic assay conditions.

Magnitude of β-glucosidase activity per termite was significantly higher in *R. flavipes* as compared to *H. indicola* in the salivary gland (T-Value = 7.09; P-Value = 0.019), hindgut (T-Value = 60.48; P-Value = 0.008), protozoa extract (T-Value = 5.20; P-Value = 0.035), and whole gut (T-Value = 9.04; P-Value = 0.012). β-glucosidase activity was found minimum and non-significant in the head (T-Value = 6.79; P-Value = 0.061) and midgut regions (T-Value = 2.82; P-Value = 0.106) of *R. flavipes* and *H. indicola* respectively as shown in Figure. 2.5.

2.3.6. Exoglucanase activity

Exoglucanase activity was determined based on V_{max} obtained by a kinetic reaction between p-nitrophenyl pyranoside cellobiose and crude enzyme dilutions. The majority of exoglucanase-specific activity is significantly localized in the hindgut region and protozoa residual as compared to other gut regions in both termite species (Figure. 2.6). In *R. flavipes*, exoglucanase activity varies significantly ($F = 58.89$ *(df = 6)*; $p = 0.001$) in throughout gut. It

was maximum in protozoa (4.55±0.74 µmol/min/termite), whole gut (5.89±0.19 µmol/min/termite), and hindgut homogenate (2.37±0.05 µmol/min/termite) whereas, minimum activity was observed in endogenous gut regions (head, salivary gland, foregut, and midgut).

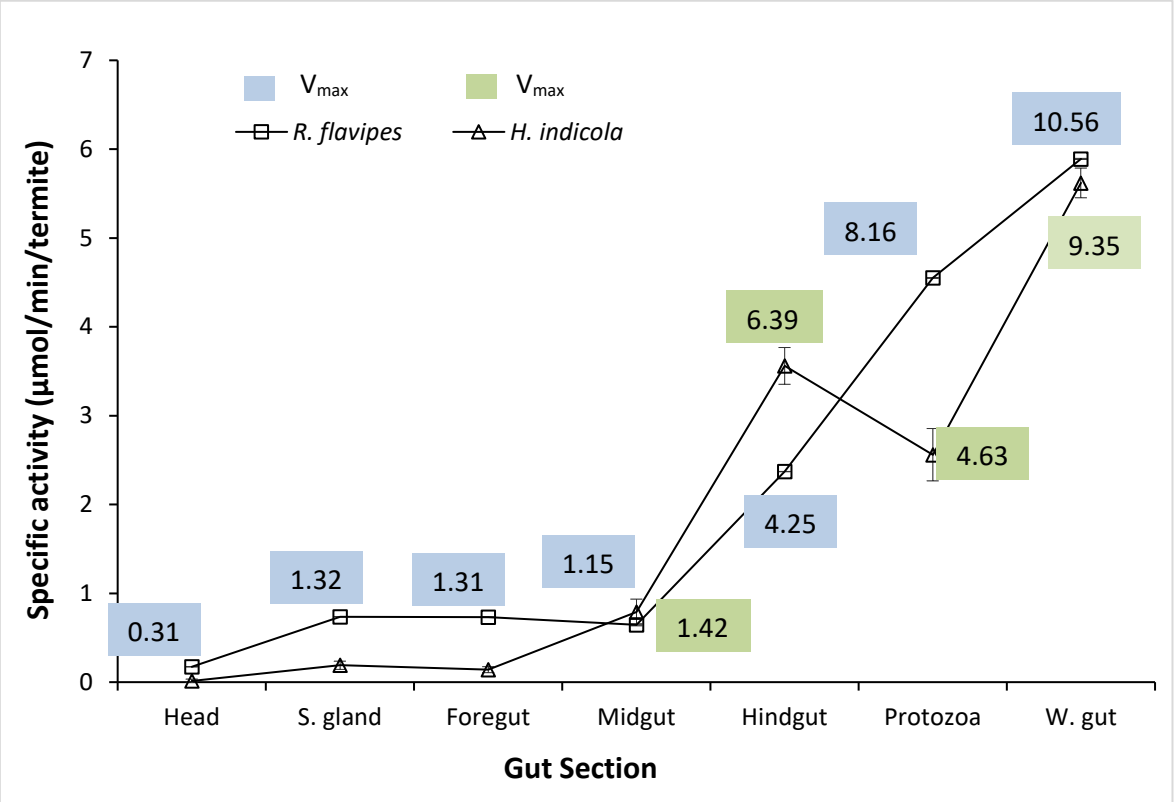


Figure 2.6. Distribution of pNPC activity in *R. flavipes* and *H. indicola* with V_{max} values indicating the velocity of the reaction.

Likewise, similar patterns of exoglucanase specific activity were observed in *H. indicola* except a significant decline curve was observed in protozoa extract. However, specific activity in hindgut was slightly more than *R. flavipes* as shown in Figure 2.6.

2.3.7. Xylanase activity

Xylanase activity was determined against the standard curve of glucose standard and quite varied (F= 15.10 *(df = 6);* P< 0.001) throughout the gut regions of the *R. flavipes* (Figure. 2.7). In *R. flavipes*, hindgut showed maximum xylanase activity (3641.57±261.26 µmol/min/ml) followed by protozoa extract (2643±190.19 µmol/min/ml), while minimum xylanase activity was observed in head (274.94±21.08 µmol/min/ml) and foregut regions (605.38±29.61 µmol/min/ml). The magnitude of xylanase activity in *H. indicola* was significantly reduced in the hindgut (T-Value = 46.49; p-Value = 0.000) as compared to *R. flavipes*.

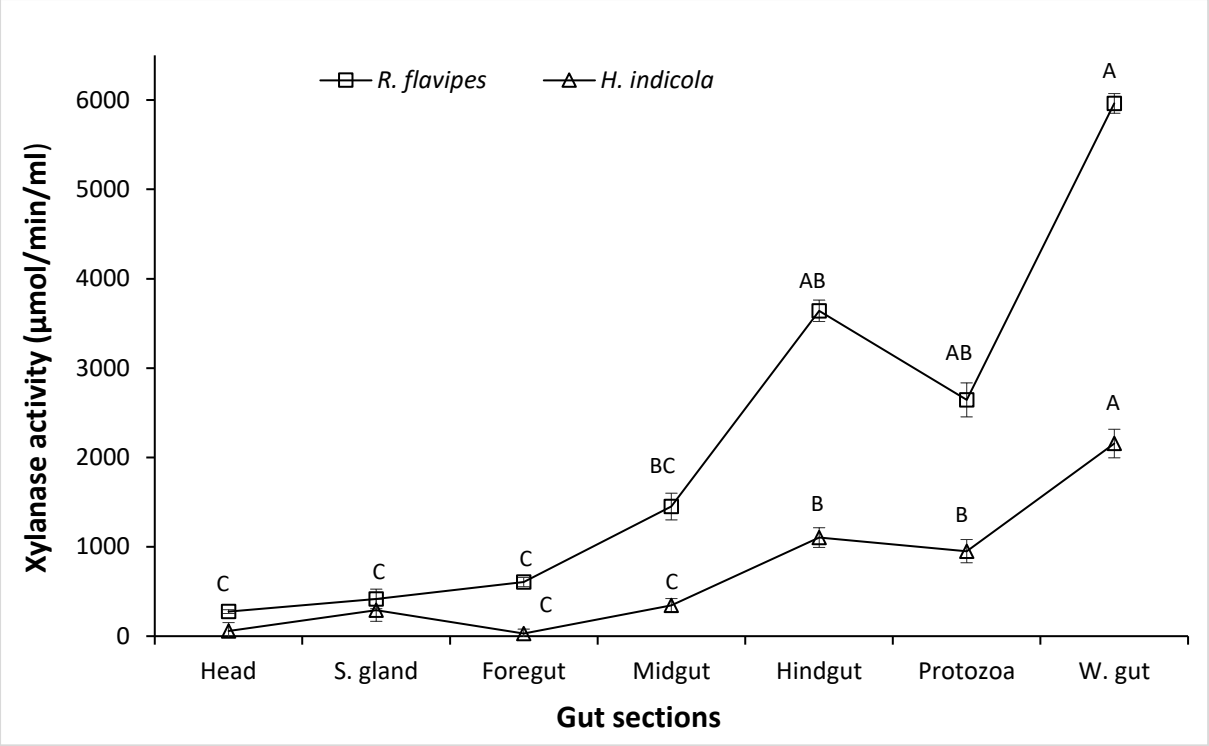


Figure 2.7. Xylanase activity throughout the gut regions of *R. flavipes* and *H. indicola*. Different words indicating significant differences among gut regions of both termite species by using Tuckey's LSD ANOVA test.

A non-significant difference (T-Value = 5.61; P-Value = 0.070) was observed among the endogenous cellulase region salivary gland of *R. flavipes* and *H. indicola*. A high magnitude of xylanase activity was observed in whole gut homogenate among both termite species.

2.3.8. Correlation analysis

The correlation between cellulase gene expression and their activities **i**n *R. flavipes* and *H. indicola* was analyzed using R language (Figure 2.8). A strong correlation was observed between β-glucosidase gene expression and β-glucosidase enzyme activity.

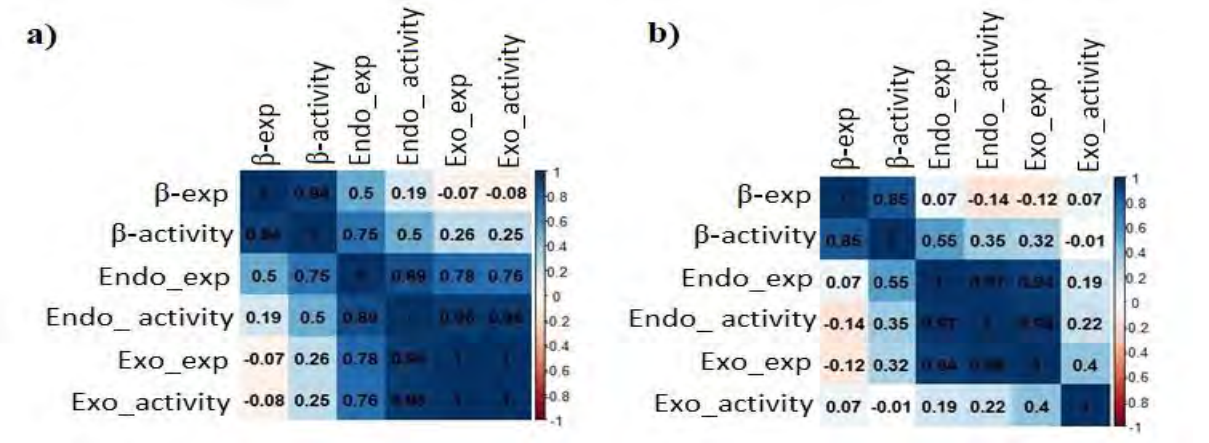


Figure 2.8. Correlation analysis of β-glucosidase, exoglucanase and endoglucanase gene expression and their activities in *R. flavipes* a) and *H. indicola* b).

The level of confidence of β-glucosidase gene expression and β-glucosidase enzyme activity in different gut regions of *R. flavipes* is equal to 0.0047, indicating a significant Pearson correlation (r = 0.94). Likewise, in *H. indicola* Pearson correlation (r = 0.85) between β-glucosidase gene expression and activity was slightly less significant (0.031) at 95% confidence**.**

The correlation between the endoglucanase gene expression and its activity throughout the gut region of *R. flavipes* was 0.89 at 95% confidence level with P-Value 0.017, whereas, in throughout gut region of *H. indicola* insignificant (P-Value = 0.714) correlation (r = 0.194) was observed between endoglucanase activity and gene expression.

In exoglucanase gene expression and exoglucanase activity, there was a significant maximum positive +1 correlation at a 95% confidence interval with P-Value 0.001 throughout the gut region of *H. indicola* (Figure. 2.8b). Similarly, a significant positive correlation +1 was observed between exoglucanase gene expression and exoglucanase activity in the whole gut region of *R. flavipes* (Figure. 2.8a)

2.3.9. Characterization of cellulolytic activities

2.3.9.1. Effect of temperature and pH

All cellulase activities were increased with the rise of temperature till 60°C for 30 min incubation period in *R. flavipes* and *H. indicola* (Figure. 2.9a-b). The major effect of temperature was noticed on the endoglucanase activity and β-glucosidase activity. The optimum temperature for all cellulase activities among both termite species lies between 40 to 60°C. After 60°C, the magnitude of cellulase activities declined significantly in *R. flavipes* than *H. indicola*.

Regarding, the buffer selection for the termite cellulase assays, two main qualities are of important consideration. First is the magnitude of activity yielded by a homogenizing buffer and second is the stability of activity at the given optimum pH of the buffer. Whole termite body activity of endoglucanase, exoglucanase, and β-glucosidase increased and optimum at a range of 5-6 pH in both *R. flavipes* and *H. indicola.* However, exoglucanase activity suddenly decreased after the pH 5.0, whereas endoglucanase and β-glucosidase activity were stable up to pH 7.0 in *R. flavipes* and *H. indicola* as shown in Figure 2.9 c-d.

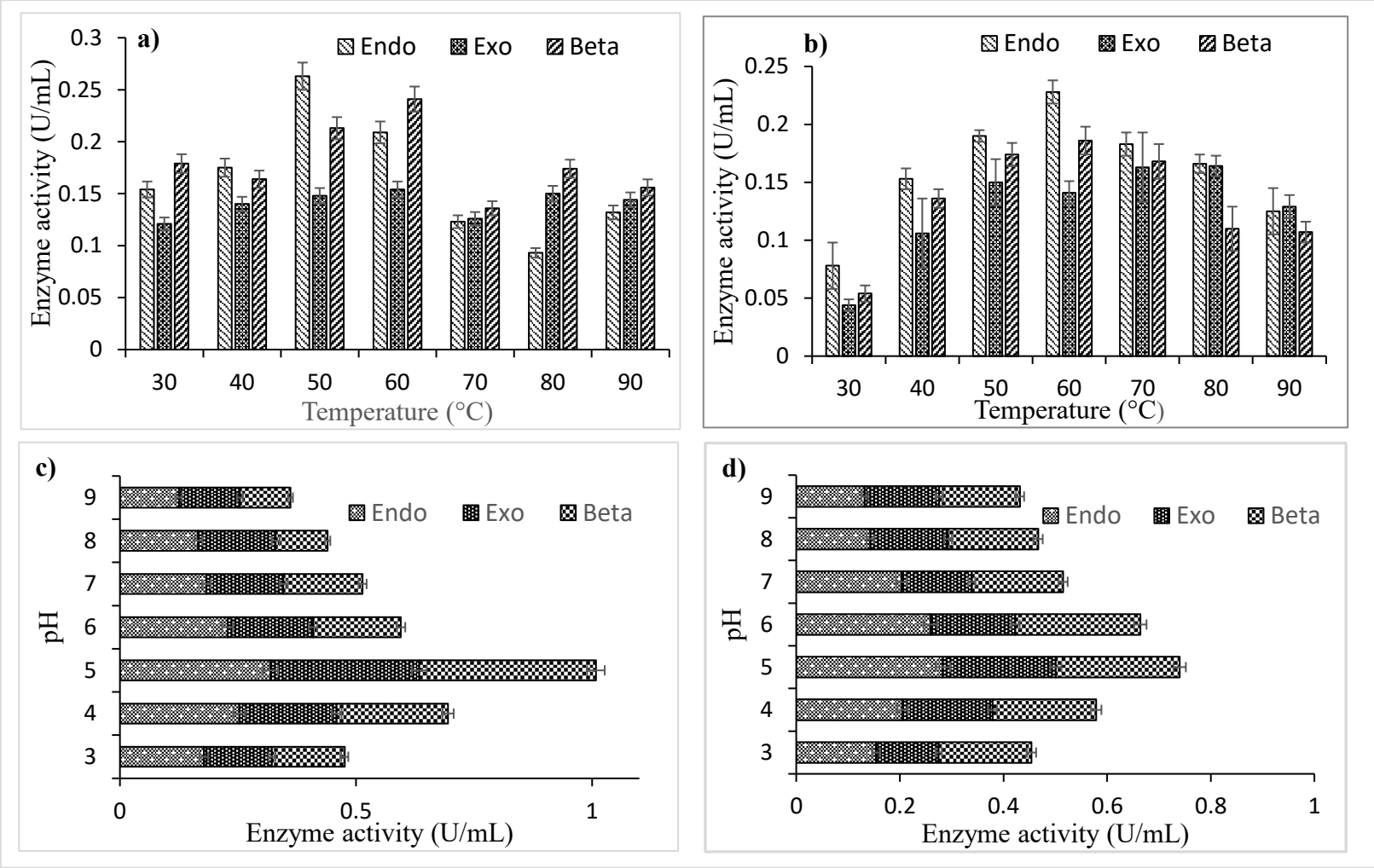


Figure 2.9. The effect of temperature (a and b) ranged from 30-90°C and pH (c, d) on the endoglucanase (Endo), exoglucanase (Exo), and β-glucosidase (Beta) activities in (a-c) *R. flavipes*; (b-d) *H. indicola*.

2.3.9.2. Effect of metal ions and surfactants on cellulase activities

Relative β-glucosidase activity both in *R. flavipes* and *H. indicola* was significantly increased in the presence of Mg^{+2}, NH_4^+, and Cu^{+2} than control. Whereas Na^+ and Fe^{+2} significantly reduced all cellulolytic activities significantly relative to control in both termite species. Endoglucanase activities did not show significant variation against the effect of metal ions both in *R. flavipes* and *H. indicola*, whereas exoglucanase activity slightly varied from the control in both termite species as shown Figure 2.10a-b. Relative β-glucosidase activity was significantly varied in *H. indicola* as compared to *R. flavipes* by metal ion solutions.

Likewise, detergents (tween-20, 60 and 80, triton x-100, SDS and surf a commercially available washing detergent (UniLiver™, Pakistan)) and surfactants (H_2O_2 and $NaClO_3$) effected the β-glucosidase activity significantly as shown in Figure 2.10c-d. Tween-60 enhanced the β-glucosidase activity up to 500% than control and SDS significantly suppressed the β-glucosidase activity <50% of control activity in *R. flavipes*. However, exoglucanase activity showed very slight variations against surfactants. In *H. indicola* commercially available detergent caused the significant reduction in the endoglucanase activity, whereas tween-60 enhanced the endoglucanase activity up to 350% which was significantly high from β-glucosidase and exoglucanase activities. Surfactants H_2O_2 and $NaClO_3$ slightly reduced the endo, exoglucanase and β-glucosidase activities both in *R. flavipes* and *H. indicola.*

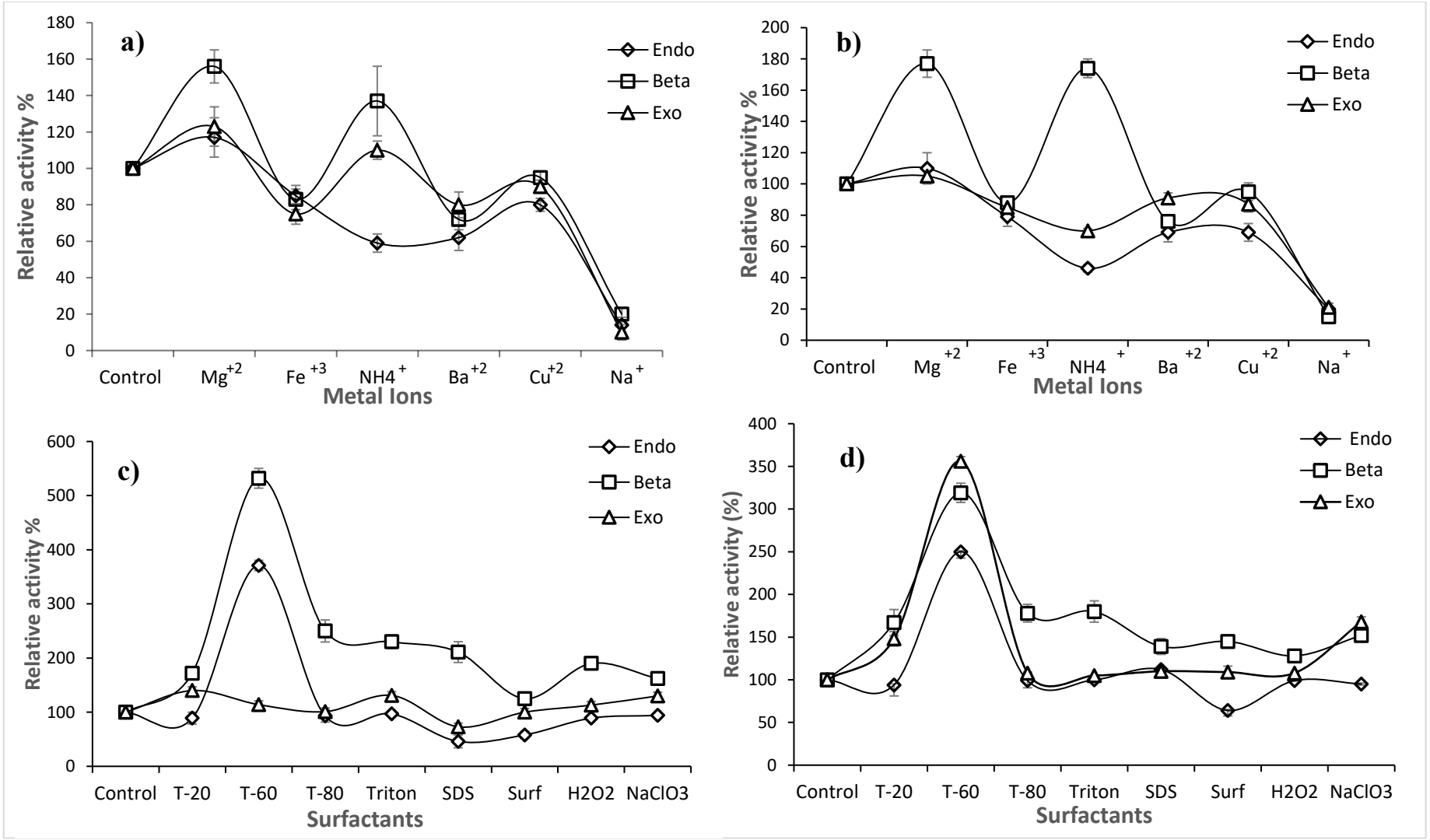


Figure 2.10. Effect of metal ions on endoglucanase (Endo), exoglucanase (Exo), and β-glucosidase (Beta) activities in *R. flavipes* (a-c) and *H. indicola* (b-d).

2.3.9.3. Effects of organic solvents

In another approach, the effect of different organic solvent on cellulolytic activities (endoglucanase, exoglucanase, and β-glucosidase) was determined in the whole gut homogenate of *R. flavipes* and *H. indicola* (Figure 2.11). Isopropyl, chloroform, benzene, ethanol, xylene, toluene, and n-hexane were incubated with enzymes. reactions. Ethanol and benzene significantly reduced the activity of endoglucanase in both termite species. Whereas chloroform and xylene showed an inverse effect and equally enhanced the activity of β-glucosidase among *H. indicola* and *R. flavipes.*

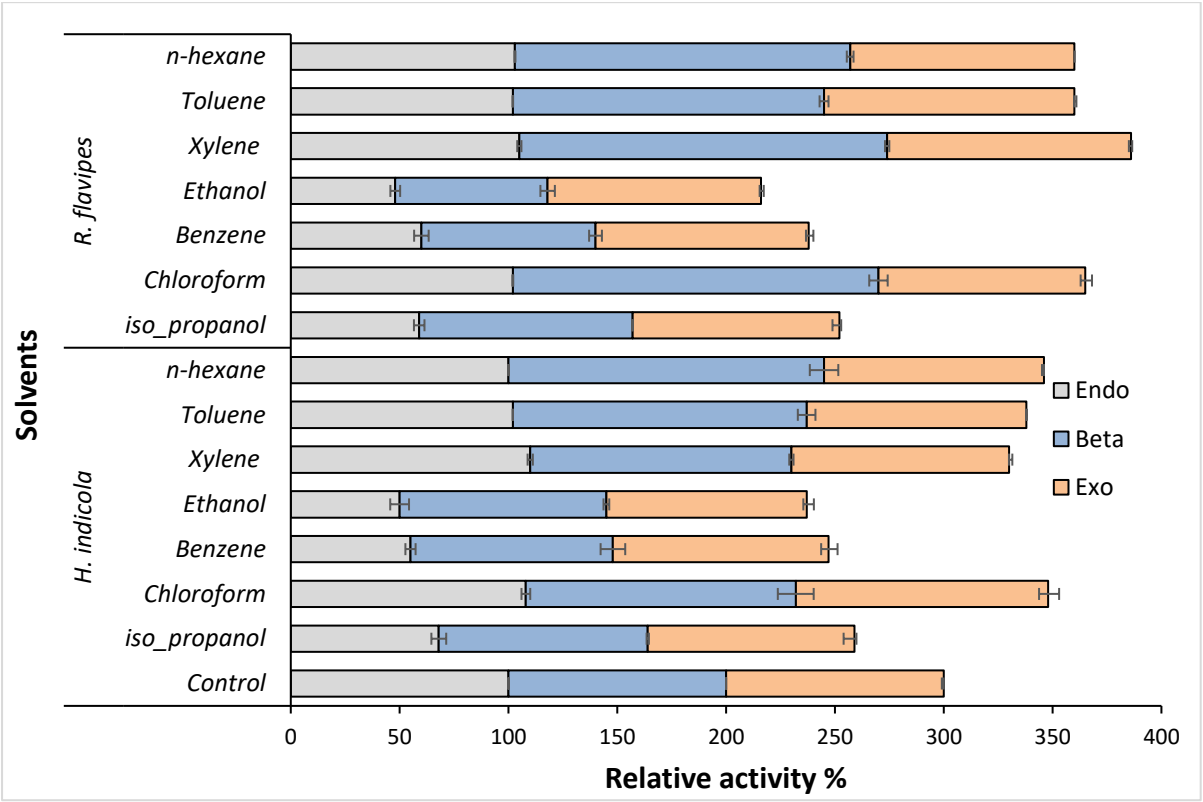


Figure 2.11. Stacked bar chart indicating the effect of organic solvents on the endoglucanase (Endo), exoglucanase (Exo), and β-glucosidase (Beta) activities in *H. indicola* and *R. flavipes*.

2.3.10. Kinetic analysis

The kinetic for the endoglucanase, exoglucanase, and β-glucosidase was determined using specific substrates CMC, pNPG, and pNPC respectively (Figure. 2.12). The effect of different concentrations of these substrates was determined on the maximum velocity (V_{max}) and reaction coefficient (K_m) of enzyme reactions. In CMCase activity, whole gut homogenate of *R. flavipes* exposed $K_m = 0.476$ and $V_{max} = 222.2$ µmol/ml/min, whereas *H. indicola* whole gut extract indicated $K_m = 1.31$ and $V_{max} = 200.0$ µmol/ml/min.

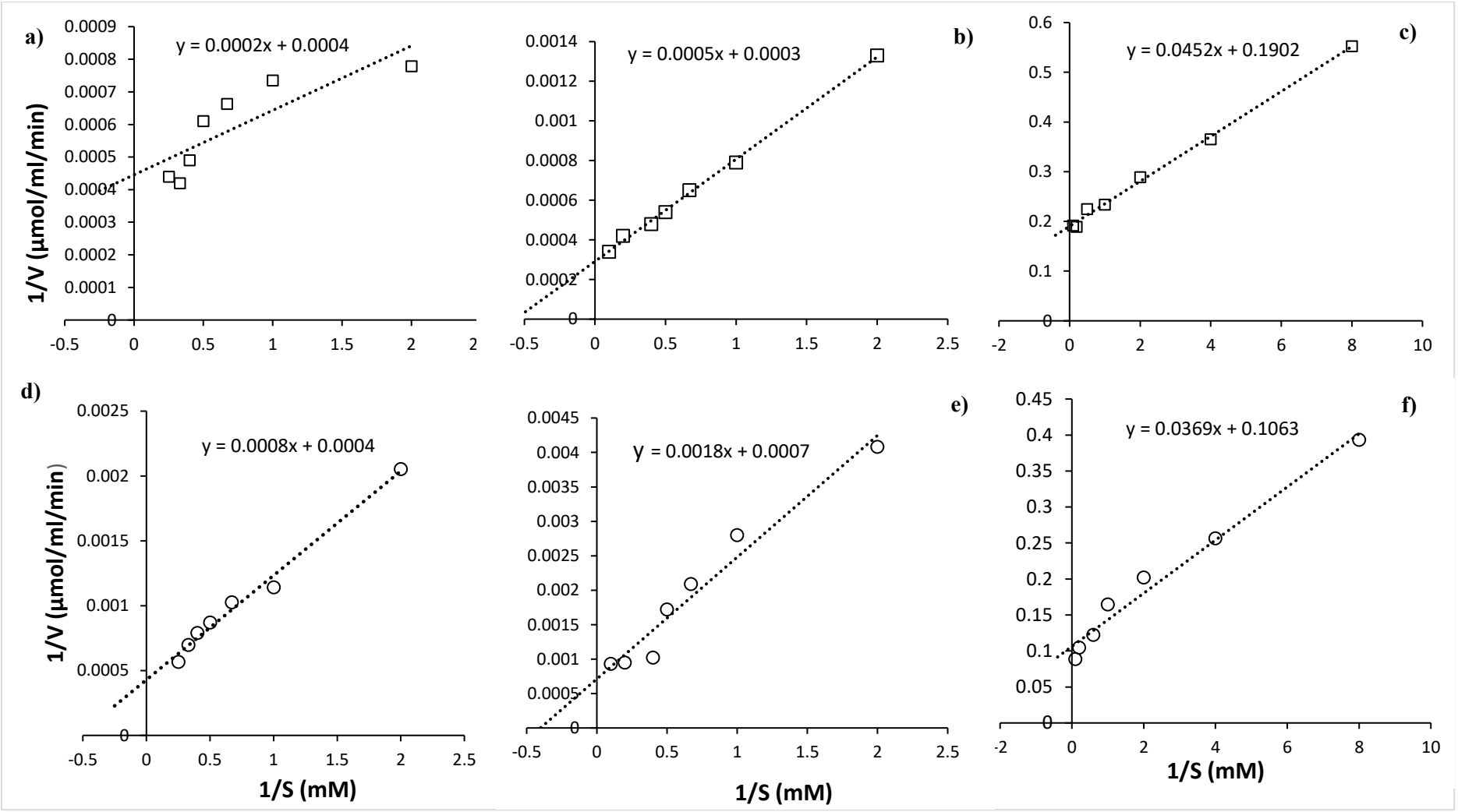


Figure 2.12. Kinetic analysis of endoglucanase **a)**, exoglucanase **b)** and β-glucosidase **c)** activities in *H. indicola* (a-c) and *R. flavipes* (d-f) respectively.

Under β-glucosidase assay condition, pNPG concentrations explored K_m = 1.67 and Vmax = 285.714 µmol/ml/min in *R. flavipes*, however V_{max} = 133.33 and K_m = 2.85 slightly varied in *H. indicola*. Likewise, under exoglucanase conditions K_m = 0.27 and V_{max} = 1.47 from *R. flavipes,* whereas K_m = 1.33 and V_{max} = 10.00 from *H. indicola.*

2.4. Discussion

Reticulitermes flavipes and *Heterotermes indicola* are lower subterranean termites causing large-scale damage to wooden structures, papers, and other products of cellulose nature. Both termite species are important economic pests and have a different zoogeographic region, a different type of wood as an energy source, which results in the variability of cellulolytic potential and distribution of cellulase activities and predicted cellulase gene expression throughout gut regions. This chapter describes the endogenous and symbiotic origin partial cellulase genes from *R. flavipes* and *H. indicola* and identified correlations between cellulase gene expression and their predicted activities throughout the gut region. Expression patterns of all endogenous and symbiotic genes across the gut regions are similar in both termites and are identical to patterns of all endogenous gene expression observed in the workers of other termite species. Especially, cellulase expression in *R. flavipes* and *H. indicola* are like individual cellulase gene studies for the termites *Coptotermes formosanus. R. speratus, Neotermes koshunensis, Hodotermopsis japonica* and *Mastotermes darwiniensis* (Slaytor *et al.,* 1997; Watanabe *et al.*, 1998; Nakashima *et al.*, 2002a; Nakashima *et al.,* 2002b; Tokuda *et al.,* 2002; Tokuda *et al.*, 2004; Tokuda *et al.,* 2007; Zhou *et al.*, 2007; Lo *et al.,* 2011; Sethi *et al.*, 2013).

The endogenous cellulase genes *β-glu* was exclusively expressed in salivary glands in both termite species, while *Exo* and the main portion of *Endo* cellulase gene were dominant in the hindgut region in *R. flavipes* and *H. indicola*. Since, foregut and hindgut are not secretory tissues (Noirot and Noirot-Timothee, 1969) that indicate, *β-gluc* gene coding the *β*-glucosidase enzyme significantly must be secreted from salivary gland, whereas, *Endo* and *Exo* must be released by symbiotic microbiota reside in the hindgut of lower termites [99]. Lower termite *R. flavipes* and *H. indicola* are quite different from higher termites regarding the distribution patterns of cellulases, since, cellulase gene expression in the higher termites has been only detected in the midgut of the digestive tract such as *Nasutitermes takasagoensis* and *Nasutitermes walker* [105]. The finding of

this study supports that the exclusive β-glucosidase activity only in salivary gland tissue of termite's gut is attributable to endogenous only β-glucosidase *β-glu* gene expression. Whereas, endoglucanase activity detected in the salivary gland and hindgut, directly support the evidence of the presence of endogenous and symbiotic endoglucanase respectively. Besides, symbiotic *Exo* and GHF11 gene high expression in hindgut contributed to the highest exoglucanase activity in the hindgut of termites, and it may also account for the exponential xylanase activity in the hindgut.

Lower termites possess a diverse cellulolytic gene pool based on their food source. Since lower termites are wood feeders and wood is rich in lignin crystalline amorphous cellulose and hemicellulose (Ljungdahl and Eriksson, 1985). High and efficient rates of lignin-hemicellulose hydrolysis can reduce the cellulolytic potential of termites by end-product inhibition [111,112]. This potential end-product inhibition supports the evidence, that why the lower termites in this study possess a diverse cellulase gene pool. This disparity in cellulase genes also exists among other cellulolytic organisms such as the fungus *Trichoderma reesei* produce multiple kinds of endo and exoglucanases [99,112]. In *R. flavipes* and *H. indicola*, endoglucanase is attributed to GHF 9, 7-5, and 45. Whereas, exoglucanase is attributed to GHF 11 and 42 in the hindgut regions. This cellulase diversity pool, provide the answer that why lower termites are an efficient wood consumer and capable to assimilate >90% of the wood glucan [20,99]. Therefore, cellulase redundancy in *H. indicola* and *R. flavipes* is a possible potential mechanism, which either responsible for the evasion of insufficient cellulose assimilation or potential end-product inhibition or combination of dual factors [99].

Lower termites showed a distinct difference in foraging strategies from higher termites, specifically regarding the role of the endogenous and symbiotic dual cellulolytic system in lignocellulose digestion. Higher termite account for 75% of total termite species in the world and all higher termites rely 100% on the endogenous cellulolytic system for their metabolic requirement since symbiont flagellates are absent in higher termites (Slaytor *et al.*, 1997). Conversely, lower termites harboring symbiotic flagellates such as *R. flavipes* and *C. formosanus*, showed dual synergetic cellulose digestion system of endogenous and symbiotic origin cellulase to meet 100% of their wood digestion requirement that indicates the implication of both endogenous and symbiotic cellulase system in lower termites (Nakashima *et al.,* 2002; Zhou *et al*., 2007; Brune, 2014).

As complete hydrolysis of lignocellulose is a multi-step process that requires a synergistic action of endoglucanase, exoglucanase, β-glucosidase, and xylanase in nature. In this chapter, we reported all these enzymes activity in *R. flavipes* and *H. indicola* termites from two different zoogeographic regions. Previously Zhou *et al*., (2007) proposed the dual cellulolytic system for *R. flavipes*, in which the liberation of simple sugar from lignocellulosic materials requires the synergistic action of endogenous and symbiotic cellulases such as endoglucanase and exoglucanases. However, they did not confer the β-glucosidase activity and expression in the whole termite gut region to detect the significant role of β-glucosidase in the complete hydrolysis of cellulose. Likewise, Nakashima *et al*., (2002b) defined a dual cellulolytic system *C. formosanus* harbor two different cellulase systems that work independently, one is an endogenous system in the midgut and the other comprise of symbiotic flagellates in the hindgut. Conversely, our findings here support the idea reported by Breznak and Brune (1994) and Zhou *et al.,* (2007), that the collaborative action of endogenous and symbiotic based multiple kinds of cellulases from termites and their endosymbionts (endoglucanase, β-glucosidase, and exoglucanase) are required for the complete hydrolysis and liberation of monosaccharides of cellulose. In addition, distribution patterns of cellulase activities in *R. flavipes* and *H. indicola* are quite similar even they belong to different zoogeographic regions. However, the magnitude of all activities was significantly high in *R. flavipes* as compared to *H. indicola*. In lower termite, high activity of β-glucosidase and endoglucanase in the salivary gland may be related to its feeding behavior as they foraged on dry wood along with the little amount of water. Endoglucanase cellulase activities remain active in foregut, midgut, and hindgut as termites continued attachment to wood fibers during digestion, but at very low magnitude. Itakura *et al*., (1997) and Inoue *et al*., (1997) reported the 77.8% activity of endoglucanase and 54.0% β-glucosidase activity in the salivary gland of *R. speratus* respectively. Through the correlation analysis, we found that cellulase gene expression highly correlated with the cellulase distribution that can be used as new strategy to inhibit the cellulase activities.

A discrete band of molecular weight 68 kDa was measured in crude and purified samples of thorax and abdomen regions in *H. indicola*. Whereas, higher termite *N. koshunensis* had a molecular weight of 60 kDa of β-glucosidase purified from the salivary glands of the termite (Tokuda *et al*., 2002). Conversely, *R. flavipes* salivary gland and hindgut regions carry the same

size protein band approximately 48 kDa that support the evidence of dual kind of cellulases such as endogenous and symbiotic endoglucanase. The correlation of endoglucanase gene expression and endoglucanase activity showed statistical significance and increased with endoglucanase activity. However endogenous β-glucosidase gene expression was negatively correlated to the symbiotic origin exoglucanase, which supports the idea of the specific distribution of endogenous and symbiotic type of cellulases.

To optimize the cellulolytic activities, we reported the optimal activities under different conditional factors such as temperature, pH, metal ions, solvents, and surfactants. By increasing temperature also increase the internal energy of the reaction, this may break the weak bond of three-dimensional proteins of enzymes leading to thermal denaturation of the protein. The optimum temperature for all cellulolytic activities calculated in both species' ranges between 40 to 65°C and it started diminishing after 65°C. Tokuda *et al*., (2002), explained the effect of heating on β-glucosidase from *N. koshunensis* that resisted temperature lower than 50°C. The cellulase activity in both species was maximum at a pH range of 5-6. of species *R. flavipes* and *H. indicola* activity remained constant over pH 7. The pH in the midgut of both species displayed persistent enzyme activity from pH 4-7. The hindgut of species *R. flavipes* demonstrated higher endoglucanase and exoglucanase activities at pH 5-6. While *H. indicola* exhibited higher activities at pH 5 and then activities drop afterward. This change in enzyme activity to pH may be due to the ionization at the side chain of amino acid in the active site and as well whole enzymes not only affect the size but also a charge of protein including positioning of the substrate (de Albuquerque Lima *et al.*, 2014). Effect of pH on the major component of β-1, 4, endoglucanases was reported in (Tokuda *et al.*, 1997). The optimal pH in *N. takasagoensis* was 5.8 and 60% of maximum activity recorded over a range of pH 5.0 - 9.2. The β- glucosidase extracted from salivary glands of *N. koshunensis* indicated optimal activity at pH 5.6, furthermore, 50% of maximum activity was ranged between 4-7 pH (Tokuda *et al*., 2002).

This study also implicated that cellulase activities (endoglucanase, exoglucanase, and β-glucosidase) among both species were stimulated in the presence of Mg^{2+}. But the endoglucanase and -glucosidase activities were inhibited due to the presence of Na^{+}. On the other hand, NH_4^{+}, $Ba^{+,}$ $Fe^{2+,}$ and Cu^{2+} slightly reduced the relative activity from the control. Van Giang and Trang (2016) and Gaur and Tiwari (2015) studied the effect of metal ions against cellulase enzymes of

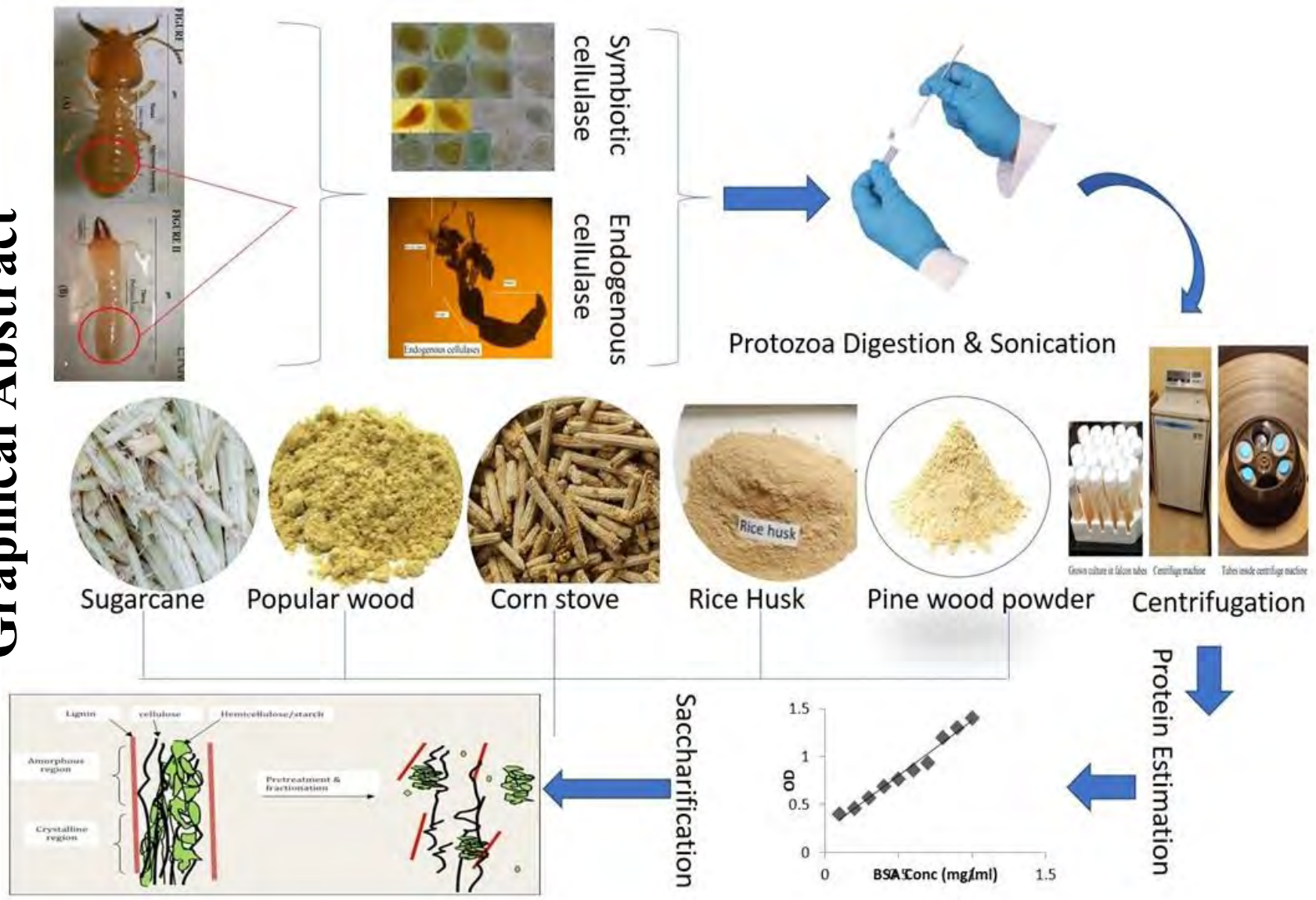
Bacillus subtilis and *Bacillus vallismortis* RG-07. In both cases, Mg^{+} ion enhanced the cellulase activity by increasing the substrate binding ability of the enzyme. Inhibition of activity by Na^{+} and reduction in cellulase activity by Ba^{+}, Cu^{2+}, $Fe^{2+,}$ and NH_4^{+} was also stated in *Ametermes eveuncifer* (Silverstri) soldiers and *B. subtilis*, that support the results of this study (Fagbohunka *et al* ., 2016; Van Giang and Trang, 2016).

An increase in enzyme activities in the presence of organic solvents may be due open conformation of enzymes. This happened many be due to the interface provided by non-polar hydrophobic solvents resulted in the stimulation of enzyme activity. It was also demonstrated earlier that cellulases activity was enhanced in the presence of oxidizing agents like H_2O_2 and sodium hypochlorite (Gaur and Tiwari, 2015). Purified cellulases from the three parts salivary, midgut, and hindgut were incubated with isopropyl, chloroform, benzene, ethanol, xylene, toluene, and n-hexane. Cellulase activities were reduced in the presence of ethanol, benzene, and isopropyl in both species.

2.5. Conclusion

- Lower termite *R. flavipes* has significantly ($p < 0.05$) higher endogenous and symbiotic cellulases activity than *H. indicola*.
- The salivary glands were the main site to produce endogenous cellulases (β-glucosidase and endoglucanases) in both lower termites.
- The gene expression of different cellulases significantly correlated with their exclusive cellulolytic activities measured in all gut regions (head, salivary gland, foregut, midgut, and hindgut).
- Despite of different zoogeographic zones showed the similar single unified cellulolytic system in lower termites, whereby, endogenous and symbiotic origin cellulases act consecutively and collaboratively across the entire digestive tract of *H. indicola* and *R. flavipes*.

Graphical Abstract



Potential of endogenous and symbiotic cellulases from the subterranean termites for the saccharification of agriculture lignocellulosic feedstock

Abstract

This chapter investigated the protein fraction-level *in vitro* and *in vivo* saccharification potential to biofuel producing feedstocks sugarcane bagasse, pinewood, cottonwood (*Populus* sp.), corn stover, rice husk by the guts of two lower termite species. The focus of this chapter was (i) to measure saccharification potential of feedstocks as compared to control diet (filter paper) by termites' gut of *Reticulitermes flavipes* and *Heterotermes indicola*, (ii) evaluate gut lignocellulase activities after no-choice feeding on feedstocks, and (iii) to determine impacts of feedstock on foraging activities and survival behavior.

Sugarcane bagasse, pinewood, cottonwood, and corn stovers were saccharified at a significantly high level as compared to the control diet and rice husk. The gut protein extract from both termite species saccharified all lignocellulose material regardless of feedstock loading. However, *R. flavipes* showed significantly ($p<0.05$) high saccharification potential than *H. indicola*. Consumption of the sugarcane bagasse, pinewood, and corn stover was maximum by *R. flavipes*, whereas *H. indicola* exposed cottonwood and sugarcane feedstocks as the most palatable food source with 100% survivability. This study hypothesized that feeding on various feedstocks will show variable impacts on enzyme activities since an adaptive mechanism enables the termites and their endosymbionts for the optimized consumption of variable food sources.

Results supported the "diet adaptation" hypothesis by indicating β- glucosidase, exoglucanase, and xylanase activities significantly highest for sugarcane bagasse, pinewood, and cottonwood as compared to rice husk and control diets in both termite species. These results are important in the perspective of physiological changes in termites that lead to gut microbial environments changes and 100% survivability on exclusive sugarcane bagasse, pinewood, and cottonwood feedstocks. The guts of both termite species exhibit the clear saccharification of all feedstocks that validates the *H indicola* and *R. flavipes* systems as a potential source for lignocellulose biomass digesting enzymes, especially β- glucosidase, exoglucanase, and xylanases.

3.1. Introduction

Lignocellulosic is the most abundant and variable natural biomass that offers a promising source for biofuel and bioenergy production. The complex structure of lignocellulose is based on generally three major components such as lignin, cellulose, and hemicellulose. Cellulose is a major source of many renewable products and byproducts, which is encapsulated by the complex structures of lignin and hemicellulose polymers. In nature, subterranean lower termites with physiological capabilities occur throughout the tree of life to perform efficient degradation of lignocellulose [13,113].

Different types of cellulases, hemicellulases, and other accessory enzymes from subterranean termites belonging to the glycoside hydrolases (GHs) family involved in the physiological degradation of lignocellulose materials [10,12,114]. The dissociation of lignin is important to increase the access of cellulase to cellulose and hemicellulose. The complete hydrolysis and depolymerization of cellulose occur with the synergistic action of endoglucanases, exoglucanases, and β-glucosidase. Aspeborg *et al.*, (2012) revealed that lignin and hemicellulose are not only depolymerized by the action of endoglucanases, cellobiohydrolases, and xylanases belonging to glucohydrolase family 5 (GHF 5), but also GHF 9 endoglucanases of current study focused termite species *R. flavipes* are involved in the breakdown of internal glucose residues of hemicellulose (Zhou *et al*., 2007; Scharf *et al*., 2011).

In natural wood-feeding insects, particularly lower subterranean termites have the inherent enzymatic capability to degrade the lignocellulose due to which termites have gained attention for biofuel and bioenergy production world widely [22,116]. Lower subterranean termites harbor gut microbiota which helps the termite to degrade lignocellulose efficiently. *Nasutitermes takasagoensis*, *N. exitiosus* and *N. walkeri* (Hogan *et al*., 1988; Tokuda *et al*., 2012) from higher, and *Coptotermes heimi, C. gestroi, Reticulitermes speratus, R. flavipes,* and *Neotermes koshunesis* from lower termites have been studied comprehensively for cellulase genes to its functions to elucidate their digestive mechanism (Nakashima *et al*., 2002; Tokuda *et al*., 2002; Tokuda *et al*., 2004; Franco Cairo *et al*., 2011; Zhang *et al*., 2012; Peterson *et al*., 2015; Scharf, 2015a)**.** There is an ample evidence of endogenous cellulase actions in lower termites that help the independent action of symbiotic cellulases in lignocellulose digestion (Watanabe *et al*., 1998; Tokuda *et al*.,

2007a; Zhou *et al*., 2007). However, Scharf *et al*., (2011ab) proposed a clear collaborative and synergistic relationship between termites and their endosymbiont that achieve efficient hydrolysis of lignocellulose digestion. Cellulases belonging to several GH families have been reported in termites by determining their cellulolytic potential against different substrates. Conversely, there is inadequate information about lignin digestion by wood-feeding insects such as subterranean termites [17,120] that is considered a major limiting factor in biofuel production from agriculture lignocellulosic feedstock (Yang and Wyman, 2008; Yang *et al*., 2012).

Previous studies revealed that subterranean termite *Reticulitermes flavipes* is a model bioreactor to study digestive mechanisms involved in lignocellulose breakdown in termites (Scharf, 2015a,b). Likewise, *Heterotermes indicola* is also an economically lower subterranean termite predominant in South Asia and can be used as a model system for lignocellulose digestion mechanisms. Since, besides of endogenous cellulolytic system, both these lower termite species harbor a diverse profile of eukaryotic and prokaryotic organisms that are analogous to a fermentation bioreactor [7,8,22,82].

Sethi *et al.,* (2013b) reported that *in vitro* digestion of pine dust (a complex lignocellulose substrate) using recombinant belonging to termite lignocellulases demonstrated their role in lignocellulose saccharification. Likewise in a study, termite gut transcriptome analysis after feeding on corn stover and soya bean residue was characterized to investigate the breakdown of the agriculture feedstocks (Rajarapu *et al*., 2015). They revealed a reduction in symbiotic microbiota in termites after corn stover and soybean residue feeding to the brown paper towel, however active enzymatic profile slightly changed. In another study, Rajarapu and Scharf, (2017) performed the saccharification and protein level responses against the second generation agriculture lignocellulose feedstock such as corn stover and soya bean residues by termite gut microbial bioreactor. They have identified changes in protein profile of *R. flavipes* upon corn stovers and soyabean feeding. In this chapter, a comparative saccharification potential of different agriculture lignocellulosic feedstocks were investigated by subterranean termites *R. flavipes* and *H. indicola*. All assay on *H. indicola* was conducted in the lab of entomology, Quaid-i-Azam University, Pakistan, while study on *R. flavipes* was conducted in the lab of urban entomology, Purdue University West Lafayette IN, USA.

Sub objectives of this study were as follows: (i) to determine *in vivo* feeding and *in vitro* saccharification of agriculture feedstock by termites and their endogenous and symbiotic lignocellulases respectively, (ii) to explore the impacts of agriculture feedstocks on the endogenous and symbiotic protein quantities, (iii) identify mechanisms involved in the digestion of agriculture feedstocks in termite gut using different enzyme activity assays. My hypotheses regarding these objectives were as, "endogenous and symbiotic cellulolytic system in these termites can saccharify the various feedstocks at variable potential under *in vitro* condition". In addition, key enzyme activities would be augmented according to the "diet-adaptation hypothesis" [13,125].

3.2. Materials and Methods

3.2.1. Agriculture feedstocks and chemical substrates

In complex lignocellulosic material wood powder of cottonwood *Populus eumericana* (PE) and pinewood *Pinus roxburghii* (PR), whereas, in agriculture feedstocks corn stovers (CS) "AGAITI-2002 variety", sugarcane bagasse (SC) "SPF 338 variety" and rice husk (RH) of "Basmati 385 variety" were obtained from National Agriculture Research Centre (NARC), Pakistan. All the feedstocks certified by NARC free of insecticide application where they were grown for experimental purpose and used as natural substrates for the saccharification. While filter paper (FP) was used as a control di*et al*ong with five above mentioned experimental treatments.

All feedstocks were dried at 65°C, commercial milled to 0.5 mm particle size. For key enzyme activities assays, all substrates were obtained from Sigma-Aldrich® (USA) and Carbohydrate Synthesis Limited (UK). All these agriculture feedstocks were tested qualitatively to determine the composition of cellulose, hemicellulose, and lignin, which was varied in each feedstock source (Figure 3.1).

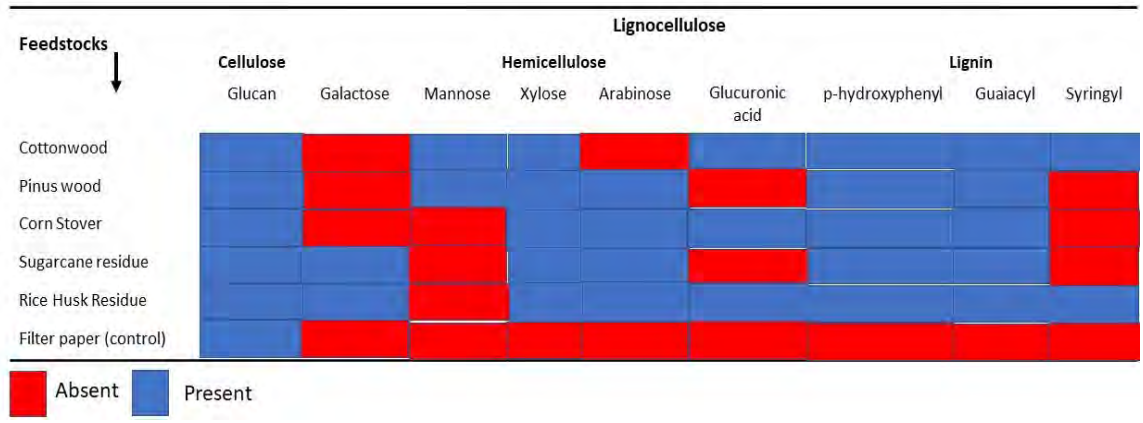


Figure 3.1. Qualitative analysis of general lignocellulosic contents of different feeding stocks used in this study.

3.2.2. No choice termite feeding assays

No choice feeding bioassay was adopted as followed by Karl and Scharf, (2015) and Rajarapu *et al*., (2015).

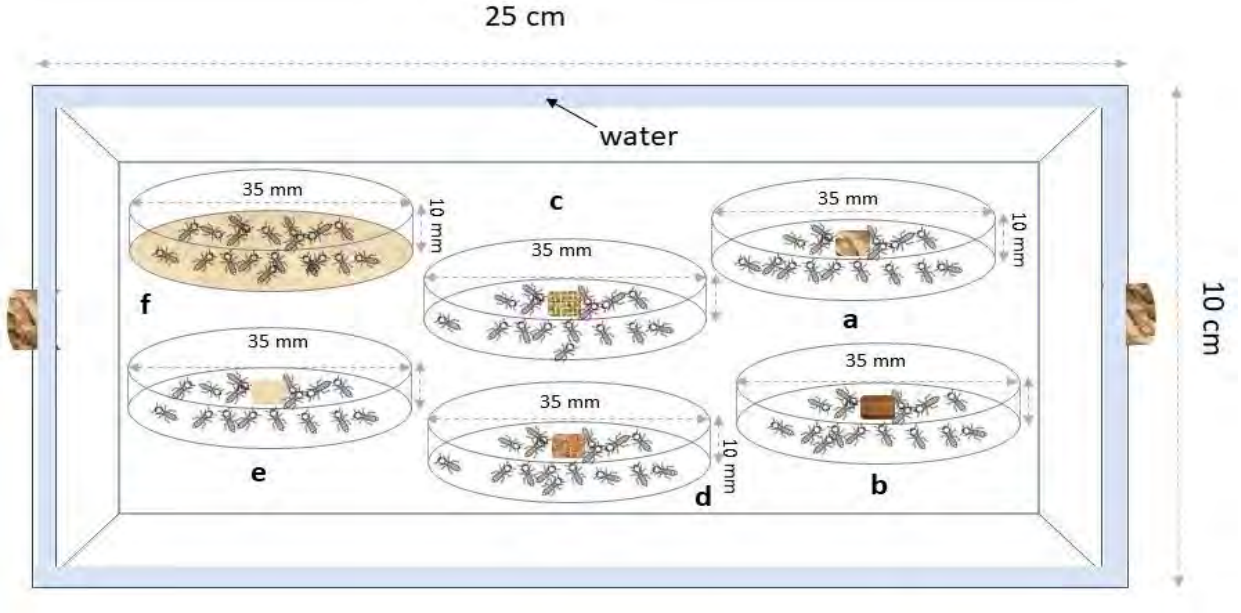


Figure 3.2. Sketch of no-choice feeding bioassay with different cookies of lignocellulases feeding stocks a) *P. eumericana*, b) *P. roxburghii*, c) corn stove, d) sugarcane bagasse, e) rice husk, and f) filter paper (control) in a humidity control acrylic chamber under 26°C and 75±5% relative humidity condition.

In this bioassay, two termite colonies *R. flavipes* and *H. indicola* were used against three replicates of each diet to provide a total of six biological replicates (Figure 3.2). Termites were first augmented in labs for 15 days to get clear their gut from wood debris and then used for experiments. Experiments on *H. indicola* carried out in Pakistan, while *R. flavipes* in the US. Each

milled diet was saturated with approximately 300 µl nano pure water, compacted into cube form, and dried at 65°C for two days. All diet samples were pre-weighted and released approximately 45 workers and 5 soldiers in a small Petri dish of 35 mm. All dishes were placed in a plastic sealable container in dark at 30°C; 70% relative humidity and added some moist paper towels to prevent termites from desiccation. After 12 days, diet sample cubes were dried at 65°C for 12 hr and re-weighed. The percent weight loss for diet samples and filter paper (control) exposed to termites was calculated using the formula (Sotannde, Yager, *et al*., 2011).

$$\%\, Weight\ loss = \frac{W_b - W_a}{W_a} \times 100$$

3.2.3. Termite gut dissections and crude enzyme extraction

After 12 days of feeding assays, 25 workers were dissected under a stereo trinocular microscope, whole guts were removed with sterilized forceps in 100 µl 0.1% phosphate-buffered saline (PBS). All termite guts were divided into host fractions (salivary gland, foregut, and midgut) and symbiotic fractions (hindgut) and placed in 500 µl of sodium acetate buffer pH 5.5 and sodium phosphate buffer pH 6.5 respectively (Scharf *et al*., 2011; Karl and Scharf, 2015).

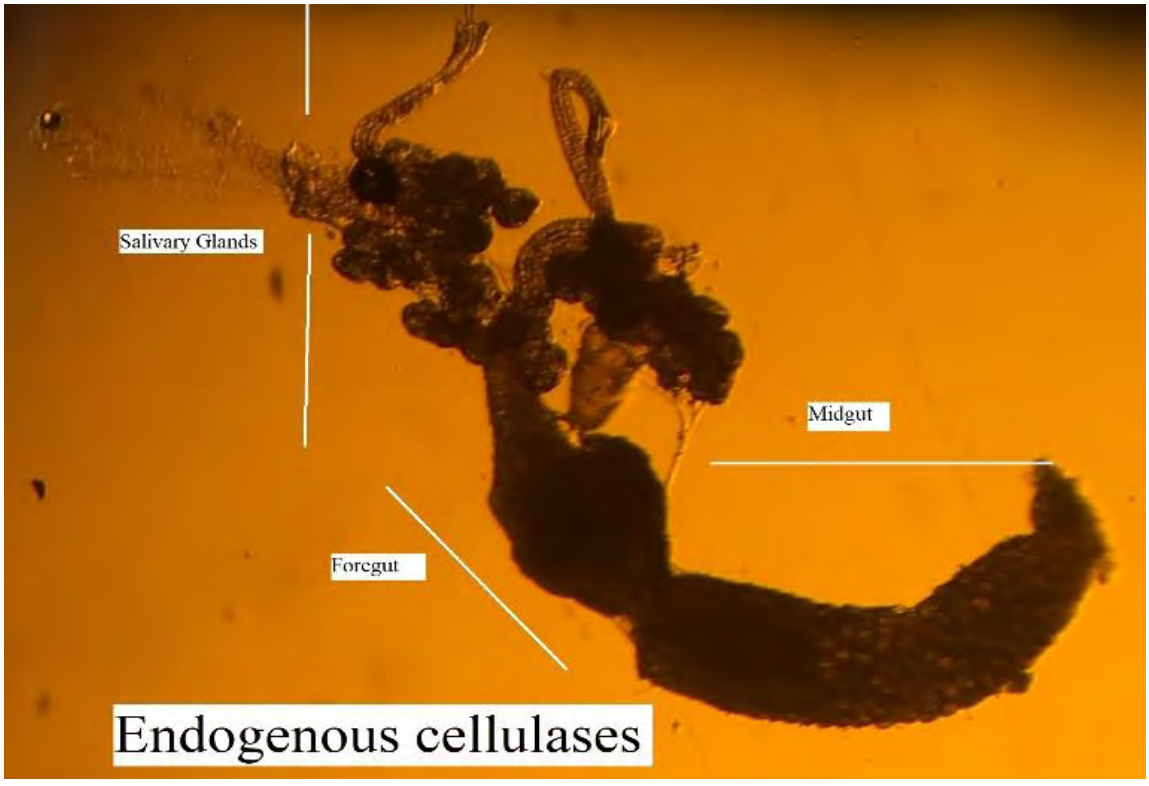


Figure 3.3. Host fraction indicating endogenous enzyme source in *R. flavipes*

The respective termite gut tissues were digested by glass homogenizer and centrifuged at 15000 rpm at 4°C for 20 min. Outstanding supernatants were pipetted out into a new autoclaved PCR 1.5 ml tube and saved at -80°C until used in enzyme activity assays and native SDS-PAGE analysis. The protein content of each supernatant was determined using the Bradford reagent assay kit (Thermo Scientific ^{TM}, USA) with bovine serum albumin as standard. Host (Figure 3.3) or symbiotic fraction (Figure 3.4) of protein supernatants 10 µl from each diet sample replicate added in triplicate to microplate with 90 µl of Bradford reagent and read the plat at 595 nm using a spectrophotometer

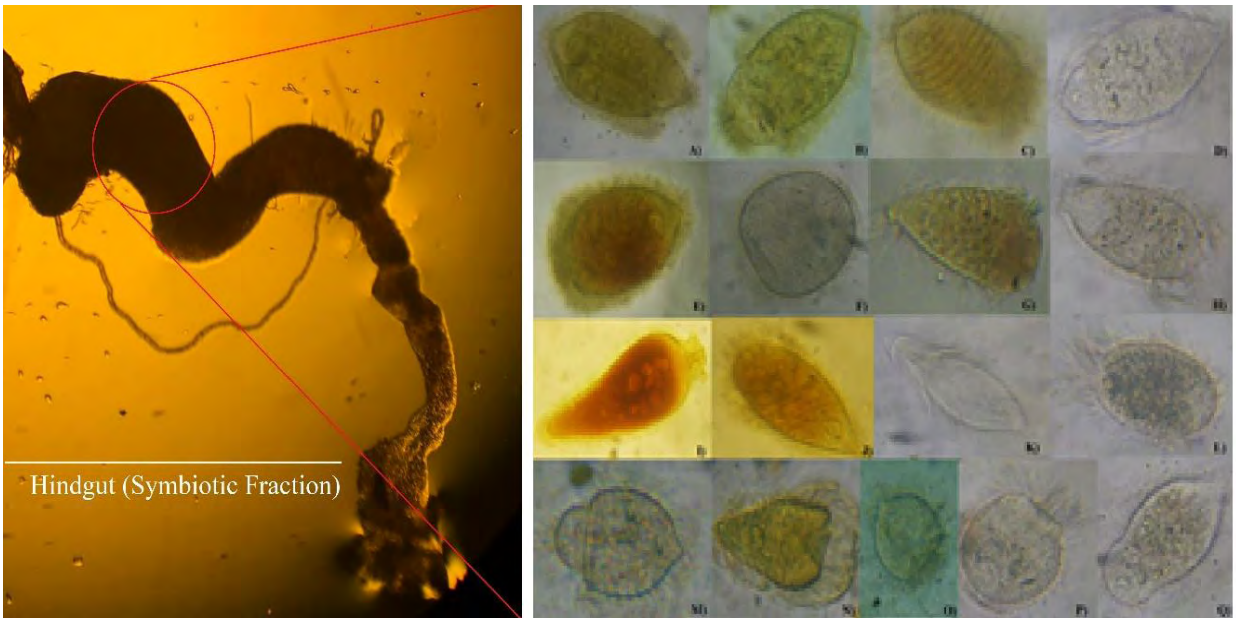


Figure 3.4. Symbiotic fraction indicated by hindgut of *H. indicola* along their flagellates.

3.2.4. *In Vitro* saccharification

Saccharification assays were adopted as followed by Rajarapu and Scharf, (2017). Approximately 50 mature active termite workers were isolated from *H. indicola* and *R. flavipes* lab colonies and dissected per trial replicate to collect guts at 4°C. Clarified protein extract was prepared as mentioned in the previous section (2.2.3) and saved for *in vitro* saccharification assays. Lignocellulose material milled pine wood, cottonwood (*Populus* sp), sugarcane bagasse, corn stovers, rice husk, and Whatman No. 1 filter paper (control) at the range of amount (0.1, 0.5, 1, 1.5, and 2% w/v) were used at different time interval incubation (2, 4, 8, 12 and 24 hours) to identify optimum saccharification incubation period. *In vitro* saccharification was carried out in

Eppendorf tube with the total reaction of the assay was 650 µl, containing 150 µl supernatant fractions (host and symbiotic) and 500 µl 0.1 M HEPES of pH 6.5 buffer carrying optimum concentration of feeding `stock substrates. All Reactions were set within 1.5 ml Eppendorf for 04 hours and placed in a shaking incubator at 37°C and 200 rpm (Rajarapu and Scharf, 2017). Reactions were stopped by adding 5 mM ethylenediaminetetraacetic acid since EDTA used to inhibit metalloenzyme and increase the color stability indication for glucose detection reagent, which carries H_2O_2 that might be worked on by gut metalloenzymes (Scharf *et al.,* 2011b). A colorimetric method by using glucose and maltose detection kit (Thermo Scientific™, USA) was used to quantify the liberated glucose against a glucose standard curve (1-0.01 mg/ml). Colorimetric assays were performed to estimate the cellulolytic and hemicellulolytic potential of the host and symbiotic fraction using exclusively or mixed in equal amounts for whole activity estimation using different substrate models [13,99,125,127].

3.2.5. Enzyme activity responses to lignocellulose feedstocks

Cellulases and hemicellulases activities (*i.e.* endoglucanase, hemicellulase, exoglucanase, β-glucosidase, β-xylosidase, β-mannosidase, and β-arabinosidase) were determined using synthetic and natural substrates (Table 2.1). Carboxymethyl cellulose (CMC), wheat arabinoxylan (WA), and beechwood xylan were used as substrates to determine cellulase and hemicellulase activities. All activity assays were performed using aliquoted gut fraction supernatants from the diet assay dissections.

3.2.5.1. Cellulose/hemicellulose substrates

The potential of cellulases and hemicellulases was estimated by performed the enzyme activity assays using six different model substrates (Table 3.1). Carboxymethyl cellulose (CMC), beechwood xylan, and wheat arabinan substrates were used to evaluate the activity of endoglucanase and hemicellulases using the 3, 5 dinitro salicylic acid (DNSA) method as reported by Karl and Scharf, (2015). Reactions were incubated for 60 minutes at 30°C and the mixture was contained on 90 µL of substrates and 10 µL crude gut supernatant. 100 µL of DNSA solution was added to stop the reaction and incubated at 100°C for 15 minutes. Reactions were cold on ice to measure the absorbance of reducing sugar at A_{660} using a spectrophotometer (SmartSpec 3000, Bio-Rad USA). The resultant absorbance was analyzed by linear equation using glucose as

standard. The unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg glucose per minute/ml.

3.2.5.2. *p*-Nitrophenyl substrates

The substrate *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-glucopyranoside, p-Nitrophenyl-β-D-xylopyranoside (pNPX), and p-Nitrophenyl-β-D-mannopyranoside (pNPM) (pNPC; Sigma, St. Louis, USA) at 10 mM was used to determine the exoglucanase activity of crude extract of subterranean lower termites *H. indicola* and *R. flavipes*. The substrate was diluted with 0.1 M sodium acetate buffer of pH 5.5. The reaction was initiated by mixing 100 µl of crude with 400 µl of the substrate and 500 µl of 0.1 M acetate buffer of pH 5.5 was used as the reagent blank. The reaction was incubated for 30 min at 45 °C and stopped by adding 1 ml of 0.6 M Na_2CO_3. The hydrolysis product was *p*-nitrophenyl (pNP) that was appeared in the form of yellow color. The absorbance of the reaction was read at 420 nm using a spectrophotometer (SmartSpec 3000, Bio-Rad USA). One unit of exoglucanase (U) was defined as the amount of exoglucanase that releases 1 µmol of pNP per minute under standard assay conditions.

3.2.6. Statistical analysis

Feeding activity and enzyme activity data were expressed in weight loss (milligram) per termite and specific activity units on a per-protein basis respectively using the statistical Program JMP version 16.0 (Minitab). All data were normalized using a Shapiro-Wilk test and all class variables (feedstocks, termite colony, replicates, and enzyme fractions) were checked against the responses (specific enzyme activity, diet loss, and termite mortality) to determine significance difference (α = 0.05) using one-way ANOVA test.

To investigate if lignocellulose digestion in termites occurs via additive (*i.e.* collaborative) or more than collaborative (i.e., synergistic) processes, I compared expected (additive interaction between endogenous and symbiotic cellulases) vs observed (whole gut extract) lignocellulose digestion in termites. Here, expected specific-enzyme activity (A_E) was determined using the following formula: $A_E = A_{Endo} + A_{Symb}$

Where, A_{Endo} = observed specific-enzyme activity in termites due to endogenous cellulases; A_{Symb} = is observed specific-enzyme activity because of symbiotic cellulases.

Table 3.1. Enzyme activities of endogenous and symbiotic fractions supernatants with their respective substrates after lignocellulose no-choice feeding assays.

Enzyme activity	Substrates (abbreviations)	Final assay Concentration	Solubility	References
Endoglucanase	Carboxymethyl cellulose (CMC)	1%	Sodium Acetate Buffer in dH_2O	(Rajarapu and Scharf, 2017; Karl and Scharf, 2015)
Hemicellulase	Wheat arabinoxylan (WA)	2%	Sodium Acetate Buffer in dH_2O	[13,125]
	Beechwood xylan (BX)	2%	Sodium Acetate Buffer in dH_2O	
Exoglucanase	p-Nitrophenyl-β-D-cellobioside (pNPC)	5 mM	Dimethyl sulfoxide (DMSO)	[13,125]
β-glucosidase	p-Nitrophenyl-β-D-glucopyranoside (pNPG)	5 mM	Dimethyl sulfoxide (DMSO)	[13,125]
β-xylosidase	p-Nitrophenyl-β-D-xylopyranoside (pNPX)	5 mM	Dimethyl sulfoxide (DMSO)	[125]
β-mannosidase	p-Nitrophenyl-β-D-mannopyranoside (pNPM)	5 mM	Dimethyl formamide (DMFM)	[13,125]
β-arabinosidase	p-Nitrophenyl-β-D-arabinopyranoside (pNPA)	5 mM	Dimethyl sulfoxide (DMSO)	[13,125]

Final assay concentration was prepared in sodium acetate buffer (0.1 M, pH 6.5)

3.3. Results

3.3.1. No-Choice feeding assays

3.3.1.1. Foraging activity

Termite species *R. flavipes* consumed significantly varied ($p < 0.05$) amounts of feeding stocks including *Populus eumericana* (PE), *Pinus roxburghii* (PR), Corn stovers (CS), rice husk (RH), and Sugarcane (SC) as compared to control filter paper (FP) likewise, bodyweight of termites also developed variations in their body weight (Figure 3.5a). A similar trend was observed in termite species *H. indicola* against feeding preferences of feedstocks except for *Populus eumericana* (PE), for which *H. indicola* showed higher preference than *R. flavipes* (Figure 3.5b).

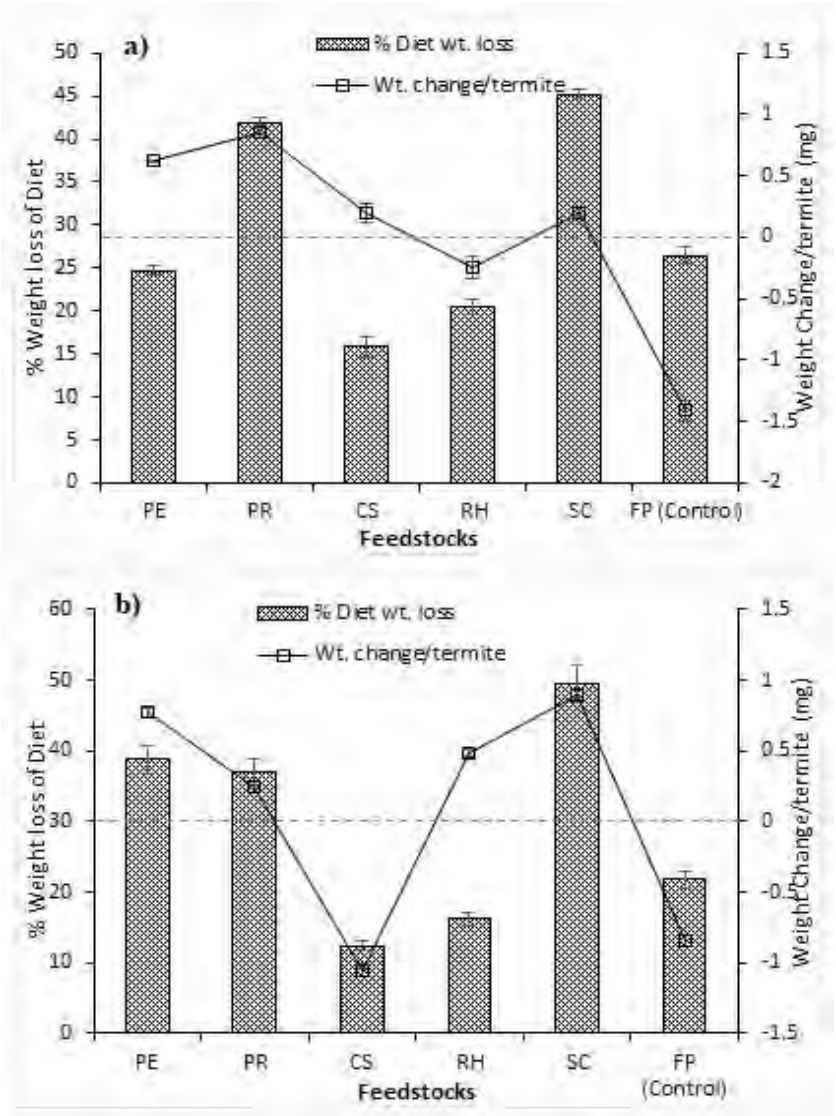


Figure. 3.5. Termite feeding assay results indicating percent weight loss of feedstocks and change in termite for *R. flavipes* a) and *H. indicola* b) after 14 days of continuously feeding.

However, body change in *H. indicola* was significantly ($p < 0.05$) reduced for the Corn stovers (CS) feedstock as compared to other feedstocks. Sugarcane (SC) and *Pinus roxburghii* (PR) feedstock increased body weight of both termite species *R. flavipes* and *H. indicola* after 14 days of continuously feeding assays. Whereas Corn stovers (CS) was the least preferable feedstock *H. indicola* termite species.

3.3.1.1. Survivability

Both termite species showed a high survival rate on all feeding stock for 14 days of continuous feeding assay (Figure. 3.6).

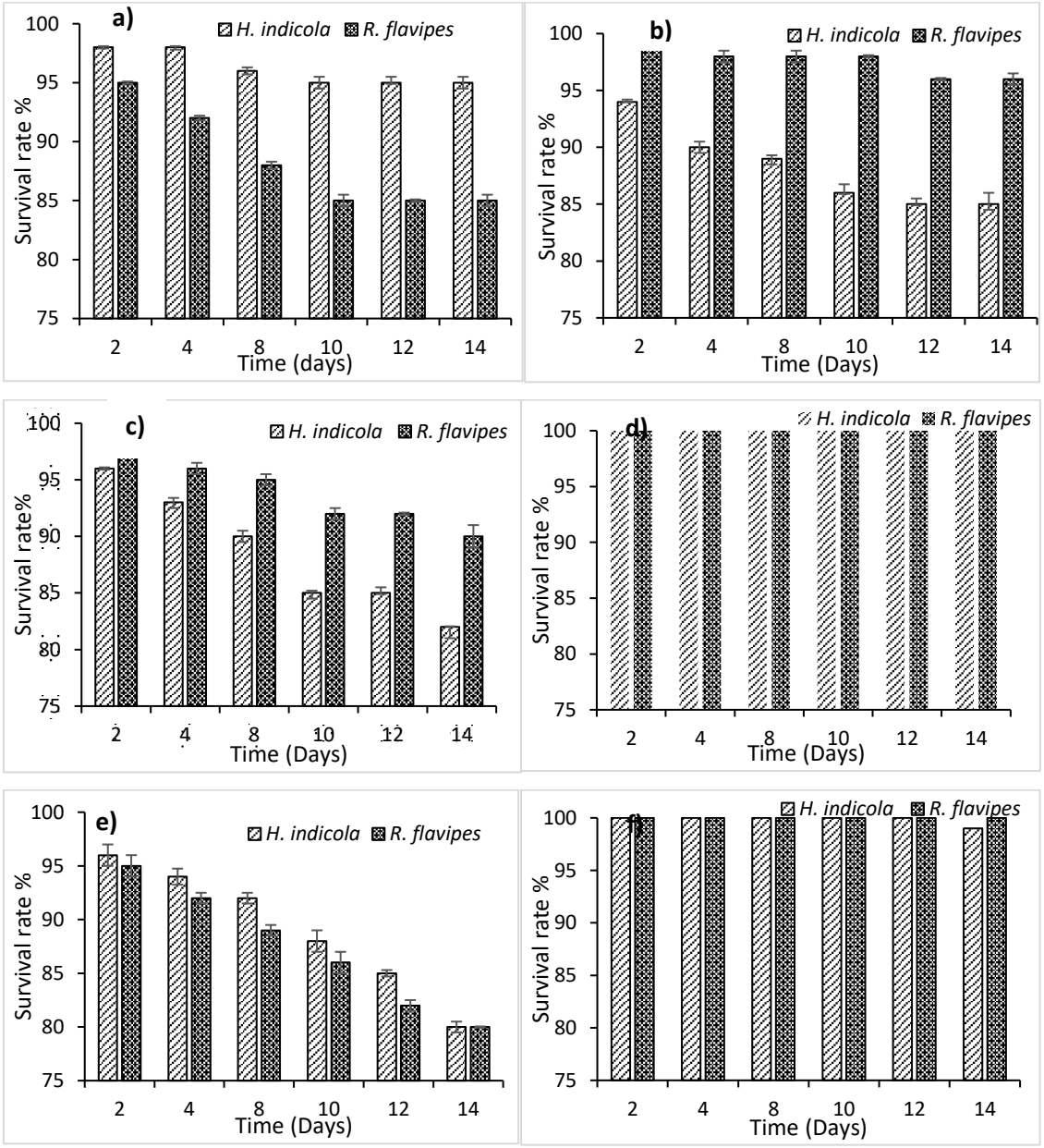


Figure 3.6. The percent survival rate of *H. indicola* and *R. flavipes* by feeding on lignocellulose diets a) *Populus* (cottonwood), b) pine wood, c) corn stovers, d) sugarcane residue, e) rice husk as compared to f) control (filter paper).

Termites *H. indicola* and *R. flavipes* showed a 100% survival rate on sugarcane bagasse and filter paper followed by *Populus* and pine wood-feeding stock. Diets of corn stovers and rice husk also showed successful termite survivability, however, it is a little lower than the percent survival rate of termites observed on sugarcane and control feeding stocks. *R. flavipes* showed an insignificant ($p>0.05$) higher survival rate than *H. indicola* across all diets treatments.

3.3.2. Optimization assays

In optimization assays, there was an unpredictable and significant ($p<0.05$) reduction in the release of detectable glucose levels during incubation lasting longer than 4 hours in all feeding stocks for each concentration against *R. flavipes* (Figure. 3.7).

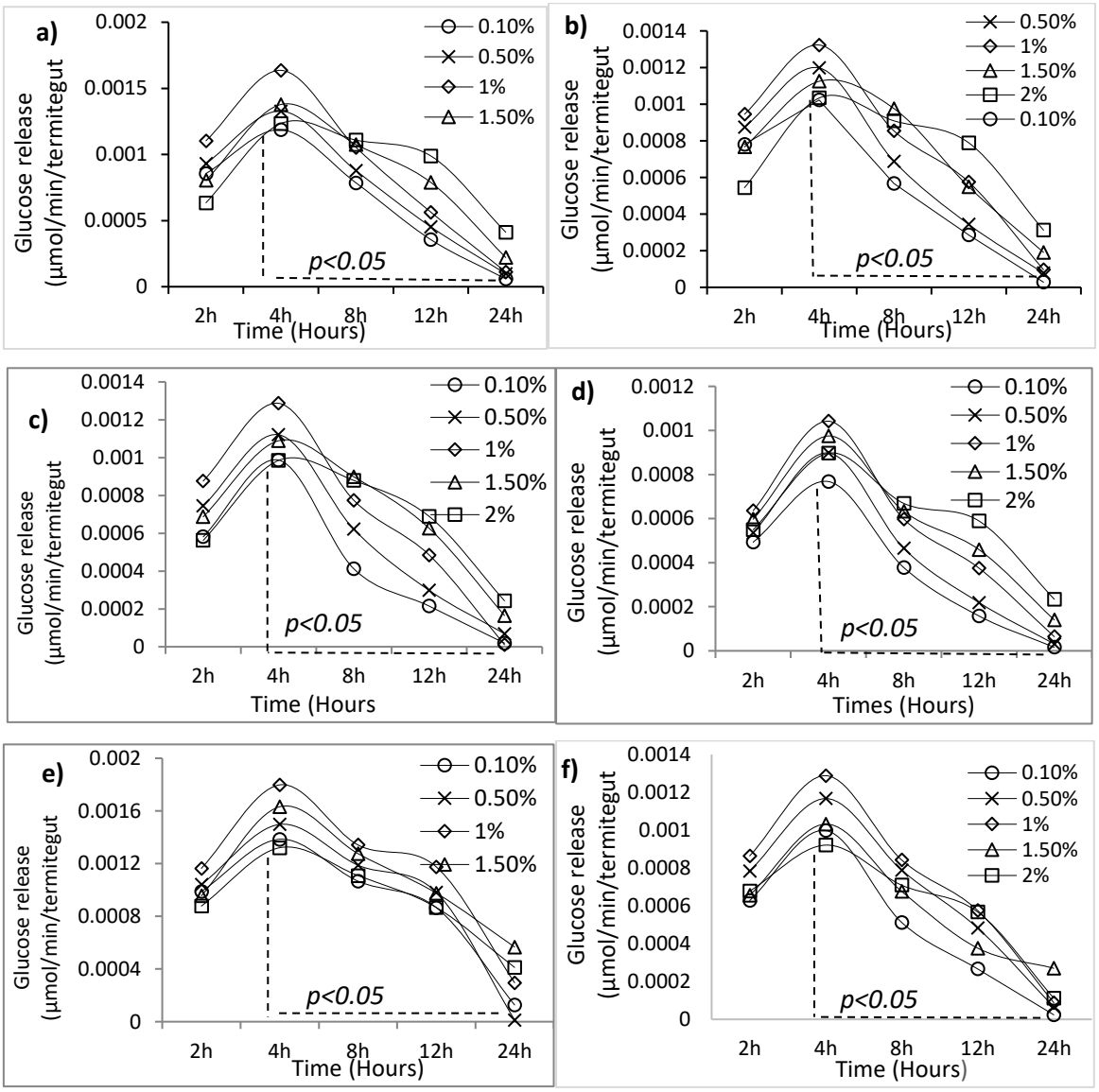


Figure 3.7. *In vitro* saccharification assay optimization by *R. flavipes* whole gut extract at the different time interval of incubation against feeding stocks a) Popular wood (cottonwood), b) Pinewood, c) corn stovers, d) rice husk, e) sugarcane residue, f) filter paper (control) at different concentrations (0.10-2.0%)

3.3.3. *In Vitro* saccharification

A significant difference (*F= 454.0 (df= 5), P= 0.000*) between glucose liberation was observed among the wood lignocellulose substrates by the symbiont fraction of *R. flavipes*.

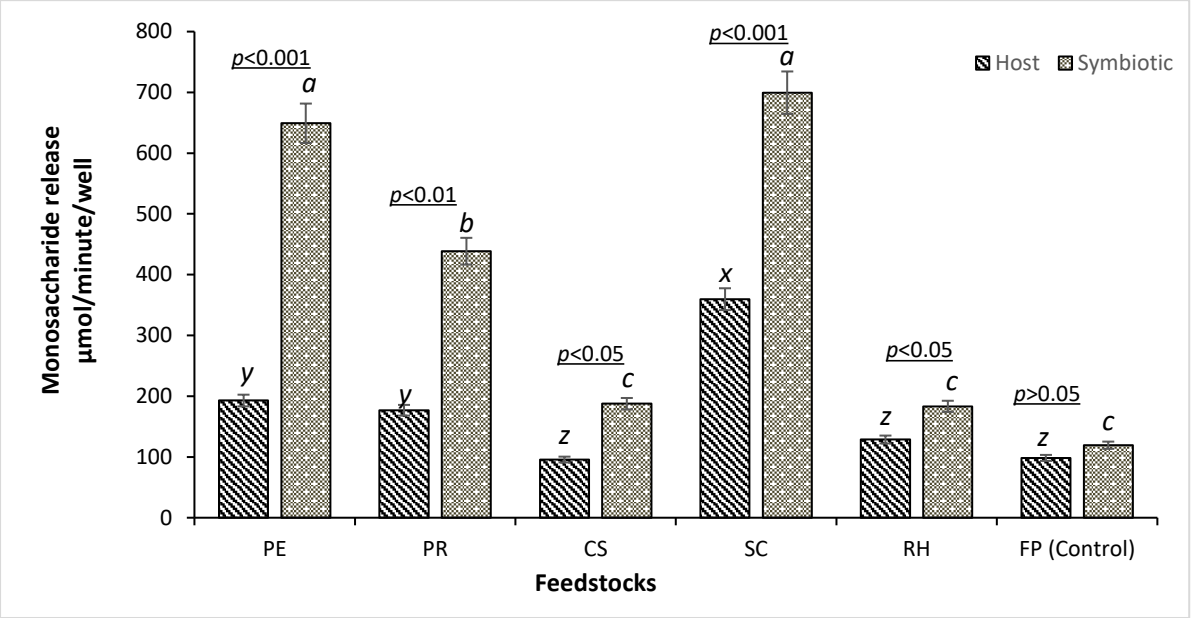


Figure. 3.8a. Monosaccharide released after 12 hours in vitro saccharification assay from the feeding stocks *of Populus eumericana wood* (PE), *Pinus roxburghii* (PR), Corn stovers (CS), Rice husk (RH) and Filter paper (FP) as a control by host and symbiotic fractions of *R. flavipes*.

All feedstocks released significantly (P< 0.05) a high number of monosaccharides than control feeding stock (filter paper) except corn stovers (SC) (187.63±11.03 µmol/min/well) and Rice husk (RH) (183.13±5.25 µmol/min/well) against the cellulase from symbiont fraction of *R. flavipes*. Sugarcane bagasse (SC) and Poplar wood (PR) feedstock liberated the maximum amount (699.88±45.66 and 649.12±11.27 µmol/min/well) of monosaccharides respectively (Fig. 3.8a). Whereas pinewood dust released slightly less number (438.67±13.67 µmol/min/well) of monosaccharides than sugarcane and Poplar wood, but significantly more ($p < 0.005$) than control (filter paper), rice husk, and corn stovers. The same trend of monosaccharide liberation was observed by the host fraction of *R. flavipes* against all feeding stocks, but the amount of monosaccharide reduced significantly (p< 0.05) in the host fraction as compared to the symbiotic fraction of cellulases (Figure 3.8a).

In the case of Pakistani lower subterranean termite, *H. indicola* showed a varied trend of monosaccharide liberation from all feeding stocks by the host and symbiotic cellulase fractions (Figure. 3.8b).

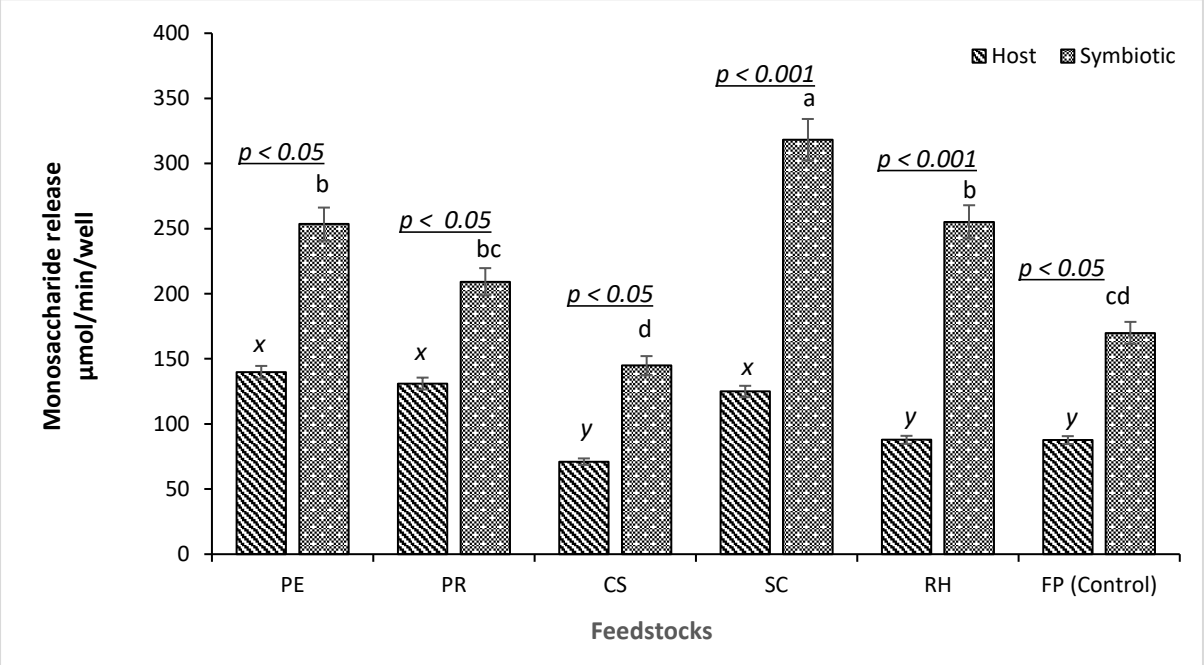


Figure 3.8b. Monosaccharide released after 12 hours in vitro saccharification assay from the feeding stocks *of Populus eumericana wood* (PE), *Pinus roxburghii* (PR), Corn stovers (CS), Rice husk (RH) and Filter paper (FP) as a control by host and symbiotic fractions of *H. indicola*.

Maximum monosaccharide liberation was calculated from the feeding stock of sugarcane (318.83±23.08 µmol/min/well) and rice husk (255.11±22.01 µmol/min/well) followed by poplar wood (253.04±14.84 µmol/min/well) against the action of symbiotic cellulase fraction. A significant difference (*F= 41.49 (df= 5), P= 0.000*) in monosaccharide liberation was observed from the tested feeding stocks and control by a symbiotic fraction. The amount of monosaccharide liberation by *H. indicola* cellulase fractions was reduced >50% than *R. flavipes* from the sugarcane and poplar feeding stock. However, host cellulase fraction showed significantly ($p<0.05$) less liberation of monosaccharides than the symbiotic fraction in all feeding stocks. Rice husk feeding stock was more palatable to *H. indicola* than *R. flavipes*.

To determine if cellulose digestion occurs via additive (collaborative) or more than additive (synergistic) in subterranean termites, the observed (whole gut) and the expected fractions (host + symbiotic) monosaccharide liberation from different feeding stocks by *H. indicola* and *R. flavipes* (Figure. 3.9). The trend of observed and expected monosaccharide liberation in *H. indicola* and *R. flavipes* was approximately same. But observed monosaccharide liberation was significantly ($p < 0.005$) higher than expected monosaccharide (1.4-1.6-fold).

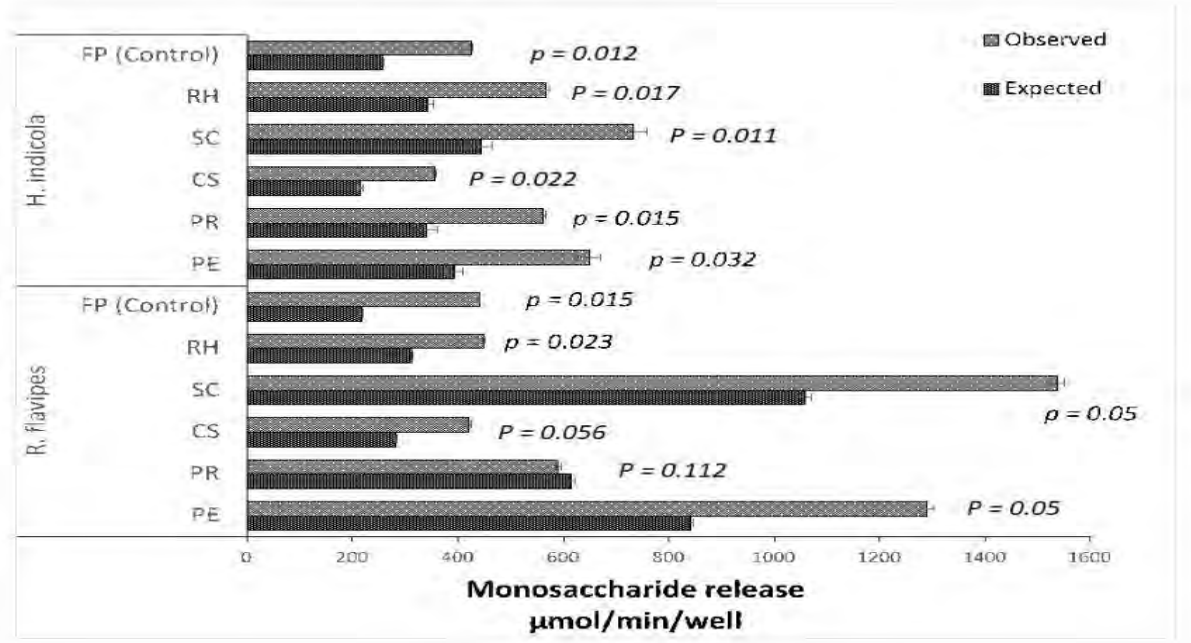


Figure. 3.9. Comparison of observed and expected monosaccharide liberation from different feeding stocks by *H. indicola* and *R. flavipes*. Expected values were determined by adding the host and symbiont fraction results, whereas, observed monosaccharides were liberated by whole gut extracts.

3.3.4. Impact of lignocellulose feeding on the cellulolytic activities of subterranean termites

The impact of lignocellulosic feeding stocks (*Populus* sp. pinewood, corn stover, a sugarcane residue, rice husk, and filter paper) on the enzyme activities by the whole gut extract of *R. flavipes* after 14-days of continuous feeding on various feeding stocks is summarized in Table 3.2. endoglucanase and hemicellulase activities did not vary significantly ($p= 0.067$; $p = 0.057$) respectively, across the feeding stocks treatment.

All other enzyme activities exhibited differences at varying degrees across feeding stocks ($P< 0.05$). All enzyme activities were highest with sugarcane bagasse and Populus wood, while minimum with corn stovers and rice husk feeding stocks. β-Glucosidase activity was maximum per unit than other enzyme activities. cottonwood, a sugarcane residue, and corn stovers were diets associated with the most variable enzyme activity, whereas pine wood. Rice husk and filter paper were the diets associated with the least variable enzyme activity.

Table 3.3. is indicating the impact of feeding stocks on the cellulase activities in *H. indicola* after 14-days diets treatments. *H. indicola* showed a similar trend in enzyme activities except for the magnitude of activities was higher in *R. flavipes*. Endoglucanase and xylanase activities did not show any significant difference ($p = 0.072$; $p = 0.112$) across the feeding stock

Table 3.2. Carbohydrate-based enzyme activities (μmol/min/mg) by whole gut extract of *R. flavipes* after continuous feeding on six different feeding stocks for 14 days.

Activity	Substrate	Feeding stocks						
		Cottonwood	Pinewood	Corn Stover	Sugarcane bagasse	Rice husk	Filter paper	p-Value
Endoglucanase	Carboxymethylcellulose	111.50±8.71^{a}	95.70±10.24^{a}	87.01±6.09^{a}	122.55±4.42^{a}	82.19±2.51^{a}	97.16±1.47^{a}	0.057
Hemicellulase	Beechwood xylan	22.18±0.50^{b}	16.10±1.02^{bc}	8.83±0.22^{e}	36.31±2.25^{a}	06.71±1.00^{ef}	13.13±0.33^{cd}	0.005
or xylanase	Wheat arabinoxylan	03.68±0.67^{ab}	02.54±0.05^{ab}	02.10±0.05^{ab}	7.79±0.05^{a}	01.67±0.01^{bc}	01.76±0.01^{bc}	0.067
Exoglucanase	pNP-cellobioside	17.41±1.66^{b}	11.33±0.67^{bc}	08.16±0.50^{c}	27.73±0.55^{a}	07.73±0.25^{c}	9.27±1.33^{c}	0.009
β-Glucosidase	pNP-glucopyranoside	392.10±08.22^{b}	373.15±12.10^{b}	244.25±2.45^{c}	642.25±23.20^{a}	169.50±5.67^{d}	123.22±6.45^{de}	0.001
β-arabinosidase	pNP-arabinoside	10.62±0.67^{ab}	08.72±1.09^{abc}	04.38±0.05^{cd}	14.15±1.03^{a}	02.35±0.05^{d}	02.10±0.02^{d}	0.010
β-xylosidase	pNP-xyloside	05.54±1.22^{ab}	03.84±0.02^{bc}	02.90±0.05^{cd}	10.50±0.50^{a}	02.22±0.02^{cd}	02.93±0.01^{cd}	0.052
β-mannosidase	pNP-mannoside	32.22±1.33^{a}	17.24±0.33^{b}	11.21±1.12^{b}	33.25±11.00^{a}	09.88±1.22^{b}	10.01±0.05^{b}	0.026

treatment. Whereas, β-glucosidase, β- arabinosidase, and exoglucanase showed significant differences ($p< 0.05$) at varying degrees across the diet treatments. All activities were highest with sugarcane residues, *Populus,* and pinewood whereas, rice husk and corn stovers were least preferable feeding stocks by *H. indicola*. The β-glucosidase activity was observed maximum in all diet treatments by *H. indicola*. Overall, cottonwood, rice husk, and Corn Stovers were the diets associated with the most variable enzyme activity, whereas sugarcane residue, pinewood, and filter paper were the least variable enzyme activity. Xylanase activity showed the least variation across the feeding treatments in *H. indicola*.

3.3.5. Response by host and symbiont fractions of termites across diet treatments

Relative enzyme activity influenced by host and symbiont fractions and paired *t-test* that revealed the significant difference of cellulase activity distribution among host and symbiotic fractions in *R. flavipes* after feeding on six various diets is summarized in Figure 3.10. Most enzyme activities were observed in the host fraction (salivary gland, foregut, and midgut), such as endoglucanase, β-glucosidase, β-arabinosidase, and β-mannosidase activities predominantly occurred in the host fraction across all diets. Two exoglucanase and xylanase activities were higher in the symbiotic fraction (hindgut) after 14-days feeding on sugarcane residue, Populus, and pinewood and filter paper feeding stocks. Whereas corn stovers and rice husk did not show a significant difference ($p> 0.05$) between host and symbiotic fractions. In some activity and diet combinations, enzyme activity was equally distributed among host and the symbiotic fraction of *R. flavipes*, such as β-arabinosidase showed combination with pine wood, filter paper, and corn stovers. All feeding stocks also revealed significant effects on gut fraction enzyme activity distribution.

In the case of *H. indicola*, relative enzyme activity influenced by cellulase host and symbiotic fraction after 14-days feeding on various feeding stocks is summarized in Figure 3.11. A similar contribution by cellulase fractions in all activities after 14 days of continuous feeding was observed in *H. indicola* as *R. flavipes*, except the magnitude of activities was significantly lower in *H. indicola* as compared to *R. flavipes*. Paired t-test showed significant ($P < 0.05$) differences between host and symbiotic factions of *H. indicola* for enzyme activities after various diet treatments. Numerous activities across all feeding stocks treatments occurred in the host fraction (salivary glands + foregut + midgut), such as endoglucanase and β-glucosidase activity mainly occurring in the host fraction across the various feeding stocks.

Table 3.3. Carbohydrate-based enzyme activities (µmol/min/mg) by whole gut extract of *H. indicola* after continuous feeding on six different feeding stocks for 14 days.

Activity	Substrate	Feeding stocks						
		Cottonwood	Pinewood	Corn Stover	Sugarcane	Rice husk	Filter paper	p-Value
Endoglucanase	Carboxymethylcellulose	99.33±2.50^{a}	90.20±1.33^{a}	72.21±2.01^{a}	104.42±3.25^{a}	68.05±1.40^{a}	77.01±2.70^{a}	0.072
Hemicellulase	Beechwood xylan	14.50±0.50^{b}	09.10±0.02^{b}	05.31±0.02^{e}	19.01±0.25^{a}	05.70±0.10^{c}	03.50±0.05^{c}	0.015
or xylanase	Wheat arabinoxylan	02.50±0.33^{b}	01.24±0.05^{b}	01.34±0.01^{b}	5.29±0.05^{a}	01.07±0.01^{b}	02.65±0.02^{b}	0.112
Exoglucanase	pNP-cellobioside	12.42±0.78^{b}	08.30±0.70^{bc}	04.65±0.05^{c}	14.52±0.50^{a}	04.35±0.20^{c}	05.70±050^{c}	0.019
β-Glucosidase	pNP-glucopyranoside	366.42±05.50^{b}	325.50±07.22^{b}	167.55±5.20^{c}	440.25±12.75^{a}	122.40±3.75^{b}	93.15±5.20^{b}	0.001
β-arabinosidase	pNP-arabinoside	08.50±0.50^{b}	05.52±0.50^{b}	02.80±0.01^{b}	12.50±1.25^{a}	02.50±0.02^{b}	01.95±0.01^{b}	0.010
β-xylosidase	pNP-xyloside	03.25±0.52^{a}	02.45±0.02^{a}	01.50±0.01^{a}	05.60±1.20^{a}	01.50±0.11^{a}	01.35±2.50^{a}	0.132
β-mannosidase	pNP-mannoside	25.22±0.50^{a}	15.50±0.01^{b}	10.50±0.50^{b}	30.50±05.50^{a}	06.22±0.25^{b}	08.50±2.05^{b}	0.116

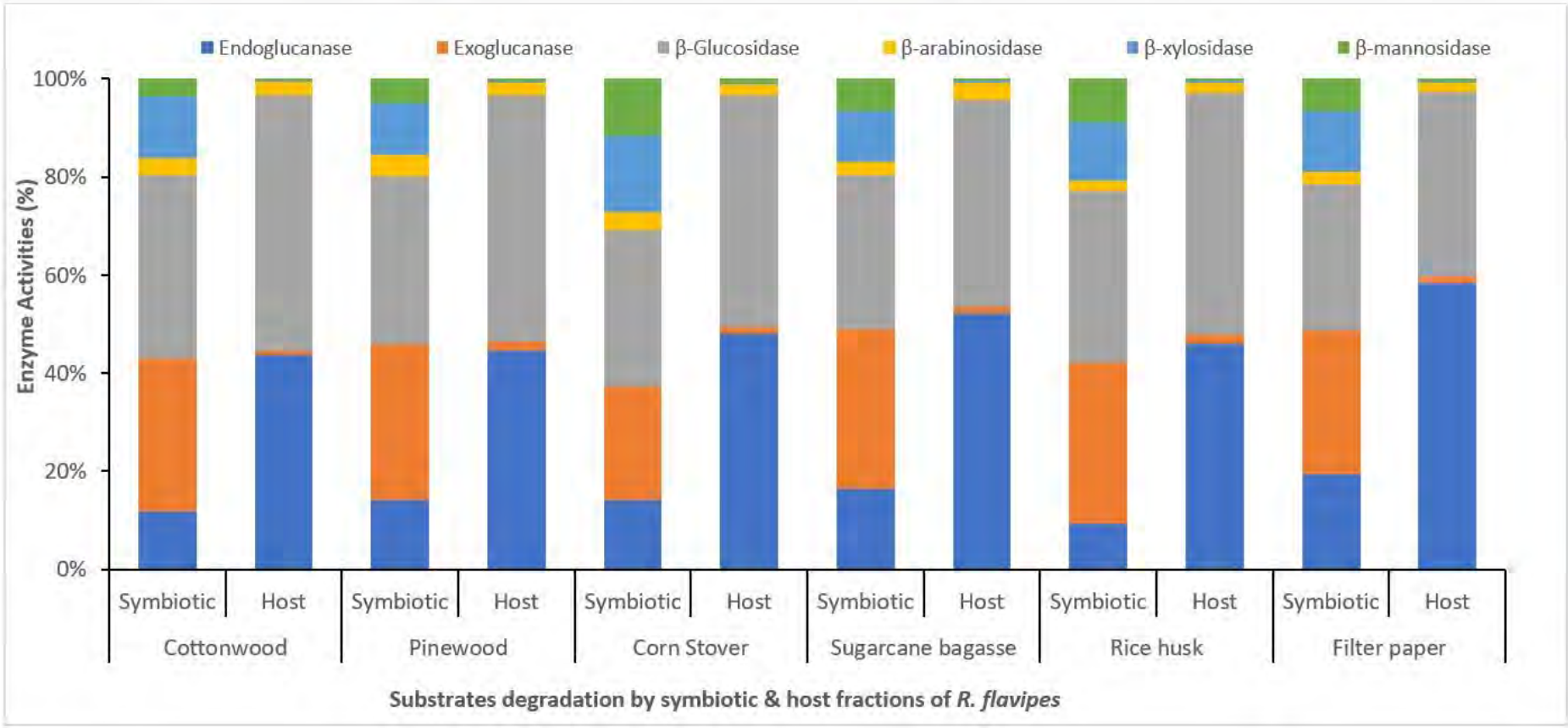


Figure 3.10. Cellulolytic activities (µmol/min/mg) by host and symbiont fractions of *R. flavipes* after continuous feeding on six different feeding stocks for 14 days.

Exoglucanase, β-xylosidase, and β-mannosidase were significantly ($p< 0.05$) higher in the symbiont fraction after feeding on sugarcane residue, *Populus*, pinewood, corn stovers, and filter paper (control) feeding stocks. In addition, three activity-diet combinations of β-arabinosidase

with Populus wood, corn stovers, and filter paper feeding stocks, which had activity equally distributed between host and the symbiotic fraction of *H. indicola*

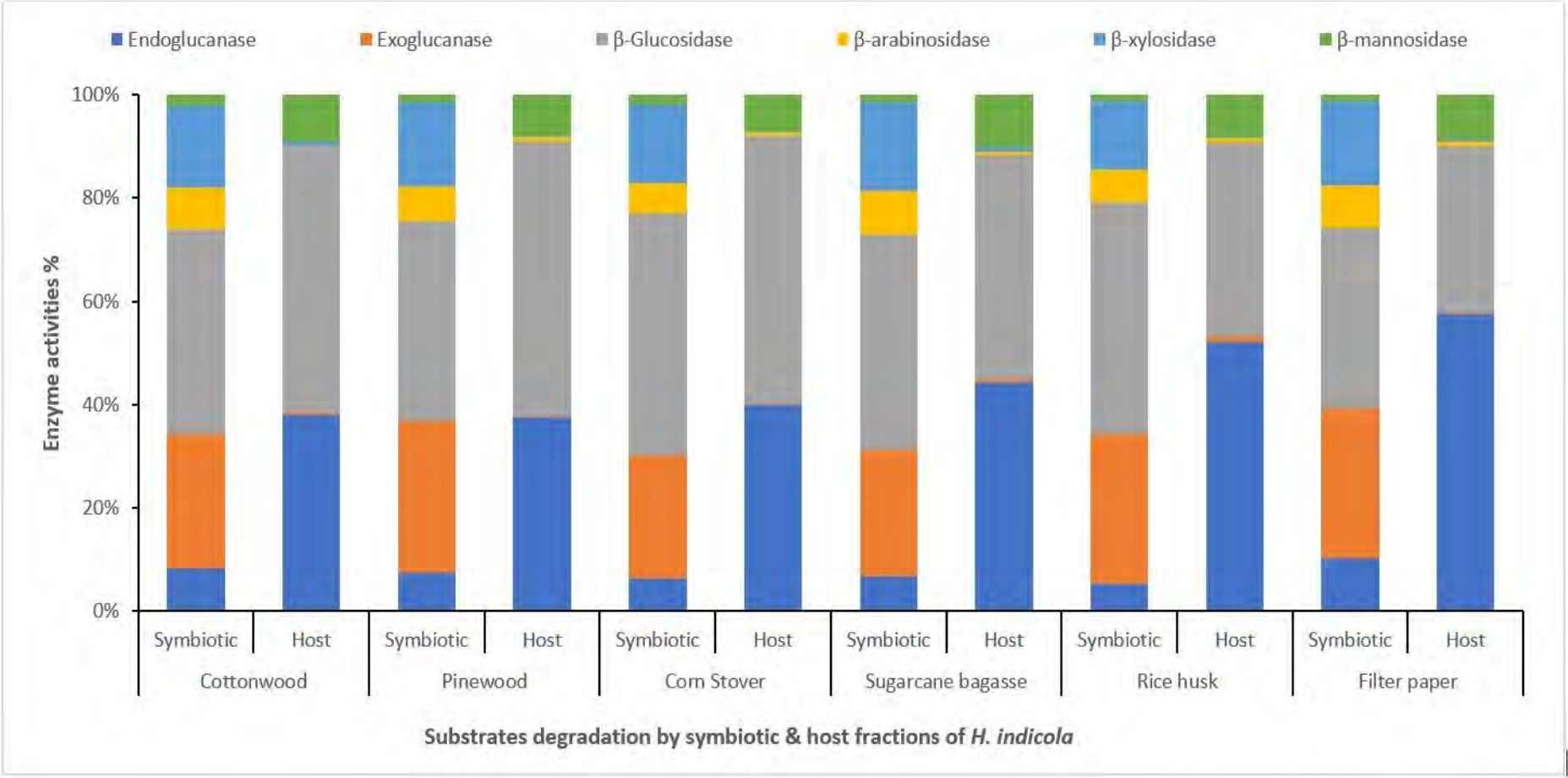


Figure 3.11. Cellulolytic activities (µmol/min/mg) by host and symbiont fractions of *H. indicola* after continuous feeding on six different feeding stocks for 14 days.

3.4. Discussion

Nowadays, biofuel production from lignocellulosic biomass has become a critical need of the energy sector in the world, and saccharification is considered a major limiting phase in it. However, there are some already existing pretreatment methods to digest the lignocellulose matrix, such as physical, chemical, physicochemical, and biological methods which are expensive and in some cases produce hazardous by-products [13]. Keeping in view such drawbacks, there is a critical need to develop efficient and eco-friendly pretreatment strategies for lignocellulose degradation [17,128]. Saccharification via enzymatic system modeled after enzymatic digestions in insects specifically on wooden feeding stocks have efficacy in the pretreatment of the lignocellulosic matrix (Ke, *et al*., 2011; Rajarapu and Scharf, 2017). Subterranean termites studied here, *H. indicola* and *R. flavipes* from different geographic regions have shown an excellent source of the potential enzymatic mechanism involved in the saccharification of lignocellulosic biomass via diet adaptation strategies. In this chapter, the enzymatic mechanisms were studied of *R. flavipes* and *H. indicola* guts fed on feeding stock sugarcane residue, cottonwood and pinewood, corn stovers, and rice husk relative to filter paper (control) to elucidate the enzymatic activities responding to the respective feeding stocks. In previous studies, Rajarapu *et al*., (2015) and Scharf, (2015c) identified novel transcripts responsible for saccharification and shifts in gut microbial profiles after feeding on different diets including corn stovers and soybean residue. In this study, saccharification by the host and symbiotic fractions of termites and response to feeding on diets (sugarcane residue, cottonwood and pinewood, corn stovers, and rice husk) relative to filter paper (control) were evaluated in the form of high foraging activities and survival rates among *R. flavipes* and *H. indicola*. Saccharification assays indicating the release of glucose by termites' guts after all diet treatments approve high foraging activities and survivability during feeding bioassays.

Overall saccharification capabilities, foraging activities, and potential lignocellulose degrading enzyme activities have similar trends in both termite species except the magnitude of activities was higher in *R. flavipes* than *H. indicola*. However, feeding treatments showed variable key results, such as sugarcane residue, *Populus,* and pine wood-feeding stocks were most favorable, whereas. corn stovers and rice husks were least palatable relative to filter paper (control) across both termite species. Key differences in saccharification and lignocellulose digesting enzyme activities by termites were observed as given below in detail.

3.4.1. Saccharification of feeding stocks by termite guts *(In vitro)*

Results of the present study showed a distinct capability of *R. flavipes* and *H. indicola* guts to saccharify feeding stocks sugarcane residues, *Populus* and pinewood, corn stovers, and rice husk relative to filter paper (control) *In Vitro*. These results are supported by *in vivo* no-choice feeding bioassays in which termites showed high foraging activities with 100% survival rate for 12-days when held with all feeding stocks except rice husk in case of both species and it is agreed with previous studies of Rajarapu and Scharf, (2017); Rajarapu *et al*., (2015). *In vitro* saccharification, sugarcane, cottonwood, and pinewood residue released significantly more glucose relative to filter paper (control), whereas, corn stovers and rice husk released low glucose than control and it may correlate with the deceptive proteolytic effects on termites' enzymes composition, as well as the desertion of gut endo microbes in response to rice husk feeding [13,124,125].

Optimization assays indicated that longer incubation time significantly inhibits/cutoff the enzymatic saccharification across the feeding stocks against both termite species. These findings agree with the study reported by Rajarapu and Scharf (2017), in which they found an unexpected reduction in detectable glucose level during longer incubation than 4 hours across corn stovers and soya bean residues, however, the co-incubation of antimicrobial agent sodium azide did not influence the saccharification process over time. These results reflect that incubation time is a more important factor for glucose production in saccharification assays rather than antimicrobial agents (microbial consumption) of released glucose in reactions. Another reason for such reduction in glucose production could be the production of non-detectable by-products form the further conversion of released glucose that is non-detectable by the colorimetric method that is used to detect glucose (Carlson *et al*., 2009; Fuchs *et al*., 2011) or the accidental conjugation of glucose with secondary metabolites present in plant-based feedstocks (Rajarapu and Scharf, 2017).

Production of inhibitory agents in enzymatic saccharification from lignocellulosic feeding stocks is another major apprehension in biofuel production. Such as lignin monomers reside in feeding stocks have the potential to inhibit cellulase activities [19,132,133]. Numerous inhibitory elements have been identified during the pretreatment of feeding stocks via physical and chemical methods (Howard, *et al*., 2003; Huang *et al*., 2011; Jönsson *et al*., 2013). Furthermore, the exploration of metabolite profiles of the enzymatic saccharification reaction can uncover the inhibitory components from in the hydrolysis of agriculture feeding stocks used in this study.

3.4.2. Impact of feeding stocks on cellulolytic activities

Activities of eight different carbohydrate degrading enzyme classes in termite digestive tract as shown in Tables 3.2-3.5, after continuous feeding for 14 days on six distinct feeding stocks (cottonwood, pinewood, corn stovers, a sugarcane residue, rice husk, and filter paper) having variable lignocellulose composition (Figure 2.1). The purpose of these activities was to evaluate the "diet adaptation" hypothesis which was also adopted by Karl and Scharf (2015). According to this hypothesis, the impacts of continuous feeding on different feeding stocks could lead to a variety of enzyme activities that are involved in digesting or detoxifying the feeding stocks [125]. Such variability in enzyme activities can result in hot/symbiotic variations in their protein profile or due to dietary changes among endosymbionts of termites. The applied goals of these activities were to detect the highest cellulase activity for specific feeding stocks and to define host vs symbiotic associations with specific lignocellulose feedstocks for industrial decomposition purposes.

Three main cellulases (endoglucanases, exoglucanases, and β-glucosidase) activities in the present study showed both anticipated and unexpected results for *R. flavipes* and *H. indicola*. These results are like earlier findings reported by Karl and Scharf, (2015) wherein the impact of five different diets (soya bean residue, corn stovers, beechwood xylan, pinewood, and filter paper) on *R. flavipes.* Lignocellulosic material decomposed synergistically by host fraction containing endoglucanase and β-glucosidase, with additive effects of the symbiotic fraction exoglucanase/CBH in both termite species. Lignocellulosic material comprises cellulose, hemicellulose, and lignin in which cellulose polymer is highly abundant and exists in amorphous and crystalline forms due to naturally occurring gaps in hydrogen bond structures [23]. In the present study, endoglucanase activity did not vary remarkedly across the diet treatments which indicates a consistent and continuous production of endoglucanases in both termite species irrespective of substrate residues. Since endoglucanase breakdown the internal glycosidic linkage in the cellulose polymer primarily (Loqué *et al*., 2015; Pauly and Keegstra, 2010). An unexpected outcome of endoglucanase activity in which feedstocks showed a non-significant impact on endoglucanase activity in termites even having the largest number of celluloses. It may be due to bulk depositions of disaccharide glucose molecules at active site of endoglucanase, which block its activity (Scharf *et al*., 2015). In both termite species, host fraction was the main source of endoglucanase activity that agree with previous studies [9,78,99,125], however, Zhou *et al*., (2007) reported some endosymbiotic origin endoglucanases

gene in the hindgut of *R. flavipes*. In the case of exoglucanase symbiotic fraction of both termite species was the main source for their activity. These enzymes also termed as cellobiohydrolases (CBHs) are produced by endosymbionts (protists) in lower termites which act on reducing ends or amorphous cellulose polymers (Zhou *et al*., 2007; Tartar *et al*., 2009; Scharf *et al*., 2011; Sethi *et al*., 2013b; Karl and Scharf, 2015). Therefore, exoglucanase activity mainly occurred in the termite' symbiotic fraction that was anticipated. However, *R. flavipes express* >40 protists' exoglucanase encoding genes belong to the GHF7 glycohydrolase family and making it complicated [9,123–125]. Watanabe and Tokuda (2010b) reported that exoglucanases are the only lower termite gut-associated cellulases that are capable of the degradation of crystalline cellulose. In contrast to endoglucanase activity, exoglucanase activity varied markedly across the diet and highest against sugarcane and pinewood feedstocks in *R. flavipes* and cottonwood was most palatable in the case of *H. indicola*. These results may be due to diet associated gene expression or due to change in endosymbiotic communities that have been experimentally verified especially for GHF7 gene expression in *R. flavipes* by Sethi *et al* (2013) and Raychoudhury *et al* (2013).

β-glucosidases hydrolyze the exoglucanase/CBH generating glucose monomers which involve in exoglucanases/CBH activity blockages (Tokuda *et al*., 2007). Cellulosic substrates extracted from different agriculture sources vary in the crystalline structure, size, shape, and amorphous contents (Montanari *et al*., 2005; Rajarapu and Scharf, 2017). The highest activity of β-glucosidase was obtained with sugarcane residue, cotton and pinewood diets fed termite guts and termites on rice husk and corn stovers showed the least activity that may be due to qualitative disparities in lignocellulose composition among feedstocks. This variance in results may be due to the presence of glucosidase inhibitors in corn stovers and rice husk feedstocks (Ximenes *et al*., 2010; Karl and Scharf, 2015). These inhibiting compounds could be the phenolics and proteases exist in each lignocellulose feedstocks. β-glucosidases activity was maximum in host fraction that agrees with prior functional and sequencing studies data [9,23,99,101] and t-test showed a significant difference between both fractions of each termite species (Tables 3.2 and 3.3). both termite species β-glucosidase activity was significantly ($p<0.05$) highest than all other activities. Another interesting outcome from cellulase activities was that filter paper with >98% pure cellulose form did not uplift the levels of endoglucanase, exoglucanase, and β-glucosidase activities as compared to sugarcane, pinewood, and cottonwood residues. That indicates potential evidence for non-cellulosic material digestion by *R. flavipes* and *H. indicola*. In nature lignin and some kind of hemicellulose have considered

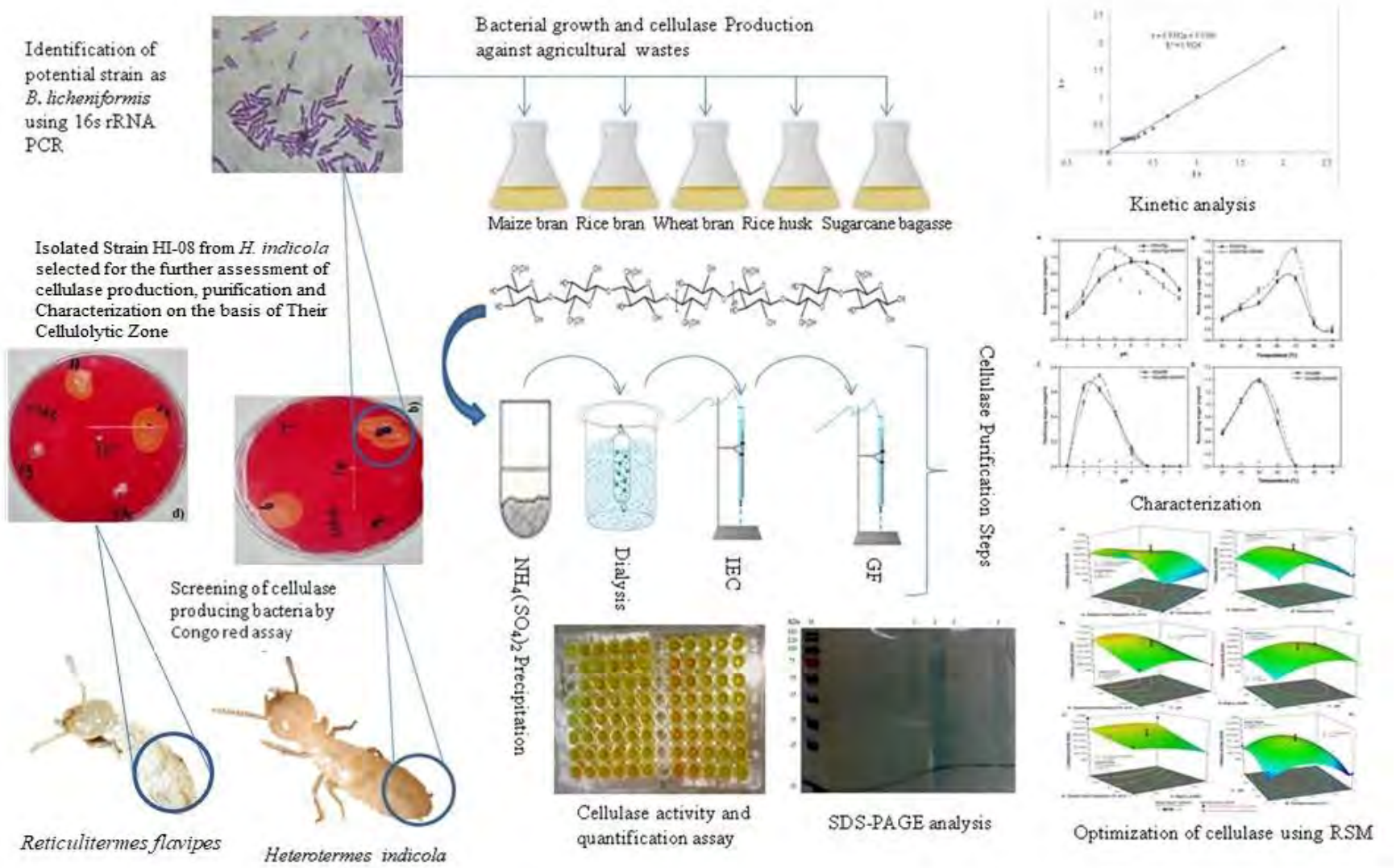
for natural recalcitrant of lignocellulosic biomass [21,138]. This could be the reason for the highest cellulase activities in lignin and hemicellulose rich sugarcane residue, cotton and pinewood, corns stovers, and rice husk relative to control barrier-free diet filter paper. An alternative reason for this lack of cellulase activity on filter paper (cellulose-induced) diet could be feedback inhibition by released glucose monomers or could be slow substrate turnover (Sun and Scharf, 2010b; Scharf *et al*., 2011).

In lignocellulosic material, the degradation of hemicellulose surrounding the cellulose polymer is critical to increasing the enzyme access to cellulose. Hemicellulose is a heterogeneous polysaccharide abundantly comprising of xylose, mannose, glucose, galactose, and arabinose [13]. The trend of β-mannosidase and β-arabinosidase activities was significantly enriched in the host fraction of *R. flavipes* and quite similar to a previous study by Karl and Scharf, (2015). According to Karl and Scharf (2015), these results could be potentially driven by termite endogenous cellulase system that preferentially acts on *p-nitrophenyl* substrates. Tartar *et al* (2009) has also reported that both termites and their endosymbionts encode the mannosidase gene in the sequencing data of *R. flavipes*. Brune (2014) added that β-mannosidases are contributed by hindgut protists in lower termites such as *R. flavipes*. In another study by Rajarapu and Sharf, (2017) reported a significant reduction in β-mannosidase while *R. flavipes* fed on soybean residue that could be due to a decrease in protist population. But in the case of β-arabinoside activity differences in gut fractions of *R. flavipes* are more confusing, since there is no previous report of host-derived arabinosidases in *R. flavipes*. Conversely, in *H. indicola* β-arabinosidase activity preferentially occurred in symbiotic fraction and this could be due to different feeding preferences in termites of different geographic regions. Further studies especially on whole genome sequencing and its annotation in both termites will shed light and elucidate these fraction-specific disparities regarding enzyme activities. Xylanase and β-xylosidase activities were significantly high in endosymbiotic fraction as it was anticipated. The impacts of lignocellulosic feedstocks on the hemicellulase activities are not surprising in that sugarcane, cottonwood, pinewood, and corn stovers feeding led to the highest activities by the whole gut of termites, while rice husk showed the minimum impact on hemicellulase activities. Since, all plant lignocellulose, especially pinewood, cottonwood, and sugarcane residues are mannan and arabinose rich (Pauly and Keegstra, 2010; Aspeborg *et al*., 2012; Rajarapu *et al*., 2015; Michelin *et al*., 2016).

3.5. Conclusion

- The results revealed the capability of lower termites' gut to saccharify lignocellulosic feedstocks sugarcane, cotton and pinewood, corn stovers, and rice husk, substantiating the efficacy of termite gut system as a bio-sourced of enzymes for enzymatic saccharification or pretreatment of agricultural wastes.
- Sugarcane bagasse was the most and highly palatable feeding stock for both termites' enzymatic system that likely would extend to microenvironments of fermentation chamber.
- Moreover, this study identified certain lignocellulase activities that might be responsible for the complete digestion of all feeding stock by supporting the hypothesis "diet-adaptation".

Graphical Abstract



Cellulase production and role in biomass conversion by bacterial endosymbionts isolated from the hindguts of subterranean termites

Abstract

Bacteria are potential microorganisms involved in the enzymatic hydrolysis of agricultural wastes by producing cellulases and other valuable chemicals. Different ionic solvents at different pH and temperature are also involved in the pretreatment of agricultural wastes to enhance the saccharification by cellulases. However, the remaining and conditioning of pretreatment buffers strongly inhibit the cellulase activity. Because of this, a symbiotic cellulolytic strain was isolated from the gut of building infesting termite species *Heterotermes indicola* and *Reticulitermes flavipes* to produce ionic-solvent tolerant cellulase. After screening by Congo red, strain HI-08 was identified as *Bacillus licheniformis* using 16S rDNA sequence analysis. It was accounted for maximum cellulase production on 2% sugarcane bagasse (1156 U/mL). The crude cellulase was purified by $(NH_4)_2SO_4$ precipitation, affinity and size exclusion chromatography, with 12.02 purification fold and 32.33% overall recovery. Approximately 55 kDa molecular weight of gel-purified cellulase was revealed by SDS-PAGE analysis. The optimum temperature and pH for maximum cellulase activity were determined as 45°C and 7.0, respectively. Kinetic analysis of purified cellulase showed the K_m and V_{max} be 2.24 mg/mL and 454.05 µg/mL/min, respectively. Statistical optimization using RSM indicated that sugarcane bagasse was the most useful agricultural waste for cellulase production, which proved its candidature in industrial bio ventures.

4.1. Introduction

The demand for bioenergy has been sharply increased in the world due to high concerns about the scarcity of fossil fuels, the necessity of a sustainable economy, and a clean environment. Cellulosic biomass is the most profuse renewable biological and inexpensive resource in the world with energy content ($3-4/GJ) (Lynd *et al*., 2008; Zhang, 2008; Zhou *et al*., 2009). The hydrolysis of cellulosic materials can promote the local economy, environment, and national energy security (Zhang, 2008). The hydrolysis process has been dependent on thermo-chemical and acid-reliant processes for decades, which has also been blamed for environmental contamination and cost-intensive processes (Hamelinck *et al*., 2005). However, enzymatic hydrolysis is a more environment-friendly and low-cost approach because of a contemporary breakthrough in metabolic engineering, molecular genetics, and enzymatic engineering in modern science (Lynd

et al., 2008). In addition, the enzymatic hydrolysis process of biomass conversion, recovery efficiency, and the cost strongly depends on the fermentation competency of the enzymes and microorganisms (Piškur *et al*., 2006). Cellulases are the enzymes that hydrolyze the cellulosic material into ethanol by the breakdown of β-1, 4 linkages, and subsequent fermentation. Naturally, a group of cellulases, endo-1, 4-β-glucanases, exo-1, 4-β-glucanases, and β-glucosidase that synergistically hydrolyze cellulose into soluble sugars and glucose (Lynd *et al*., 2002) mediates complete hydrolysis of cellulose.

Currently, the trend of cellulase research in the world has been emphasized mostly on fungi, like basic yeast *Saccharomyces cerevisiae* in bio-ethanol production because of its unique properties of genetically modifiable, high conversion ability, and alcohol tolerance (Pourramezan *et al*., 2012). However, the upholding of the fungal mass culture and mounting concentration of aromatic compounds in the industrial conversion of biomass into bioethanol can modify fungal biochemistry and inhibit its application (Piškur *et al*., 2006). Therefore, isolation of an ideal cellulase-producing microorganism with a high capability of biomass conversion and resistance against different inhibitors can enhance the efficiency of the hydrolysis process (Lynd *et al*., 2008).

Some insects, like termites, dung beetles, wood roaches, and crop-eating pests use the lignocellulosic materials as their food source with high efficiency by humiliating the cellulose into glucose (Sun and Scharf, 2010). Among these insect termites are more efficient in the hydrolysis of lignocelluloses due to high adaptive diverse intestinal tract, a dual hydrolysis system enables them as highly resourceful natural bioreactors (Brune, 2014). Gut microbiota in lower termites comprises a diverse range of protozoa, bacteria, nematodes, spirochetes, and fungi that are considered essential for complete cellulose digestion into glucose (Watanabe and Tokuda, 2010). Furthermore, numerous studies have been conducted on the isolation of flagellates and bacteria with their cellulolytic potential from lower termites' species such as *Coptotermes formosanus, Hodotermopsis sjostedti, Reticulitermes seperatus, Neotermes koshunensis,* and *Mastotermes darwiniensis* (Tokuda *et al*., 2007; Yuki *et al*., 2008; Lo *et al*., 2011; Sethi *et al*., 2013; Ni and Tokuda, 2013). Therefore, termites are an attractive source as novel cellulolytic microorganisms and cellulases for the conversion of biomass into biofuel at the industrial level.

In Pakistan *Heterotermes indicola* Wasmann, and United States *Reticulitermes flavipes* Kollar is considered as the most destructive lower termite species to wooden structures in urban settings (Su, 2002; Manzoor and Mir, 2010). The hindgut of both termite species is enlarged than the midgut and houses a diverse range of protozoa and bacteria. Symbiotic cellulase system produces more complex cellulolytic enzymes. Thus, it possesses strong hydrolytic activity (40-88%) against carboxymethyl cellulose (CMC) substrate as compared to endogenous cellulase (40-85%) of total gut activity (Tokuda *et al*., 2005). Furthermore, several intestinal bacteria have been isolated from different termite species that play an important role to degrade the cellulose fibers (Brune, 2014). It is, therefore, considered that the hindgut of *H. indicola* and *R. flavipes* may represent an ideal prospecting resource for identifying potential microorganisms, which can be used for cellulase production in biomass conversion application. This chapter was focused on screening and identifying the potential cellulolytic bacterium from the hindgut of wood-feeding termite species *H. indicola* and *R. flavipes* in Pakistan. The cellulase production, purification, and optimization were carried out to understand enzyme kinetics for the confirmation of cellulase candidacy in different bio-applications.

4.2. Materials and methods

4.2.1. Microorganism

The workers of *H. indicola* were collected from infested storerooms in the Biological Science Department of Quaid-i-Azam University, Islamabad Pakistan (33.747680°N, and 73.138161°E) in monsoon season (July to September) of 2018. Whereas a field colony of *R. flavipes* was collected at Purdue University campus West Lafayette IN, the USA in monsoon (2019) using cardboard baits. Approximately, 25 guts of each species were stabbed using sterile (70% EtOH washed) forceps and needles from workers of both termite species aseptically transferred into Erlenmeyer flask containing 25 mL enrichment media and incubated at 37°C for 72 h. The enrichment media was contained on following components dissolved in dH_2O at pH 7.0: 1% CMC, 0.6% NaCl, 0.5% $(NH_4)_2SO_4$, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4$, 1% yeast and 1% nutrient broth. After enrichment 10 µL of culture was spot inoculated on CMC supplemented agar plates in replicate of three and incubated at 37°C for 24 h to get pure and discrete colonies of bacteria. Among the 15 isolated strains, the potential strain was selected based on the clear zone produced on 0.1% Congo red-stained CMC-agar plates and subjected to further cellulase production and characterization.

The strains with >1 cm hydrolysis zone was considered as significant and selected for cellulase production.

The selected strain was identified by using standard protocols of morphological and biochemical techniques described in Bergey's Manual for Determinative Bacteriology (Holt *et al*., 1994). The molecular identification of the selected strain was performed by 16S rRNA. gene sequencing. In brief, DNA was extracted using the phenol-chloroform method (Marmur, 1961). The PCR mixture was prepared by adding: 2 µL of genomic DNA template, 5 µL 5X PCR buffer, 4 µL 50 mM $MgCl_2$, 1 µL 10 mM dNTPs, 1 µL of each forward (UnF 5′-CCAGCAGCCGCGGTAATACG-3′) and reverse primer (UnR 5′-GGACTACCAGGGTATCTAAT-3′) (Barghouthi, 2011), 0.5 µL *Taq* polymerase enzyme and dH_2O to make a total volume of 50 µL.

PCR reactions were carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, extension at 72°C for 1.5 min and a final extension for 10 min. The hold was at 4°C and the PCR product was confirmed by gel electrophoresis on 1% agarose gel and purification was carried out using Gene JET Gel (Thermo Scientific®, EU) kit. The concentration of purified DNA was estimated by nanodrop (Nano Drop® ND-1000, Thermo Scientific, USA), and the purified PCR product was sequenced commercially. The consensus sequences were constructed using MEGA6 [141].

4.2.2. Preparation of crude enzyme and assay

The crude enzyme was obtained by transferring the 24 h old cultures to falcon tubes, sonicated for 10 min, and centrifuged at 8000 rpm for 15 min. The resultant supernatant was collected as extracellular crude cellulase and cells were discarded. Cellulase activity was determined by measuring the amount of reducing sugar released from the enzyme and substrate (CMC) reaction through DNSA (3, 5-dinitrosalicylic acid) method (Miller, 1959). In this method, the reaction was conducted by adding 500 µL of the substrate (1% CMC; 0.1M phosphate buffer pH 7.0), 100 µL of the crude enzyme, and 400 µL dH_2O in a test tube. The reaction was incubated at 45°C for 30 min and stopped by adding the same amount (1 mL) of the DNSA solution. Then the reaction was

boiled in a water bath for 10 min followed by cooling in ice at 5 min for stabilization. Optical density was measured using a spectrophotometer (SmartSpec^{TM} Plus BIO-RAD, USA) at 546 nm.

The activity of the cellulase was determined by drawing standard curve at different concentration of glucose (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 mg/mL). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg glucose per minute mL^{-1}.

4.2.3. Cellulase production on agro-wastes

The effect of five agricultural waste materials (maize bran, sugarcane bagasse, wheat bran, rice bran, and rice husk) on the production activity of cellulase was determined by the growing culture in cellulolytic broth media supplemented with 2% (w/v) agro-waste, 0.2% K_2HPO_4, 1% peptone, 0.03% $MgSO_4$ and 0.25% $(NH_4)_2SO_4$. The culture was incubated in rotary shakers (100 rpm) at 45°C for 36 hrs. The enzymatic production activity was determined by the DNSA method (Miller, 1959) as discussed in the enzyme assay section above.

4.2.4. Purification of cellulases

The resultant supernatant from crude enzymes of the selected strain was subjected to purification. All purification measures were carried out at 4°C. The cell-free supernatant was precipitated overnight with ammonium sulfate $(NH_4)_2SO_4$ at 8000 rpm for 10 min and the pellet was recovered at 60 and 80% of saturation. The precipitated cellulose was resuspended in 0.1 M phosphate buffer pH 7.0 and dialyzed in the same buffer using a dialysis tube with pore size <10 kDa. The recovered cellulose was dissolved in fresh buffer, assayed for activity, and protein estimation. The dialyzed cellulase was subjected to a column of ion exchange resins Roti® change (1×4 Roth®, Germany; Cat No. 6849.1) equilibrated with 0.1 M phosphate buffer (pH 7.0). The desired fractions were allowed to attach with a matrix for 1 h at 4°C and eluted with a linear gradient of NaCl (0.1 to 0.4 M) at a flow rate of 0.5 mL/min. Approximately 1 mL for each fraction was collected and used for enzyme activity and protein estimation assay. Active fractions were pooled and subjected for the further purification step.

The pooled fractions of cellulase were further purified by gel filtration chromatography. The Sephadex G-75 column prep grade (0.7×10 cm; Luer Lock column, Sigma-Aldrich, USA;

Lot#3110) was equilibrated with the same buffer and eluted at a flow rate of 0.5 mL/min. The gel-purified enzymes were also subjected to enzyme activity and protein estimation.

4.2.5. Estimation of protein concentrations

The estimation of protein concentration in the crude precipitated and purified cellulases was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard expressed as mg mL^{-1}. The optical density was measured through a spectrophotometer at 280 nm.

4.2.6. SDS-PAGE analysis

The molecular weight of the cellulose in crude and purified form was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli *et al*. (1976) using 5% staking and 10% resolving gel. The gel was stained using 250G Coomassie brilliant blue and de-stained in de-ionized methanol acetic acid-water (Sambrook and Russel, 2001). The approximate molecular weight of cellulases enzymes was estimated by running samples in SDS-Page against standard PageRuler^{TM} pre-stained protein ladder, 10-180 kDa (Thermo-Scientific Pvt Ltd., USA).

4.2.7. Kinetic parameters

The effect of substrate concentrations was investigated on the velocity of the reaction of purified cellulase. The reaction was incubated with various concentrations of CMC ranging from 0.25 to 2.5 mg/mL under standard assay conditions. The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Line weaver-Burk plots of the Michaelis-Menten equation (Lineweaver and Burk, 1934).

4.2.8. Characterization of purified enzymes

The effect of temperature on enzyme activity was followed by incubating the reaction of cellulases with 0.1% CMC in 0.1M phosphate buffer pH 7.0 at different temperatures (25, 35, 45, 55, 65, 75, 85, 95, and 105°C) under standard assay conditions. The effect of pH on the activity of cellulases was also calculated by measuring the hydrolysis of CMC in 0.1 M phosphate buffer at different pH values ranging from 3.0 to 9.0. The CMCase activity was determined at 45°C under standard test conditions (Miller, 1959). The effect of different metal ions (0.01 M) on enzyme activity was

determined by incubating reaction with solutions of $CaCl_2$, $ZnSO_4$, $MgCl_2$, $CuSO_4$, $FeSO_4$, $NaCl_2$, $HgCl_2$, $AgCl_2$, Co and $NiCl_2$ at 45°C for 1 h.

4.2.9. Statistical optimization using response surface methodology (rsm)

Statistical optimization for cellulase production was assessed by evaluating the intensity of different factors on the production using the statistical approach second-order Box-Behnken Design (BBD) (Ferreira *et al*., 2007). Based on the results of preliminary assays during cellulase characterization, most effective independent variables such as sugarcane bagasse (% w/v), temperature (°C), pH, and $MgCl_2$ were selected for statistical optimization of cellulase with 3 different levels (Table 4.3). The experimental design was built and analyzed by subjecting the data for ANOVA, estimation of main effect and interaction, and fitting the second-order regression equation using the Design-Expert software 11.0 (Stat Ease Inc., Minneapolis, MN, USA) as described by Saini *et al.* (2015).

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j \quad (1)$$

Where, y is response (cellulase production); β_0 is the constant value; x= independent variables affecting response; βi, βii, βij are coefficients for linear, interaction, and quadratic regression respectively; and k is the total number of variables. Based on results obtained from 29 experiments of the BBD matrix, a quadratic non-linear polynomial regression equation (Eq. 2) was constructed that indicated the pragmatic relationship between response and selected variables in coded form.

$$Y = +1013.60 + 203.50A - 127.33B + 118.33C + 85.00D - 127.00AB + 58.50AC - 82.50AD - 1.25BC - 22.25BD - 9.75CD + 20.83A^2 - 452.67\,B^2 - 218.67\,C^2 - 64.42\text{-}D^2 \quad (2)$$

Where the Y= cellulase activity (U/mL); A = Sugarcane bagasse (% w/v); B = Temperature (°C); C = pH; and D = $MgCl_2$ (mM).

4.3. Results

3.3.1. Isolation, screening and identification of cellulolytic strain

Fifteen bacterial strains were isolated from the hindgut of *H. indicola* and *R. flavipes* on the nutrient agar supplemented with carboxymethyl cellulose (CMC). Only seven isolates exhibited good cellulase activity that was reevaluated on CMC agar plates stained by Congo red and NaOH solutions in triplicate (Figure. 4.1). The zones of clearance by isolates reflected their extent of cellulolytic activity. The isolates with good cellulolytic activity produced >1.0 cm zone just after 6 h of incubation at 40°C indicating an extracellular property of cellulases. Among seven strains, HI-08 indicating maximum diameter of clearance zone in all replicates, thus HI-08 was selected for further processing (Figure. 4.1).

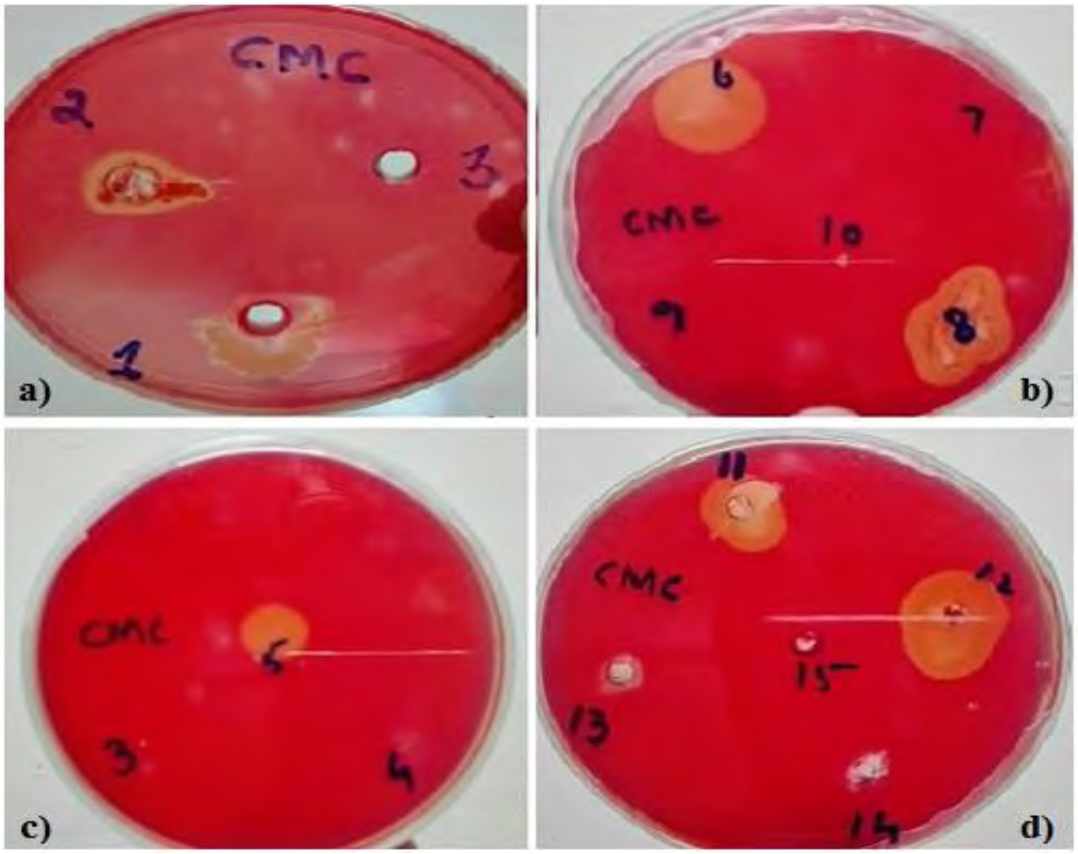


Figure. 4.1. The screening of cellulolytic bacteria cultures on CMC ager plats with Congo-Red assay in replicate of three. The zones of clearance by isolates reflected their extent of cellulolytic activity.

The competent cellulase producing isolate HI-08 was rod-shaped *Bacillus* gram-positive, motile showing high growth in aerobic and anaerobic environment with wide range of pH (5-11).

It was positive in catalase, acetyl methyl carbinol, citrate and nitrate reduction test and negative in oxidase. Based on morphological and biochemical characteristics, it was identified as *Bacillus* sp. by industrial microbiology lab, Quaid-i-Azam University Islamabad, Pakistan. The analysis of 16S rRNA gene sequence of strain HI-08 indicated its 99 % homology with *Bacillus licheniformis* strains and submitted as *B. licheniformis* HI-08 to GenBank [KY786028] (Figure. 4.2).

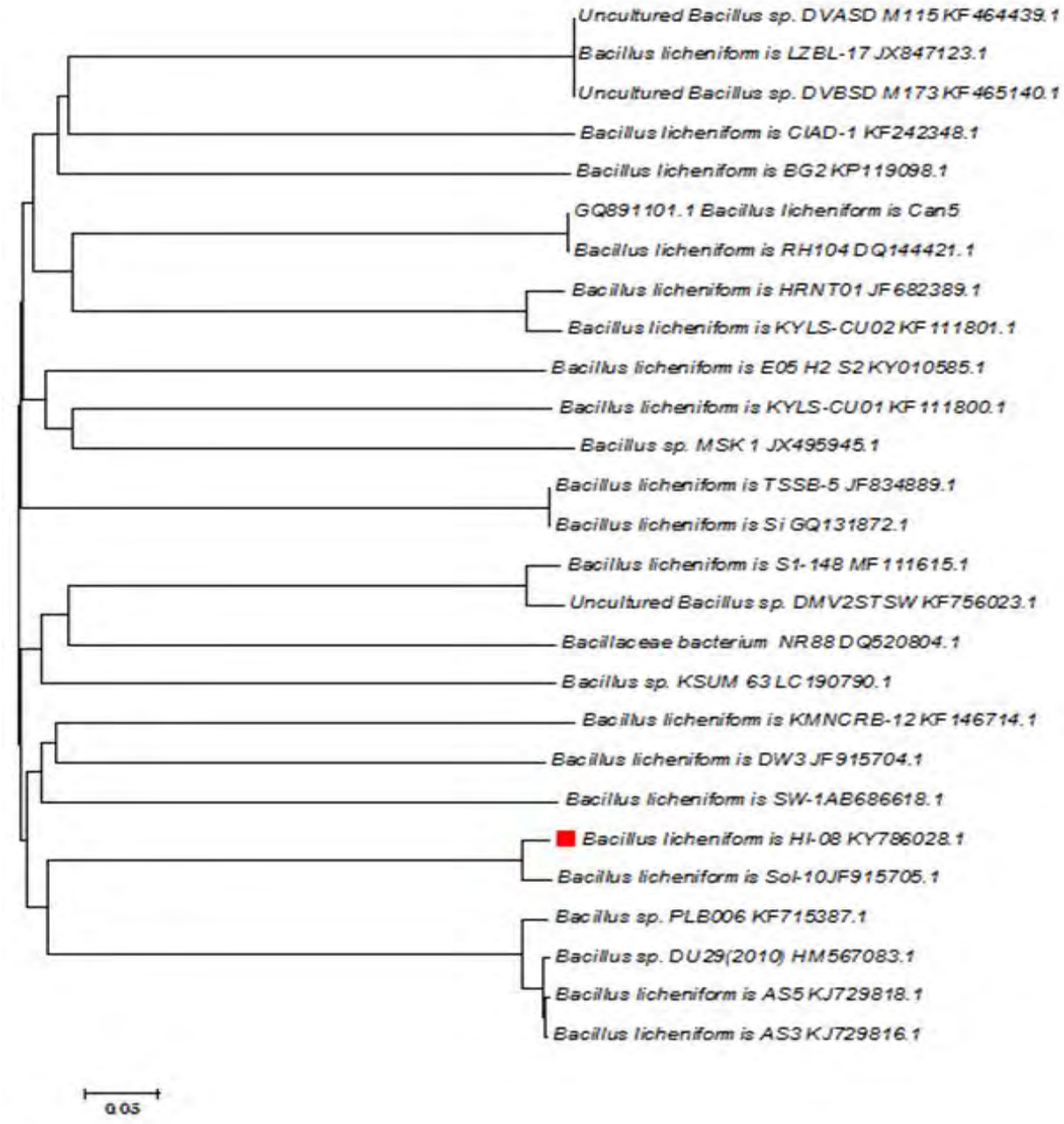


Figure. 4.2. Phylogenetic tree analysis of *B. licheniformis* HI-08 strain. Tree was constructed using neighbor-joining method using bootstrap method (1000 replicates) by Mega 6.

4.3.2. Cellulase production on agro-wastes

Bacterial strain *B. licheniformis* HI-08 showed maximum cellulase production on 2% sugarcane bagasse (1156.03 U/mL) and found it suitable substrate for highest cell growth followed by rice husk (985.09 U/mL) and rice bran (725.73 U/mL) after 36 h of incubation at 45°C (Figure. 4.3). However, the maize bran (602.33 U/mL) and wheat bran (712.56 U/mL) showed minimum cellulase production and cell growth (Figure. 4.3).

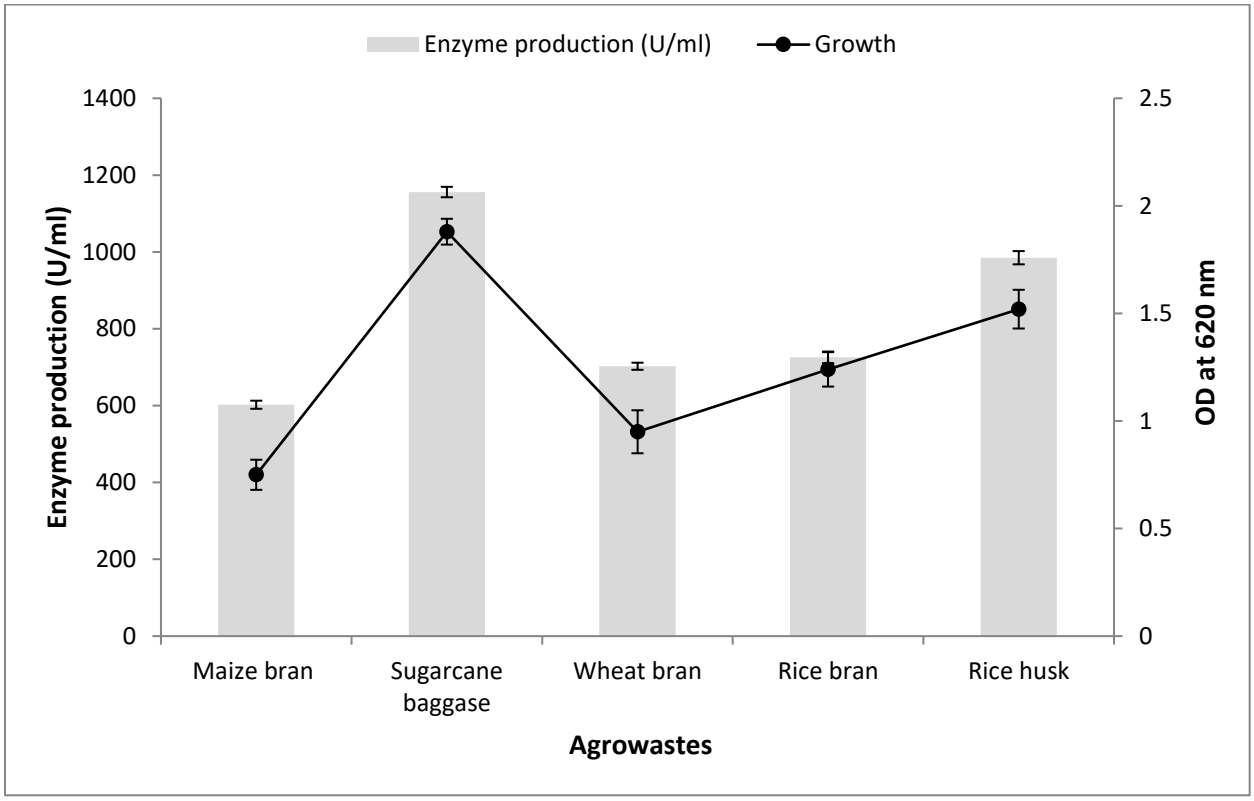


Figure. 4.3. The cellulase production and growth profile of *B. licheniformis* HI-08 against five different agro-wastes (carbon source) such as maize bran, sugarcane bagasse, wheat bran, rice bran, and rice husk (2%). Each value indicating the mean±SD.

4.3.3. Purification of crude cellulase

The crude of extracellular cellulases with maximum activity (5598.70 U/mL) against CMC was first subjected to ammonium sulfate precipitation (salt in) dialysis (salt out) for purification. The maximum pellet and cellulase activity (4036.6 U/mL) were recovered in a fraction of 80% obtained by the addition of $NH_4(SO_4)_2$. The fraction had 7.38 mg total protein and 546.88 U/mg specific activity with 72.09% recovery and 2.7 purification folds (Table 4.1).

Table 4.1. Purification steps indicating total activity, total protein, specific activity, purification fold, and percentage yield of cellulase from *B. licheniformis* HI-08.

Purification Steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	5598.70	28.54	198.14	1	100
Precipitation $(NH_4)_2SO_4$	4036.6	7.38	546.88	2.76	72.09
Ion exchange resin	3417.52	2.98	1146.81	5.78	61.04
Sephadex G-75	1810.23	0.76	2381.88	12.02	32.33

No enzyme activity and protein contents were observed in collected unbound fractions. The cellulase activity and protein contents were detected in fraction No. 9 of bounded protein that was eluted by the addition of 500 mM NaCl (Figure. 3.4a). It showed a high specific activity of 1146 U/mg with 61% recovery (Table 4.1).

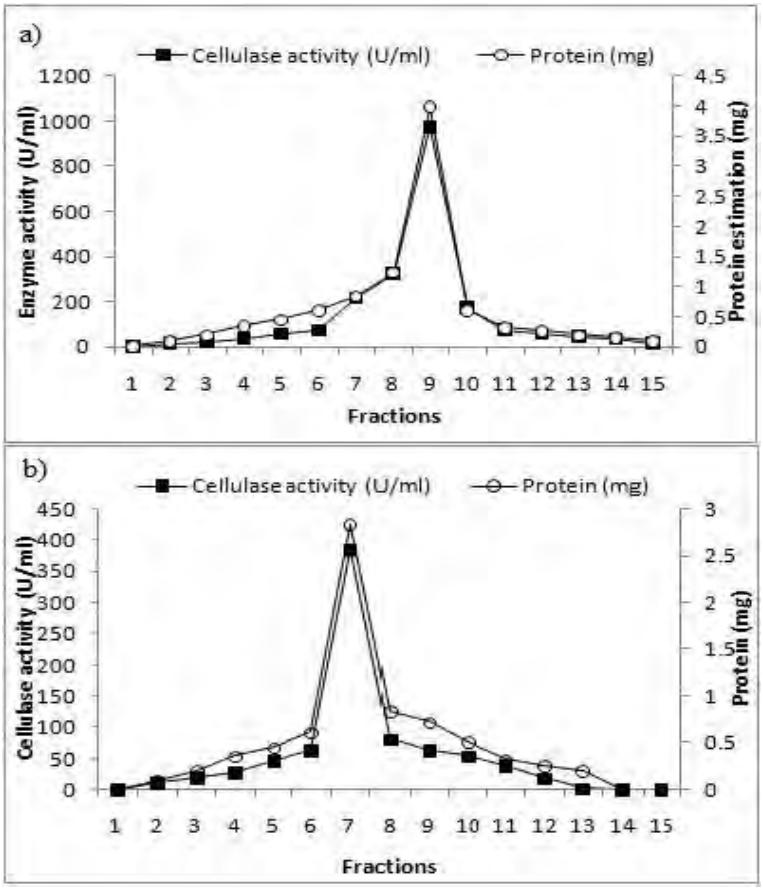


Figure 4.4. Chromatographic purification profile of cellulase from *B. licheniformis* HI-08 on ion-exchange column (a) and Sephadex G-75 column (b).

The gel filtration Sephadex G-75 was used for further purification of active fraction obtained by ion-exchange chromatography. Figure. 4.4b indicates the overlapping active peaks of protein contents and activity at fraction No. 7 on the Sephadex G-75 column. The confirmation of cellulase purity was assessed by the presence of a single band on SDS-PAGE after ion-exchange and gel filtration. The molecular weight of the purified cellulase of *B. licheniformis* was approximately 55 kDa (Figure. 4.5).

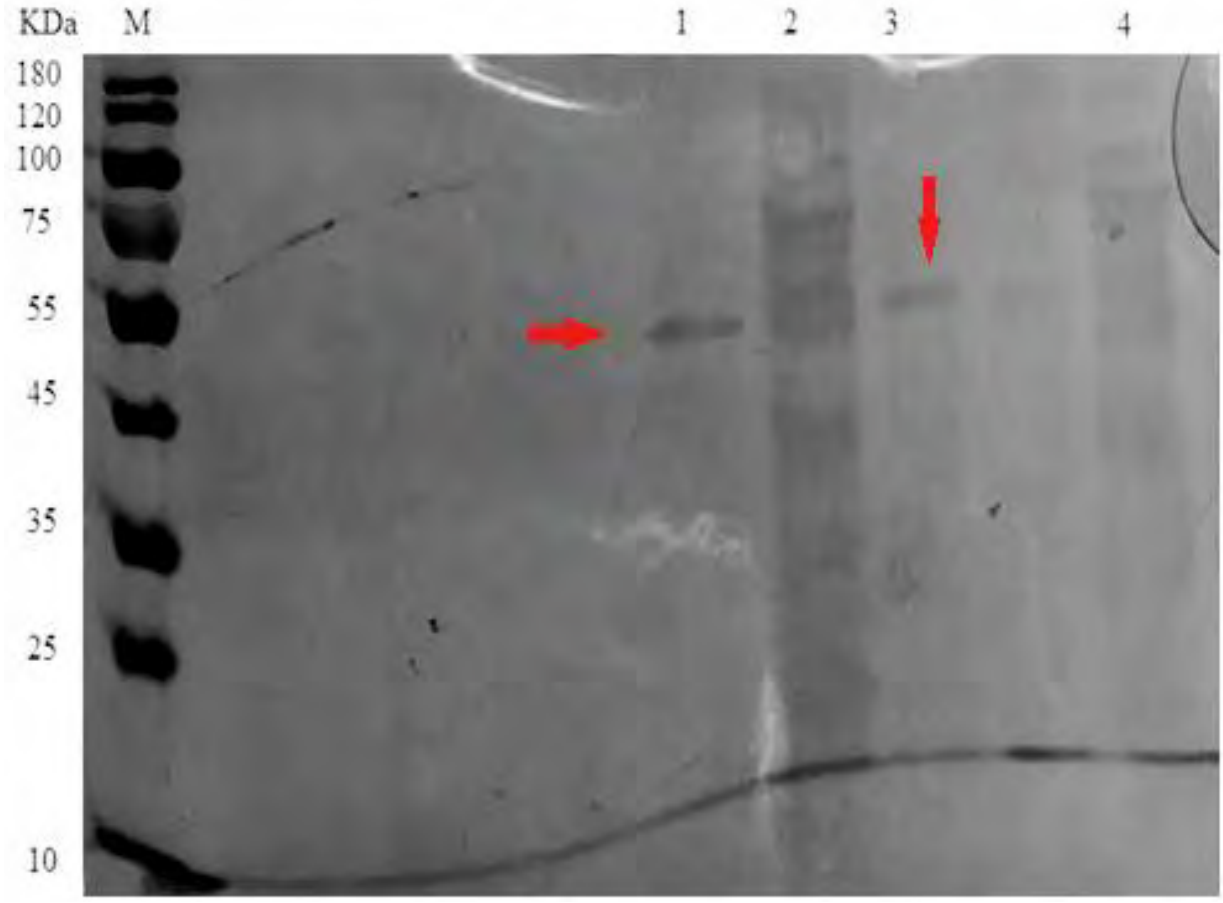


Figure 4.5. SDS-PAGE analysis of cellulase from *B. licheniformis* HI-08 obtained after different purification steps; M: Markers kDa; Lane 1: arrow indicating purified cellulase band after anion chromatography; Lane2: crude supernatant; Lane 3: arrow indicating cellulase band obtained after gel-filtration Sephadex G-75; Lane 4: concentrated fraction after $NH_4(SO_4)_2$.

4.3.4. Kinetic analysis

The kinetics of purified cellulase with CMC exposed for HI-08 the K_m and V_{max} to be 2.28 mg mL^{-1} and 454.05 µg mL^{-1} min^{-1} respectively by Lineweaver-Burk plot (Figure. 4.6).

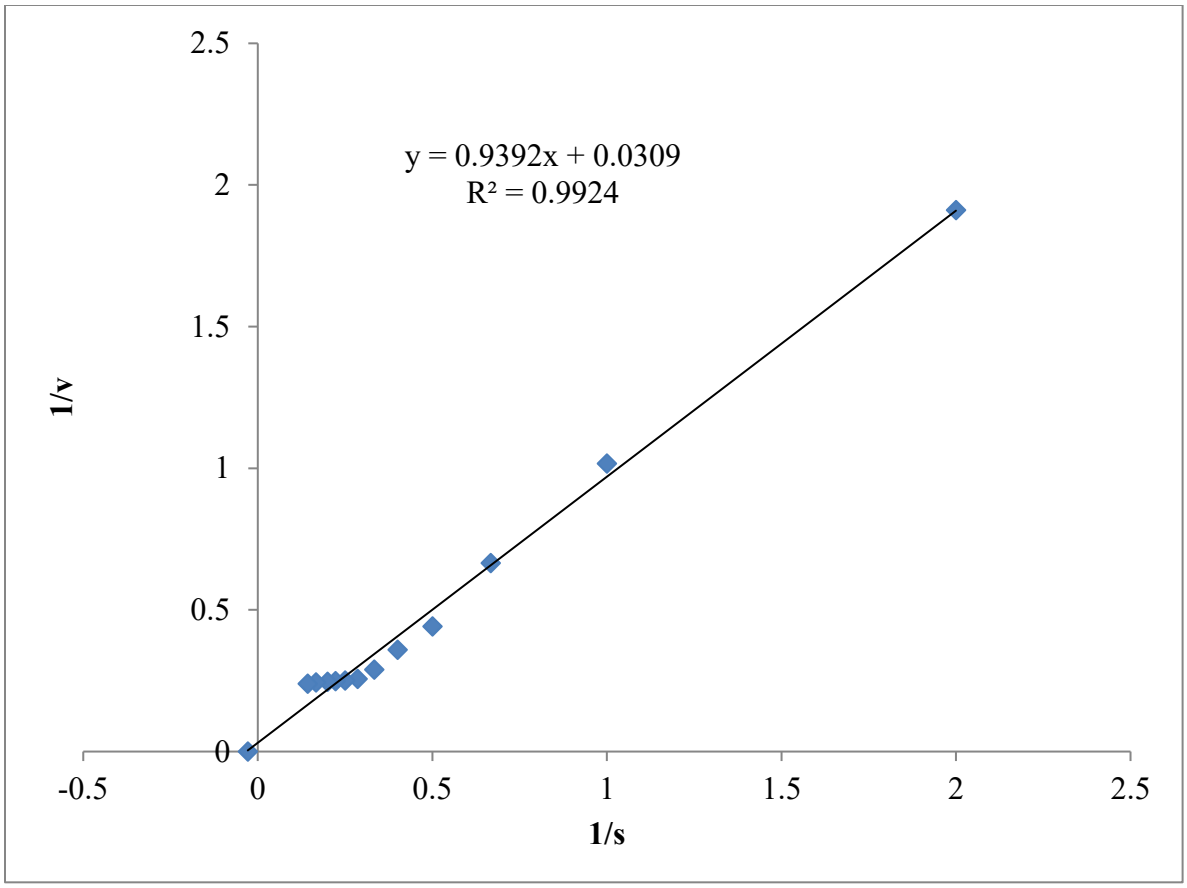


Figure 4.6. Lineweaver-Burke plot of initial velocity data of cellulase against different concentrations of CMC.

4.3.5. Temperature and pH Optimization

Purified cellulases showed optimal activity at 45°C and turned down thereafter at varying temperatures ranged from 25-105°C (Figure 4.7a). The optimal pH for the activity and stability of purified cellulase was determined by using different pH ranging from 3-10. Figure 4.7b indicates the maximum cellulase activity and stability at pH 7.0 in 1 h of incubation.

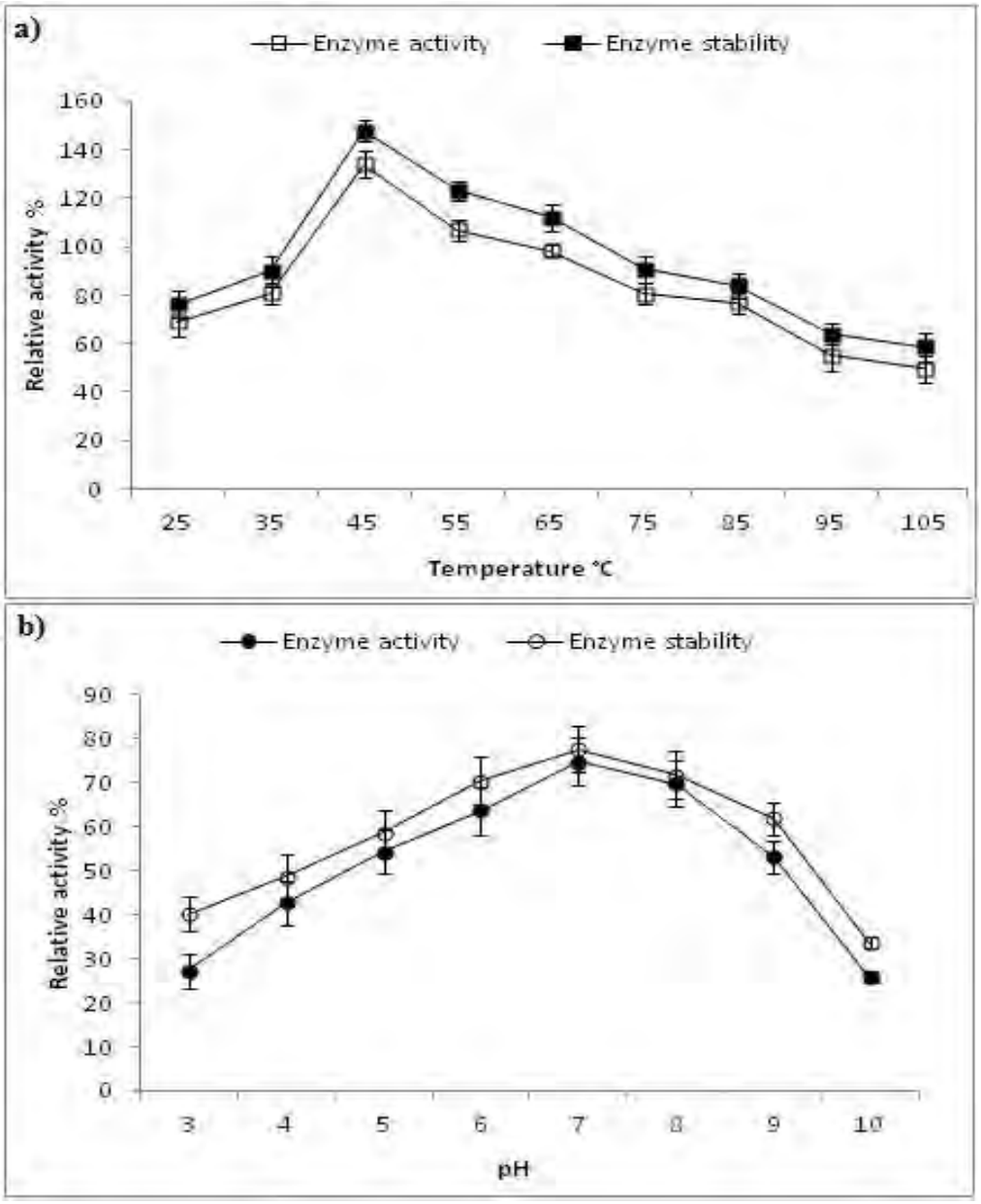


Figure 4.7. Temperature (a) and pH (b) effect on purified cellulase extracted from strain HI-08 after 1 h of incubation under standard assay conditions.

4.3.6. Effect of metal ions on cellulase activity and stability

The activity and stability of purified cellulases of strain HI-08 were enhanced in the presence of alkali metal ions such as Mg^{+2}, $Ca^{+2,}$ and Na^{+} by increasing concentration (Table 4.2). Maximum inhibition of cellulase activity was observed against mercury ion Hg^{+2} at both concentrations (10 and 20 mM) under standard assay conditions. Likewise, 20 mM of Zn^{+2}, Ni^{+2}, $Co^{+2,}$ and Cu^{+2} slightly inhibited the cellulase activity and stability as compared to control (Table 4.2).

Table 4.2: The activity and stability of the purified cellulase of *B. licheniformis* HI-08 against different metal ions at concentrations 10 and 20 mM.

Metal ions	Concentrations (mM)	Residual activity %	
		Enzyme activity	Enzyme stability
Control	0	100	100
$CaCl_2$	10	139	117
	20	205.5	175.5
$ZnCl_2$	10	88.6	62.4
	20	56	44
$MgCl_2$	10	144.4	119
	20	215.2	180.5
$CuSO_4$	10	67.5	56.0
	20	44.5	26.9
$FeCl_3$	10	121	98.8
	20	88.6	65.5
NaCl	10	134.5	102.0
	20	182.8	144.6
$HgCl_2$	10	33**	44
	20	12**	26
$AgNO_3$	10	90	78.4
	20	126.5	108
$NiCl_2$	10	88.5	77.8
	20	67	59.6
$CoCl_2$	10	85	76.6
	20	63	55

The cellulase activity and stability in absence of metal ions were considered as control (100 %). ** indicating significant inhibition of residual activity. SD= < ± 0.05 for all values.

4.3.7. Response surface methodology for cellulase optimization

In the preliminary assays, several variables were screened out during the production and hydrolysis efficiency of the cellulase; however, the optimization of the four most significant cultural and nutritional factors was carried out to enhance the cellulase production using RSM in Design Expert 11.0. The box-Behnken design was employed with an experimental layout of 29 runs at three different levels (minimum (+1), medium (0), maximum (1) against selected independent variables, and cellulase activity U/mL (response) as shown in Table 3.3. The adequacy and suitability of the second-order BBD matrix for cellulase activity were scrutinized by ANOVA and Fisher's '*F*-test (Table 4.4). The results showed that the model was found 'significant' with *F* value (25.85) and *p-Value* (<0.0011 at 95% confidence level). Besides, a 'non-significant' lack of fit value specified that model was a good fit. The highest *F*-value (15.54) and *p-Value* (< 0.05) for sugarcane bagasse

indicated that the individual effect of sugarcane bagasse on cellulase activity was significantly higher than other variables. However, a non-significant effect of interactions and squares among all factors was observed on the cellulase production, except the limited interaction was observed between sugarcane and temperature variables (Table 4.4).

Table 4.3. The Box-Behnken design at three levels of each independent variable and response of dependent variable cellulase activity (U mL^{-1}) of *B. licheniformis* HI-08*.

Run No.	Sugarcane bagasse (% w/v)	pH	Temperature (°C)	$MgCl_2$ (mM)	Cellulase activity (U/mL)
1	2(1)	7(0)	105(1)	10(0)	453
2	2(1)	7(0)	45(0)	5(-1)	1356
3	1(0)	10(1)	105(1)	10(0)	347
4	1(0)	7(0)	105(1)	5(-1)	388
5	1(0)	7(0)	25(-1)	20(1)	521
6	1(0)	7(0)	45(0)	10(0)	866
7	1(0)	7(0)	25(-1)	5(-1)	498
8	1(0)	10(1)	45(0)	5(-1)	702
9	0.5(-1)	7(0)	105(1)	10(0)	320
10	1(0)	10(1)	25(-1)	10(0)	680
11	2(1)	10(1)	45(0)	10(0)	1064
12	1(0)	10(1)	45(0)	20(1)	940
13	2(1)	3(-1)	45(0)	10(0)	432
14	1(0)	3(-1)	45(0)	5(-1)	390
15	0.5(-1)	10(1)	45(0)	10(0)	754
16	1(0)	7(0)	105(1)	20(1)	333
17	1(0)	3(-1)	45(0)	20(1)	667
18	1(0)	7(0)	45(0)	10(0)	986
19	0.5(-1)	7(0)	25(-1)	10(0)	453
20	1(0)	3(-1)	105(1)	10(0)	247
21	1(0)	7(0)	45(0)	10(0)	1034
22	1(0)	3(-1)	25(-1)	10(0)	675
23	2(1)	7(0)	25(-1)	10(0)	986
24	1(0)	7(0)	45(0)	10(0)	1096
25	1(0)	7(0)	45(0)	10(0)	1112
26	2(1)	7(0)	45(0)	20(1)	1465
27	0.5(-1)	7(0)	45(0)	20(1)	1040
28	0.5(-1)	3(-1)	45(0)	10(0)	356
29	0.5(-1)	7(0)	45(0)	5(-1)	889

*The values in parenthesis are indicating the three levels of independent factors in coded form.

Based on RSM analysis the optimal culture conditions for maximum cellulase production 393.99 U/mL were predicted as; Sugarcane bagasse 2.0% (w/v), pH 7.0, temperature 45°C, and

$MgCl_2$ 10 mM. The validation of the model was conducted using model condition in 96-well plate exhibited cellulase activity of 400 U/mL that was 1.9-fold higher to cellulase production under non-optimized conditions. Besides, the validity of predictions and goodness of fit for the model can be further ensured in terms of *P-Value* (< 0.05) and the coefficient of determination (R^2), predicted R2 and adjusted R^2. The value for the coefficient of determination was 0.9066 that indicated the compatibility of experimental data was 90%. However, the difference among adjusted (0.7808) and predicted (0.3357) coefficients values was more than 0.2 that is indicating an insignificant correlation between experimental and predicted cellulase activities.

Table 4.4: Analysis of variance for the quadratic model to assess the cellulase activity of *B. licheniformis* HI-08 using Response surface methodology.

Source	Sum of Squares	df	Mean Square	*F*-value	*p-Value*	
Model	2.619E+06	14	1.871E+05	5.85	0.0011*	Significant
A-Sugarcane Bagasse	4.969E+05	1	4.969E+05	15.54	0.0015*	
B-Temperature	1.946E+05	1	1.946E+05	6.08	0.0272*	
C-pH	1.680E+05	1	1.680E+05	5.25	0.0379*	
D-$MgCl_2$	86700.00	1	86700.00	2.71	0.1219	
AB	64516.00	1	64516.00	2.02	0.1774	
AC	13689.00	1	13689.00	0.4280	0.5236	
AD	27225.00	1	27225.00	0.8511	0.3719	
BC	6.25	1	6.25	0.0002	0.9890	
BD	1980.25	1	1980.25	0.0619	0.8071	
CD	380.25	1	380.25	0.0119	0.9147	
A^2	2813.06	1	2813.06	0.0879	0.07712	
B^2	1.329E+06	1	1.329E+06	41.55	< 0.0001*	
C^2	3.102E+05	1	3.102E+05	9.70	0.0076	
D^2	26922.69	1	26922.69	0.8417	0.3744	
Residual	4.478E+05	14	31986.50			
Lack of Fit	3.728E+05	10	37281.58	1.99	0.2652	insignificant
Pure Error	74995.20	4	18748.80			
Cor Total	3.067E+06	28				

*indicating the significant (*P*<0.05) terms.

For a better perceptive of the interaction between the factors and their effects on the cellulase activity (U/mL), 3D surface plots were constructed by using coefficients of the quadratic polynomial equation. In this model, only factor sugarcane bagasse (A) and temperature (B) terms were significant for the response (*P*<0.05). Figure. 4.8. indicating the insignificant interaction of

sugarcane bagasse and temperature for the cellulase activity. By changing the concentration of sugarcane bagasse had a variant effect on cellulase activity at lower (-1), middle (0), and upper (+1) level of temperature, pH, and $MgCl_2$ (Figure. 4.8a-c). Similarly, at middle level 0 (45°C) of temperature, the cellulase activity was highest, but pH and $MgCl_2$ had no significant effect on cellulase production at any concentration of temperature indicating the insignificant interaction (Figure 4.8d-f).

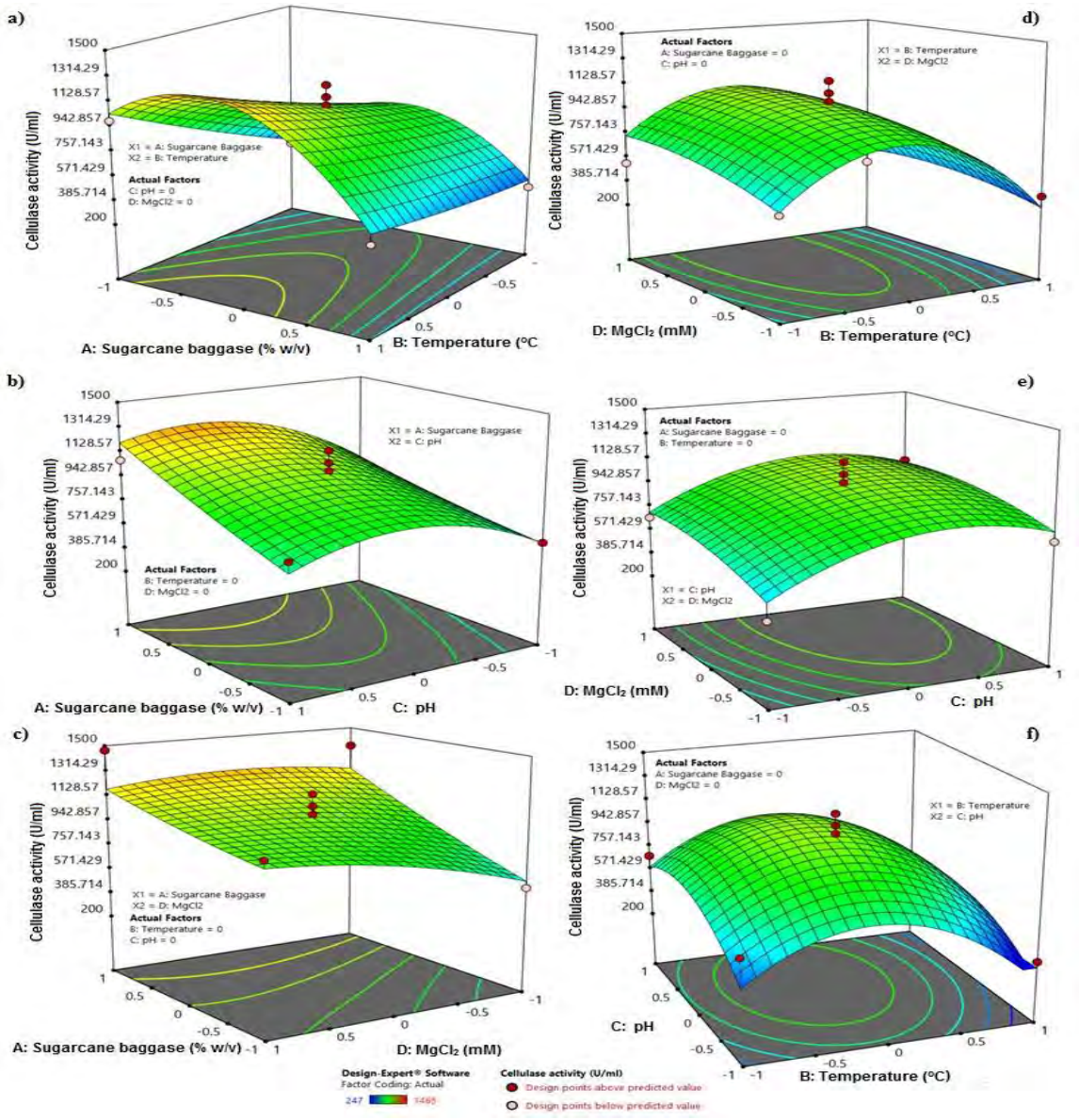


Figure 4.8. 3D surface plots indicating the effects of variables and their interaction on cellulase activity. a) Sugarcane bagasse vs temperature; b) Sugarcane bagasse vs pH; c) Sugarcane bagasse vs $MgCl_2$; d) $MgCl_2$vs temperature; e) $MgCl_2$vs pH; f) pH vs temperature.

4.4. Discussion

In earlier reports, the *B. licheniformis* has been reported from the hot springs, marine water, and composite (Annamalai *et al*., 2012; Shajahan *et al*., 2017; Marco *et al*., 2017). However, in the present study, it was isolated from the highly adaptive gut of wood-feeding termite *H. indicola* and *R. flavipes* that indicates the variability and tolerance property in *B. licheniformis* for habitat selection. During the screening test, HI-08 produced a maximum clearance zone by degrading the congo red stain and selected for further study (Figure 4.1). Gohel *et al*. (2014) reported that cellulase produces these clear zones due to the degradation of polysaccharides (CMC) into monosaccharide, which has no affinity for binding towards Congo red dye.

The agricultural waste such as sugarcane bagasse was appeared most suitable substrate for growth and cellulase production of *B. licheniformis* HI-08 followed by rice husk and rice bran. Similarly, sugarcane bagasse was also reported as the most suitable substrate for the *B. vallimortis* (RG-07) growth and enzyme production (Gaur and Tiwari, 2015). In another study, Sadhu *et al*. (2013) found the sugarcane bagasse as the most effective substrate for cellulase production against *Bacillus* sp. In contrast, Annamalai *et al*. (2012) reported that *B. licheniformis* AU01 isolated from marine water showed maximum cellulase production and growth on rice bran. This disparity suggests the bacterial habitat and isolation source affect the applicability of strain in enzyme production. Lee *et al*. (2008) reported that sugarcane bagasse, rice hulls, and rice husk were the most suitable carbon source to produce cellulase by increasing bacterial growth due to their inducible nature. Therefore, the cellulase production and growth profile of *B. licheniformis* HI-08 on different cellulosic wastes could be valuable in the production of bioethanol, as a bio-staining agent in the textile industry, single-cell protein, and many other industrial chemicals.

The purification profile indicated that specific cellulase activity was increased by increasing the purification fold of cellulases from *B. licheniformis* HI-08. The purification profile indicated 12.02 folds of purification factor and 32.33 % yield with the highest specific activity of 2381.88 U/mg (Table 4.1). In contrast, cellulase obtained from hot spring (NCIM 5556) and marine water (AU01) strains of *B. licheniformis* showed 42.99 and 451.9 U/mL specific activities respectively (Annamalai *et al*., 2012; Shajahan *et al*., 2017) after ion-exchange chromatography. Gaur and Tiwari (2015) reported 39.1fold purification with a 28.8% yield after gel-filtration for

cellulase purification from *B. vallismortis* RG-07. In another study, cellulase from *B. licheniformis* showed 8.66fold purification and 11.5% recovery after the gel filtration that had less hydrolysis capability from the cellulase of present study strain HI-08. Thus, the hydrolysis potential of a strain isolated from termites was higher than earlier reported strains of *B. licheniformis* isolated from hot springs, marine water, and composite (Annamalai *et al*., 2012; Shajahan *et al*., 2017; Marco *et al*., 2017). The molecular weight of HI-08 was around 55 kDa that was less than *B. vallimortis* RG-07 (80 kDa) and approximately equal to alkaliphilic *B. licheniformis* SVD1 (55 kDa) (Shajahan *et al*., 2017; Annamalai *et al*., 2012; Marco *et al*., 2017).

In the kinetic analysis, the K_m value of the cellulase obtained from HI-08 was less as compared to K_m of cellulases isolated from the *Clostridium thermocellum* Umcel5 (K_m, 16 mg/mL) (Liu *et al.,* 2009), The metagenomic study of novel C67-1 cellulase gene isolated from the rumen of buffalo (K_m 37 mg/mL) (Duan *et al*., 2009), *Salinvibrio* sp. (K_m 3.03 mg/mL) (Wang *et al*., 2009) and *Pseudomonas fluorescents* (K_m 3.6 mg/mL) (Bakare *et al*., 2005). The lower value of kinetic constant (K_m) illustrated that purified cellulase of *B. licheniformis* HI-08 has a higher affinity of cellulase against CMC. Likewise, Azadian *et al*. (2017) reported the K_m and V_{max} of cellulase from *Bacillus sonorensis* HSC7 0.186 mg/mL and 0.052 µmol/min, respectively. Sriariyanun *et al.* (2016) found the 0.80 mg/mL K_m and 1000 µM/min V_{max} for ionic liquid tolerant cellulase *Bacillus* sp. MSL2 isolated from rice paddy field soil.

The purified cellulose of strain HI-08 showed complete stability up to 50% over the range of 25-105°C during 30 min of incubation that coincides with the thermostability of *B. vallismortis* (Gaur and Tiwari, 2015). Similarly, thermostability for endoglucanase of *B. licheniformis* strain C108 was up to 100% isolated from the lake soil (Aygan *et al*., 2011). Many researchers have reported the thermostability profile of cellulase from *Bacillus* sp. range from 30-100°C can retain activity up to ≈50% (Singh *et al*., 2009). However, the cellulase from the strain HI-08 was more thermostable and can be used in extreme thermal conditions in different cellulosic applications. A similar optimum temperature was found in another strain *B. licheniformis* NCIM 5556 and AU01 isolated from hot spring and marine water respectively, in India (Annamalai *et al*., 2012; Shajahan *et al*., 2017). *Bacillus* strains MSL2, RG-07, and HR68 have optimum activity for cellulases at 50, 65, 70°C respectively (Sriariyanun *et al*., 2016; Gaur and Tiwari, 2015; Robson and Chambliss, 1989).

The pH results of *B. licheniformis* HI-08 exhibited more than 50% enzyme stability over the pH range 5.0-9.0 which was higher than previously reported cellulase stability from *Bacillus* sp. (Yu and Li, 2015; Sriariyanun *et al*., 2016). The trends of cellulase stability in the present study enhance the applicability of HI-08 for hydrolyzing the cellulosic material in a broad alkaline pH range. Similarly, optimum cellulase activity was observed in alkali stable *Gracilibacillus* sp. strain SK1, *Bacillus* sp. PKM-5430 and *Pseudomonas fluorescence* at pH 7.0 (Bakare *et al*., 2005; Lee *et al*., 2008; Yu and Li, 2015).

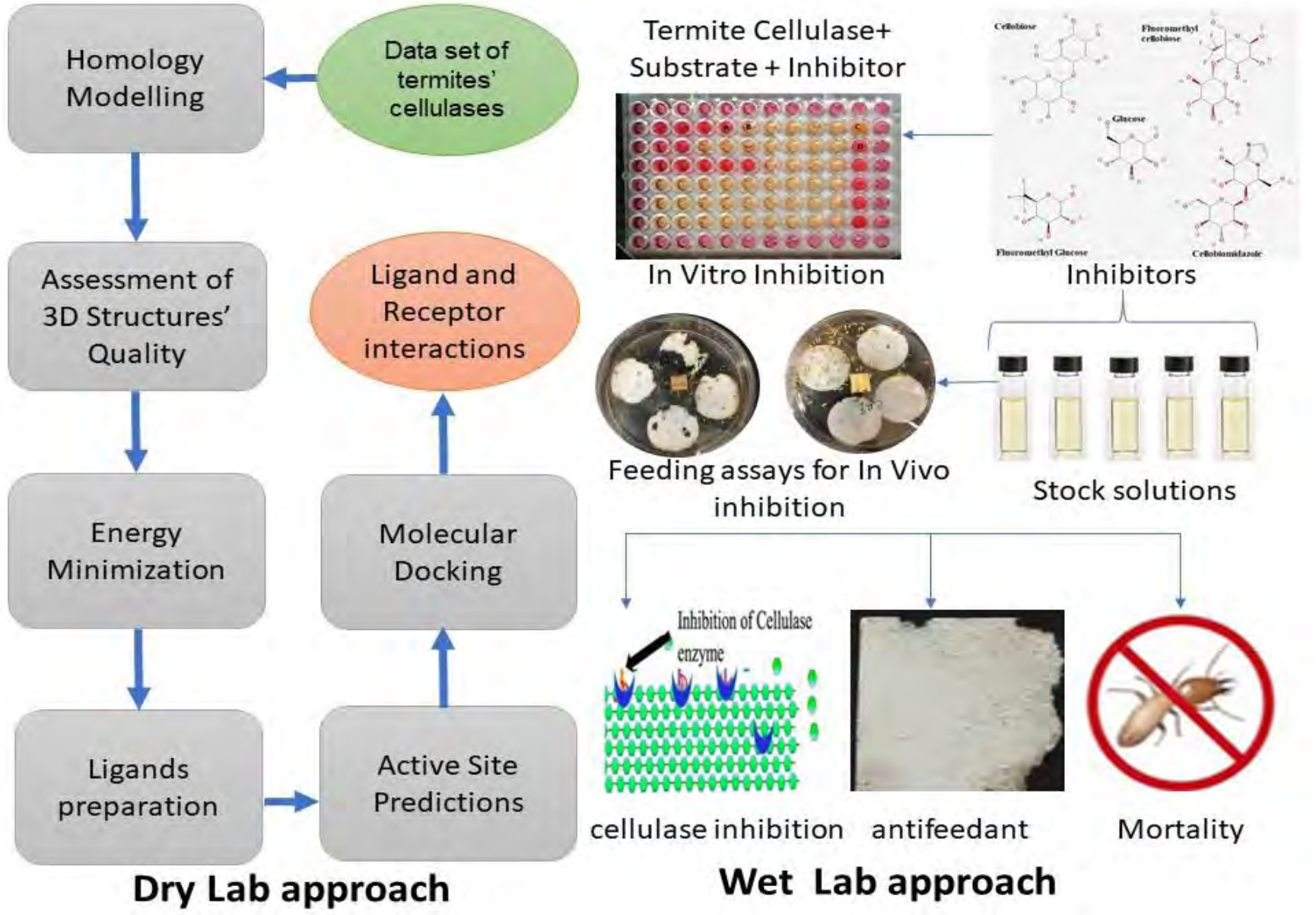
The high residual activity in the presence of alkali metal such as Mg^{2+}, Ca^{2+}, and Na^{+} indicated the extreme halotolerant and alkaline nature of cellulase from *B. licheniformis* HI-08 that was similar to other cellulases of *Bacillus* strain RG-07(Gaur and Tiwari, 2015). However, other metal ions reduced the cellulase activity and stability to an inconsistent extent such as Hg^{2+}, Zn^{2+}, Co^{2+}, $Ni^{2+,}$ and Cu^{2+}. In previous studies, the inhibition of cellulase activity due to Hg^{+2} was correlated to the binding nature of mercury with thiol, carboxyl groups, and tryptophan residues in the cellulase (Lusterio *et al.,* 1992). The inhibition due to transition group metal ions might be related to competition associated with exogenous cations and enzyme cations, causing a reduction in metalloenzyme activity (Gaur and Tiwari, 2015). The high salt and alkaline nature of cellulase from HI-08 make it a suitable candidate in the cellulosic material processing industry because during pretreatment of biomass different acidic and alkali nature salts are used and create extreme salinity conditions that might be neutralized by HI-08.

The maximum cellulase activity of *B. licheniformis* HI-08 exhibited 400 U/mL during validation assay under optimization conditions using RSM design. It was higher to maximum cellulase activity (42.99 IU/mL) of *B. licheniformis* NCIM 5556 isolated from hot spring (Shajahan *et al*., 2017). Likewise, Saini *et al.* (2015) found wheat bran 2.5% (w/v), an incubation time of 8 days, ammonium sulfate 0.53% (w/v), and avicel 0.5% as predicted variable for maximum cellulase 1.26 FPU/mL from *Penicillium oxalicum* IODBF5 using response surface methodology. In another report, Yang *et al*. (2012) found that the optimization technique enhanced 1.6-fold cellulase production in fungus *Penicillium* sp. CLF-S. This difference might be due to the isolation source of bacterial strains because lower termites use symbiotic cellulases (bacterial and protozoa' cellulase) for complete and efficient wood digestion. Therefore, *B. licheniformis* HI-08 could be a potential source for cellulase production as compared to previous studies (Annamalai *et al*., 2012;

Dave *et al*., 2015). It suggests the direct use of wild type *B. licheniformis* HI-08 for cellulase production and biomass conversion at the commercial level.

4.5. Conclusion

- The cellulase of *B. licheniformis* HI-08 is unique with respect to isolation sources that are wood-feeding termites.
- It confirmed its candidature in biomass conversion application by hydrolysis of agricultural wastes with optimal activity at temperature 45°C and pH 7.0.
- The retention of cellulase activity in presence of various metal ions indicated the organic ionic-tolerant nature of cellulose.
- The optimization model using RSM enhanced the cellulase activity by 1.8 folds with optimized conditions.
- HI-08 is a wild type strain with lucrative cellulase titers that can be further improved using mutations and other biotechnological techniques.


Homology Modelling
Data set of termites' cellulases
Assessment of 3D Structures' Quality
Ligand and Receptor interactions
Energy Minimization
Molecular Docking
Ligands preparation
Active Site Predictions
Dry Lab approach
Termite Cellulase+ Substrate + Inhibitor
In Vitro Inhibition
Inhibitors
Feeding assays for In Vivo inhibition
Stock solutions
Inhibition of Cellulase enzyme
cellulase inhibition
antifeedant
Mortality
Wet Lab approach

Inhibition of the cellulases from subterranean termites using carbohydrate-based inhibitors: Evidence from dry and wet laboratory approaches

Abstract

Cellulases are the enzymes that degrade the lignocellulosic materials in subterranean termite by dissolving the β-1,4-glycosidic bonds and provide energy to termites for living. These enzymes with great biomass conversion potentials provide life to termites that could be a significant target in termite control strategies. In this chapter, dry lab and wet lab approaches were conducted to cutoff the lignocellulose activities of termites by targeting termite cellulases including endoglucanase, exoglucanase, and β-glucosidase using ecofriendly carbohydrate-based cellulase inhibitors. Before conducting wet laboratory bioassays, in dry lab studies 3D structure of three main cellulases endoglucanase, exoglucanase, and β-glucosidase from *R. flavipes* were modeled using Modeler 9.24v and validated via Structure Analysis and Verification Server comprising on the package of different programs such as PROCHECK, Verify3D, ERRAT, and Ramachandran plot. Interactive binding of carbohydrate-based inhibitors of cellulase (Fluoromethyl glucose, Cellobiomidazole, Glucose, Cellobiose and Fluoromethyl cellobiose) with enzymes was assessed by GOLD scoring. The results of *in silco* studies revealed Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose showed higher promising binding affinities with exoglucanase and β-glucosidase as compared endoglucanase based on GOLD docking scores.

These results were supported by wet-lab studies, in which the efficacy of these inhibitors was investigated by conducting biochemical and feeding bioassays. All five inhibitors were tested against endogenous and symbiotic cellulase fractions under optimal conditions. Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose significantly ($p < 0.05$) inhibited the activities of exoglucanase and β-glucosidase during *in vitro* assays. Feeding bioassays revealed significant effects of Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose inhibitors on all three enzyme activities and subsequently reduced the gene expression levels, termite foraging activity, protozoal communities, and at end termite mortality. All these results suggest the efficacy of Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose as termite control agents and can be applied in field conditions using baiting technology.

5.1. Introduction

Subterranean termites' infestation in wood and its structures is a serious threat to the timber industry and causing damage to more than 20 billion USD annually (Su, 2002). Subterranean termites *Heterotermes indicola* in Pakistan and *Reticulitermes flavipes* in the United are the most economically important termites' species in the respective regions. Soil treatments and baiting technology are considered two important methods to control subterranean termites. In soil treatments, we use a large quantity of the liquid termiticides in liquid form that are the major neurotoxic to invertebrate and vertebrates and inhibitors of mitochondrial respiration. Besides it, the use of synthetic pesticides has been reduced the termites' attack significantly however, the breakdown of these products is not eco-friendly due to their non-targeted applications at high rates. Chemical control of termites was achieved by different methods such as treatment of soil and seedling, pressure treatment to preserve timber, space fumigation, and baiting systems (Su and Scheffrahn, 1998). Soil treatments approach by chemical insecticides have back draws in form of emerging termite resistance and environmental concerns. Other termite control methods are physical and cultural control however, these are not applicable at a major scale level. In this method, physical barriers or the removal of termite queen and mound, flooding, suffocating, and use of toxic repellents are involved. In the biological method, natural enemies of termites such as predators (ants, lizards, frogs, and mammals), parasites (nematodes), and pathogens (entomopathogenic fungi) are involved in termite control (Rath, 2000). This method is expensive and has concerns about outdoor applications and to maintain the quality of wood in service. Therefore, here is a need for termite control before their infestation, and in this sense wood species with termiticidal properties can play a major role to reduce termite attack.

In the baiting system, termite' colonies are recruited to feed the impregnated substrates by slow-acting chemical pesticides. However, the major back draw of the baiting method is that the population of termite colonies doesn't suppress so immediately. In this respect, there is a need for fast-acting bait ingredients that can reduce or eliminate the termite population immediately. As we know lower termite digest the cellulosic material with the unique capability of their collaborative cellulase action by endoglucanases, exoglucanases, and β-glucosidases with approximately 100% efficiency (Breznak and Brune, 1994; Inoue *et al*., 1997). Cellulases in lower termite inhibit mutually symbiotic and endogenous origin, where "endogenous" means to the cellulases

predetermined by termite cellulase genes and "symbiotic" mention the cellulases produced in the termite hindgut. While substantial research efforts have been directed toward the discovery and characterization of termite cellulases (Zhou *et al*., 2008), however, disproportionality minor efforts have been applied toward the termite control by novel methods such as by targeting cellulases.

In the past few years, only a few beta-glucosidase inhibitors including palladium, gluconolactone, *p*-coumaric, sinapinic and ferulic acid, conduritol B epoxide (CBE), and 1-deoxynojirimycin have been used in a different study of cellulase inhibitions (Peciarová and Biely, 1982; Dale *et al*., 1985; Fan and Conn, 1985; Gross *et al*., 1986; Legler and Bieberich, 1988; Lassig at al., 1995; Legler, 1990; Zhu *et al*., 2005; Zhou *et al*., 2008; Zhang *et al*., 2012). In the past, the prediction of cellulase active sites and the mechanism of lignocellulose hydrolysis was unclear, which was one of the major obstacles in the studies of cellulases and their inhibitors (Kawaguchi *et al*., 1996). However, the development in metagenomic and transcriptome analysis in 21 century has exposed the mechanisms involved in lignocellulose digestion and revealed the cellulase active sites by a bundle of studies (Zhang *et al*., 2012; Raychoudhury *et al*., 2013; Sethi *et al*., 2013; Scharf, 2015a, b). In this sense, Zhu *et al*., (2005) first time conducted such a study and observed minor inhibition of the cellulase in *Coptotermes formosanus* under *in vivo* condition using various carbohydrate-based in the United States. After that Scharf *et al*., (2008) conducted the project at the University of Florida, USA, and constructed a patent of three carbohydrate-based inhibitors of cellulase (fluoromethyl glucose, cellobiomidazole, and fluoromethyl cellobiose) as feeding stimulants in termites. Besides, the end product of lignocellulose biomass including cellobiose and glucose has been proposed as cellulase inhibitors that could limit the rate of lignocellulose hydrolysis in high solid conditions (Kristensen *et al*., 2009). However, all these studies were carried out in the wet lab bioassay condition.

A computational molecular docking technique is used to investigate the protein-ligand interactions and to predict the structure of the intermolecular complex formed between the compounds (Selvam *et al*., 2017). It is an important tool that places small molecules (ligands/inhibitors) in the binding sites of its target protein (receptor) and approximations its binding affinities. Interaction between ligands and protein molecules is the most interesting fact of molecular docking because of its industrial applications. The interaction feature of molecular docking based on protein and ligand structures is evaluated using energy scoring function encoded

by different algorithms; if the interaction shows the lowest energy score, it is predicted as the "best match" known as the binding mode (Ezat *et al*., 2014). Revealing the cellulase inhibition using end-product and carbohydrate-based inhibitors in enzyme engineering approaches under dry and wet lab conditions could be successful termite control tactics. However, no such studies or projects have been carried out in developing countries such as Pakistan. Cellobiose, glucose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose are structural units of disaccharide sugars and analogs of carbohydrate polymers. These inhibitors could be a milestone in environment-friendly termite control tactics since these are phago-stimulant and easy to use as non-toxic baits in field conditions.

This chapter investigated the efficacy of carbohydrate-based and end-product inhibitors by targeting the termite cellulases using dry and wet lab approaches. Following major objectives were carried out in this chapter; 1) To evaluate the binding capability of carbohydrate-based inhibitors against the receptors of termite protein structures using molecular docking tool, 2) Inhibition of termite cellulolytic activities using carbohydrate-based inhibitors under *in vitro* wet lab conditions, 3) To demeanor nourishing bio-assays with the inhibitors to check their effects on feeding behaviors of termites and their survivorship, 4) To inspect the inhibition of cellulase in survivors and to validate the feeding bioassays.

5.2. Materials and methods

Dry Lab approaches

5.2.1. Data set of termites' cellulases

The X-ray crystallography-based 3D (3-dimensional) structures of cellulases (β-glucosidase (GHF 1), β-(1,4)-endoglucanase (GHF 9), exoglucanase type cellulase (GHF 7) belonging to termite species *R. flavipes* are not available in PDB (Protein Data Bank) database. To construct structures, this protein in FASTA format was fetched from UniProtKB (https://www.uniprot.org/uniprot) with IDs A0A5A4M2Q0 (Shelomi *et al*., 2020), D9ILU1 (Scharf *et al*., 2010), and Q462G9 (Zhou *et al*., 2007) for endoglucanase, β-glucosidase, and exoglucanase respectively. These sequences were protein blasted in NCBI-BLAST (Basic Local Alignment Tool) (Altschul *et al*., 1990) to find the homologous sequences for a 3D structure template with 99% query cover and >50% identity. Homologous sequences are capable of 3D template structures for homology modeling and their atomic coordinates reports were retrieved from the PDB (Berman *et al*., 2002).

5.2.2. Homology Modelling

Each atomic coordinate file of the template along with respective target downloads from PDB and final template and target sequence alignment file (Clustal Omega) was used to construct the target protein model using an automated homology modeling tool MODELLER 9.21 (Webb and Sali, 2016). Approximately, 10 models for each enzyme were calculated from the random generation of staring structures and the superlative model with least Root Mean Square Deviation (RMSD) value was designated by overlaying the model against its template (Maiti *et al*., 2004; Selvam *et al*., 2017).

5.2.3. Quality Assessment of Structures

The generated model of each enzyme was subjected to validation for confirmation of their thermodynamic stability by removing poor rotamers and outliers in different programs (Garza-Fabre *et al*., 2013). In this view, a Structure Analysis and Verification Server (SAVES: hsttps://servicesn.mbi.ucla.edu/SAVES/) issued by the National Institute of Health (NIH) was used, which is the package of different programs such as PROCHECK, Verify3D, and ERRAT. The readability of each structure was identified and assessed by evaluating stereochemical

parameters using PROCHECK (Laskowski *et al*., 1993), ERRAT (Colovos and Yeates, 1993), Verify3D (Lüthy *et al*., 1992), and Ramachandran Plot (mention allowed region in percentages) in SAVES.

5.2.4. Energy Minimization

Energy minimization is an essential technique in optimizing the suitable space orientation of molecules since drawn structures have no stable conformations due to high energetics (Mackay *et al*., 1989). In this study, energy minimization was performed through UCSF Chimera 1.1 (Pettersen *et al*., 2004) by assigning Gasteiger charges to target structures and minimized for 1500 step in which 750 of conjugate steps and 750 of steepest descent and step size was set to 0.025 Å under Tripos Force Field (TFF) (Ahmad *et al*., 2018).

5.2.5. Prediction of active sites

The binding sites or active amino acids of modeled protein structures of cellulases (endoglucanase, exoglucanase, and β-glucosidase) were identified form UniProtKB and another source/related literature (Scharf *et al*., 2010; Shelomi *et al*., 2020; Zhou *et al*., 2007) and predicted by submitting the structures to DoGSiteScorer: An active site prediction and analysis server to determine the binding affinities between carbohydrate-based inhibitors (cellobiose, glucose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose) and termite enzymes.

5.2.5. Inhibitor's preparation

Two-dimensional structures of the five carbohydrate-based inhibitors including cellobiose, glucose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). The 2D structures of inhibitors were obtained PubChem and converted the SDF files into PDB using OpenBabel 3.1.1 version. Energy minimization of the compound was carried out in WinCoot-0.8.9 beta 2 release.

5.2.6. Molecular docking

Molecular docking is a critical assistive tool in computational biology, and it investigates the binding mode of ligands within the space of 3D protein structures (Morris and Lim-Wilby, 2008). The 3D minimized structures of inhibitors including cellobiose, glucose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose were docked with the predicted active sites of

modeled structures of endoglucanase, exoglucanase, and β-glucosidase from lower termite sp. *R. flavipes* by using Genetic Optimization for Ligand Docking (GOLD) (Verdonk *et al*., 2003). Glu399 from β-glucosidase, Asp422 and Glu431 from endoglucanase, and Glu94 and 99 from exoglucanase were selected as active site residues and 10Å was set as a defined radius for spherical space.

Genetic Optimization for Ligand Docking is a professional paid docking program, which has the ability of its scoring function to score and rank ligands according to their binding affinities. A genetic algorithm is utilized by GOLD and approximately 71% accurately detect dock ligand binding mode identical to experimentally outcomes in wet lab conditions (Jones *et al*., 1997). In this study, a scoring function of GOLD was used for molecular binding by describing two different energy terms such as Van der Waal bindings between enzyme and ligand, and hydrogen bonding for energy terms (Verdonk *et al*., 2003). Ligand and protein binding complex visualization was carried out in different software such as LigPlot (Wallace *et al*., 1995), UCSF Chimera, and Discovery Studio (DS)

5.2.7. Ligand-receptor interactions

The interactions between inhibitors (cellobiose, glucose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose) and cellulases (endoglucanase, exoglucanase, and β-glucosidase) from *R. flavipes* as docked compounds were carefully monitored by LigPlot v. 4.5.3 (Wallace *et al*., 1995).

Wet Lab approaches

5.2.8. Termite collection and augmentation

The colonies of lower termites (*Heterotermes indicola* and *Reticulitermes flavipes*) were augmented under laboratory conditions. To establish the healthy colonies of *H. indicola*, a transparent apparatus of the acrylic sheet containing specific dimensions (L×H×W= 15×20×15) was used for each termite colony. A specific amount of sterile soil/sand 2500 gram was added along with all casts of termites (500 workers, 100 soldiers, and 300 alates) in transparent apparatus and supplemented with the most palatable wood *Populus eumericana* (Rasib *et al*., 2014) as a food source. All termites transparent were covered by a black cloth to hide the termite foraging activities

from the light effect and placed in the control condition at 28±1ºC with relative humidity 70-80% using a humidifier. Moisture contents in the container were inspected twice a month (Figure 5.1). Whereas *R. flavipes* field colony was collected at Purdue University campus West Lafayette IN, the USA in summer 2019 using cardboard baits. After each collection survey, termites were brought to the laboratory and established termite colonies in a dark room within a sealed plastic box (30×24×10 cm) at 26 ± 1°C and 65±2% RH using pine wood steaks and paper towel beds.

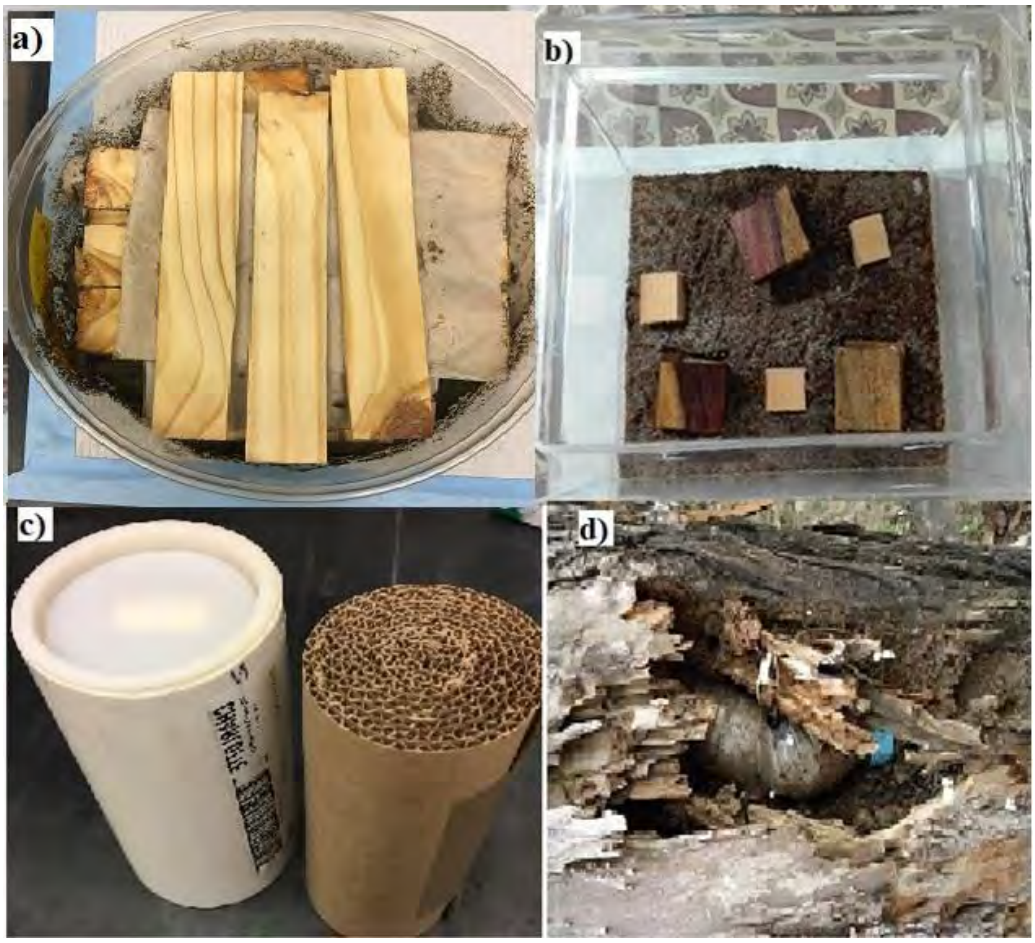


Figure. 5.1. Termites rearing in transparent cages showing successfully established termite colonies under the controlled condition for *H. indicola* (a, b) while for the collection of *R. flavipes* paper cardboard was baited under the ground (c, d).

5.2.9. Chemicals

Three major substrates carboxymethyl cellulose (CMC), p-nitrophenyl cellobioside (pNPC), and p-nitrophenyl glucopyranoside (pNPG) were used to determine the effect of inhibitors on cellulase

activities. The carbohydrate-based cellulase inhibitors glucose (analytical grade, 98% purity), fluoromethyl glucose (FMG; 95% purity), cellobiose (CB; 98% purity), fluoromethyl cellobiose (FMCB; 95% purity), cellobiomidazole (CBI; 95% purity) (Zhou *et al.,* 2008) were used from the urban entomology lab of Dr. Michael E Scharf in Purdue University, USA to determine inhibitor effect on different cellulase activities in lower termites (Figure 5.2). All carbohydrate-based inhibitor stock solutions were prepared in analytical grade methanol (Sigma, St. Louis, MO).

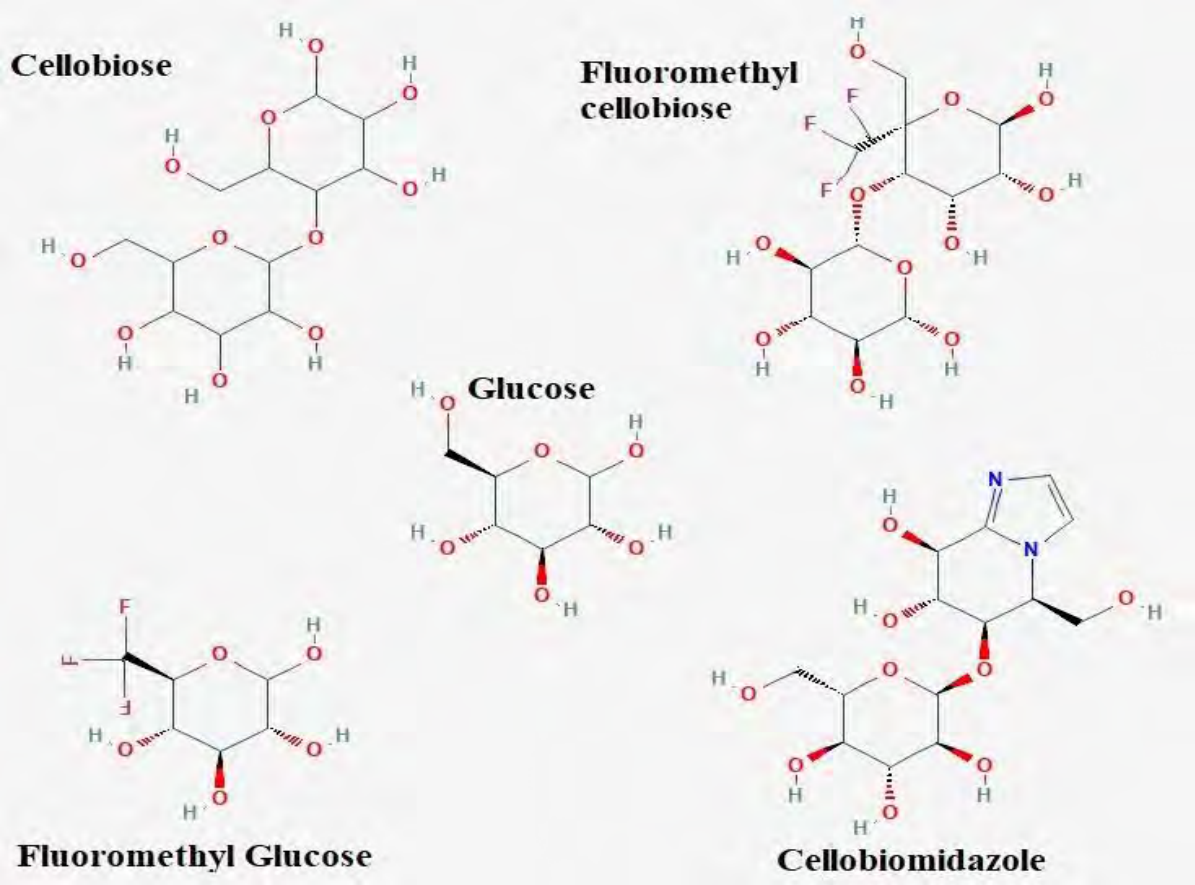


Figure. 5.2. Chemical structures of carbohydrate-based cellulase inhibitors used against wood-feeding termites.

5.2.10. *In Vitro* bioassay

The potential of the 05 carbohydrate-based inhibitors of cellulase (fluoromethyl glucose, fluoromethyl cellobiose, glucose, cellobiose, cellobioimidazole) was tested compared to two different cellulase sources of termites. It included endogenous termite cellulase extracted from the salivary gland, foregut and midgut, and symbiotic cellulases from the hindgut extract. The

effectiveness of inhibitors was identified according to Zhu *et al*., (2008) method with little modification. For potential inhibitors, a suitable concentration was identified that yielded 0-100% cellulase inhibitions. All assays were instigated by the adding 50 µL inhibitor in a 500 µL reaction comprising of 400 µL of substrate and 100 µL of enzyme mixture. The percentage of inhibition was calculated by comparing the inhibition curve to blank reagent (methanol) control to determine 50% inhibition (I_{50}) using the general linear regression equation. Each inhibition curve was created by triplicate determination of each inhibitor.

5.2.11. *In-vivo* inhibition bioassays

The same termite colonies were used for cellulase activities in no-choice feeding assay for I_{50} determination. A no-choice feeding bioassay using filter paper disks Whatman No. 1, (9.0 cm diameter) as substrate was conducting according to method Hassan *et al*., (2017) with minor modification.

Filter paper disks were pre-weighed and treated with 0.2 mL of each concentration (100, 75, 25, 12.5, and 6.25 mM) of inhibitors, air-dried at ambient temperature, and kept in Petri dishes (3×3 inches, China). Three replicates were employed for each concentration and control that was treated with solvent methanol only. 40 workers and 10 termite soldiers were released in each replicate of treatments and control. A few drops of (0.1 ml) dH_2O were periodically added onto the bottom edge of each Petri dish to keep up humidity. The Petri dishes were kept in dark at 28±1°C and 70±5 % RH, percentage mortality of termites was calculated at each treatment after 2 weeks.

$$\%\, Mortality = ODP \div TP \times 100$$

Where: TP= total population of workers; ODP= observed dead population of workers.

Filter papers were cleaned and re-weighed after oven-dried at 60°C for 12 h, and the percent weight loss of filter paper by termites was calculated by the formula (Sotannde *et al*., 2011).

$$\%\, Weight\ loss = \frac{W_b - W_a}{W_a} \times 100$$

Where: Wa= weight of filter paper after the experiment.; W_b= weight of filter paper before experiment.

In the post-feeding assay, we determined endo, exoglucanase, and β-glucosidase activities from the pooled homogenate of alive termites at the end of the experiment. The goal of this experiment was to find out the correlation between cellulase inhibition under *in vitro* assay and morality and feeding influences after *in vivo* feeding bio-assay. Crude cellulases from the living termites were also extracted as mentioned in the above crude cellulase extraction method section. Enzyme assays were conducted according to standard procedure as described above, in previous Chapter No. 1.

5.2.12. Protozoa Counts

The impacts of carbohydrate-based inhibitors (Glucose, CBI, FMCB, FMG, and CB) on hindgut protozoa were determined according to the method described by Hassan *et al*., 2017. The termite workers were fed on filter paper treated with different concentrations of inhibitors and control with MeOH as described in the feeding bioassay. After 14 days, five termites from each treatment were collected and removed their hindguts using sterilized needle and forceps. The contents of the five hindguts were pooled for one treatment as a single sample against each treatment of inhibitor and homogenized in 250 µL of 0.2% saline solution using sterilized micro-pestle.

Twenty-five microliters of the resulting gut contents were loaded onto a Neubauer hemocytometer (Bright-Line™ Sigma, USA) and protozoa numbers were calculated by determining the mean numbers of protozoa in four squares of each chamber under a digital biological trinocular microscope (Optika® B-500, Ponteranica, Italia). Counts were made for all flagellate species and the number of protozoa was calculated according to Lewis and Forschler (2014);

$$X_{=} \frac{\left(No.\ of\ cell\ counted \times Volume\ of\ saline \frac{solution}{sample}\right)}{\left(Volume\ of\ hemocytometer \times No. \frac{termites}{sample}\right)}$$

The reduction in the protozoa population was determined by comparing the resulting population with the protozoa population in the control treatments.

5.2.13. RNA extraction and qPCR

After the 14-day of feeding bioassay, 25 whole guts along with salivary glands were dissected from survived termite workers in 200 µL of RNA lysis buffer by embedding sample in ice cubes

and later stored at -80°C. Total RNA was extracted using SV Total RNA Isolation Kit (Promega; Madison, WI. USA) and cDNA was synthesized from 2 µg of total RNA using SensiFast™ cDNA synthesis kit (BioLine, USA). Quantitative RT-PCR was used to measure the transcript level of cellulase genes including endo, exoglucanase, and β-glucosidase across the inhibitor's treatments.

Reactions for qPCR were performed by preparing a mixture of 20 µL for each to replicate using SensiFAST™ SYBR® No-ROX One-Step Kit (BIOLINE, USA) containing; 10 µL of 1X SensiFAST™ SYBR® No-ROX One-Step Mix, 7 µL of nuclease-free H_2O, 1 µL each of forward and reverse primers as mentioned in chapter 1 (Table 1.1), 1 µL reverse transcriptase enzyme and 1 µL RNA sample. All gene expression for each treatment replicate were standardized to the reference NADH-*dh* gene and control MeOH. SensiFAST™ SYBR® No-ROX Mix protocol amplification conditions; initial denaturation 95°C for 2 min, denaturation at 94°C for 10 sec, annealing at 60°C for 20 sec, extension at 72°C and melting curve from 50 to 90°C with increment 0.5°C were used to amplify the target and control genes.

The quality of primers and qRT-PCR amplification efficiency for both control and target genes were validated by the existence of a single gene-specific product by sequencing of products, melting curve, and agarose electrophoresis. Simultaneously, for specific target gene the levels of relative expression in different parts of termites were standardized to reference or *NADH-dh* (control gene) and determined using $2^{-\Delta\Delta CT}$ method reported by Livak and Schmittgen (2001). ANOVA test followed by LSD *t*-test for mean separation was performed using statistical software SPSS IBM 20 to determine significant relative gene expression.

5.2.14. Statistical analysis

Wet lab assay data was plotted on an excel sheet and normalized in SPSS 26. V for each variable at the end of each experiment. The dose-dependent effect and IC_{50} of cellulase inhibitors was determined for different concentration against cellulase activities and termite mortality using One-Way ANOVA and probit analysis using SPSS 26. V.

To access which variables were most significantly associated with termite mortality, each measured variable (enzyme activity, cellulase gene expression, protozoa counts, termite weight loss, and termite feeding behavior) was used by performing multiple linear regression for termite mortality at a confidence level of 95% ($\alpha = 0.05$).

5.3. Results

5.3.1. Inhibitor target selection

Termite cellulase including endoglucanase, β-glucosidase, and exoglucanase are three major enzymes involved in lignocellulose degradation and are validated inhibitors' targets in termite control strategies. The combinatorial effect of selected enzymes degrades the lignocellulosic material efficiency in the hindgut of lower subterranean termites (Scharf *et al*., 2011a). Targeted enzymes sequences of *R. flavipes* were obtained from the UniProtKB submitted from the previous work carried out in the Urban Entomology Lab, Purdue University IN, USA by Dr. Michael E Scharf.

5.3.2. Homology Modelling

Modeler 9.25 v generated 10 initial models of each cellulase enzyme separately by using random generation and spatial resistance. The stereochemical properties of each model were used in the selection of the best model. Each selected model for each enzyme was superimposed with a template to reveal the degree of generated structures with a template by calculating the root mean square deviation (RMSD). Based on the stereochemistry of generated models, model 2 was selected for β-glucosidase, Model 1 for endoglucanase, and model 4 for exoglucanase. The modeled and energy minimized structures of β-glucosidase, endoglucanase, and exoglucanase from *R. flavipes* was developed using UCSF Chimera 1.14 (Figure 5.3).

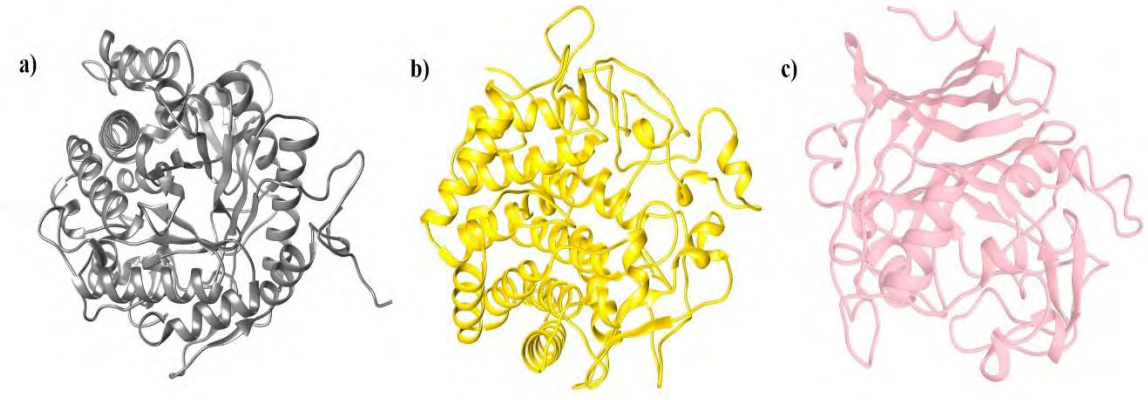


Figure 5.3. Ribbon diagram of modeled structures of enzymes a) β-glucosidase, b) endoglucanase, and c) Exoglucanase showing the α-helices, β-strands, and loops.

5.3.3. Assessment of models

The selected and targeted protein models were assessed by the general stereo-chemical parameter of SAVES online server using PROCHECK, Verify3D, Ramachandran plot, and ERRAT tools (Table 5.1; Figure 5.4).

Table 5.1. The rotamers, outliers, and Rama distribution Z-score for the selected model of each enzyme.

Beta glucosidase			
Poor rotamers	0	0.00%	Goal: <0.3%
Favored rotamers	391	97.51%	Goal: >98%
Ramachandran outliers	2	0.41%	Goal: <0.05%
Ramachandran favored	479	97.16%	Goal: >98%
Rama distribution Z-score	0.47 ± 0.35		Goal: abs (Z score) < 2
Endoglucanase			
Poor rotamers	0	0.00%	Goal: <0.3%
Favored rotamers	348	96.13%	Goal: >98%
Ramachandran outliers	4	0.87%	Goal: <0.05%
Ramachandran favored	438	95.63%	Goal: >98%
Rama distribution Z-score	0.51 ± 0.37		Goal: abs (Z score) < 2
Exoglucanase			
Poor rotamers	2	0.79%	Goal: <0.3%
Favored rotamers	246	96.85%	Goal: >98%
Ramachandran outliers	1	0.32%	Goal: <0.05%
Ramachandran favored	303	95.89%	Goal: >98%
Rama distribution Z-score	-0.63 ± 0.42		Goal: abs (Z score) < 2

Model 2 for β-glucosidase indicated strong and the highest stereochemistry with only one residue in the disallowed region, 0.0% poor rotamers, and having 0.47 Rama distribution Z-score, while Verify 3D and overall qualifying factor was 91.72% and 86.41% respectively.

Likewise, 10 possible models were generated for endoglucanase, and model no. 2 was selected for endoglucanase showed strong stereochemical properties such as 0.0% poor rotamers, no one residue recorded in the disallowed area and having Rama distribution Z-score 0.51, whereas Verify 3D and overall qualifying factor was 91.74% and 75.83% respectively. In case of exoglucanase, based on the quality assessment of structure, model

1 was selected for exoglucanase by MODELLER 9.25 since it showed robust stereochemistry with only three residues in the disallowed region, 0.79% poor rotamers, and having -0.63 Rama distribution Z-score, while Verify 3D and overall qualifying factor was 87.22% and 57.41% respectively.

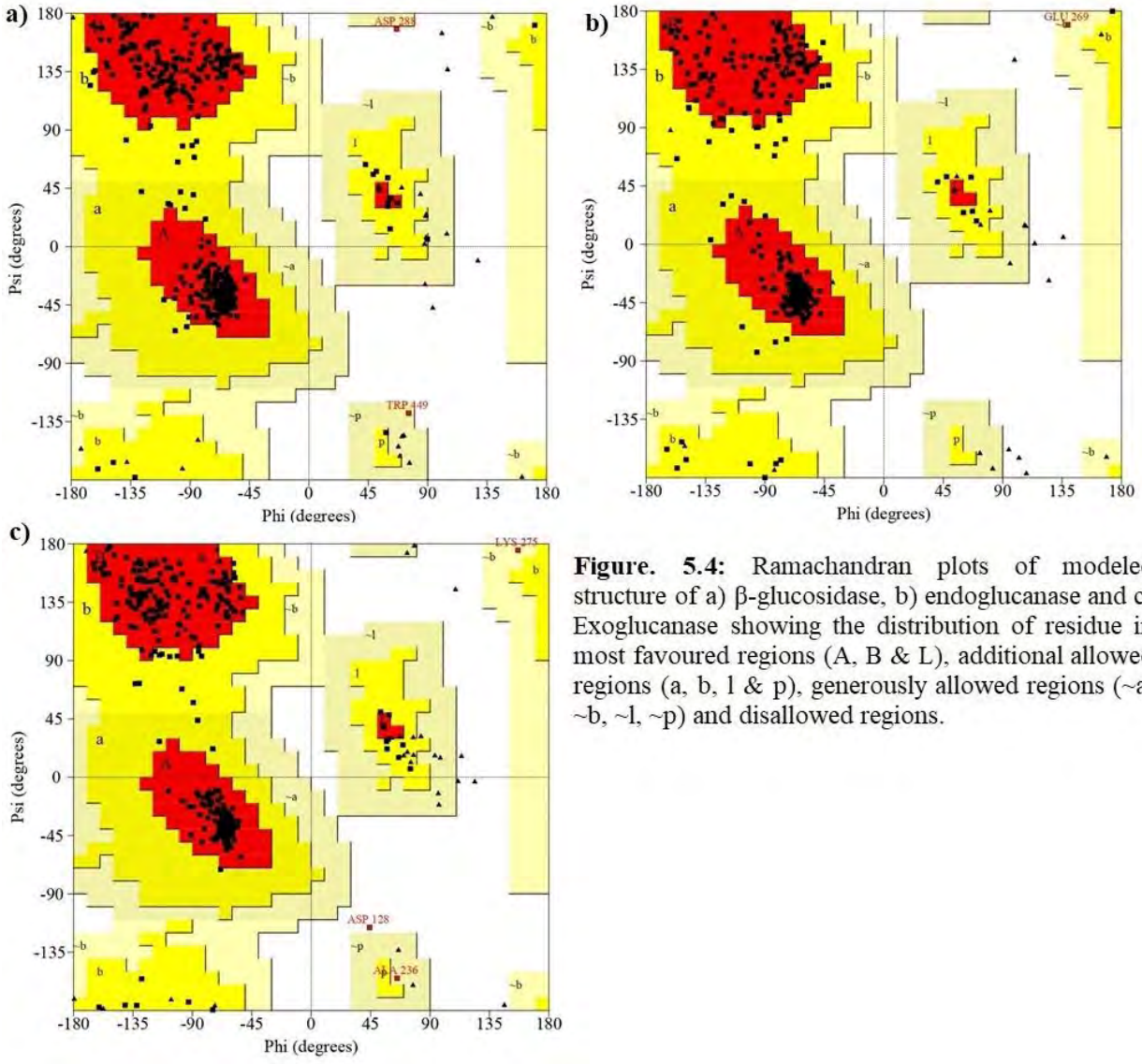


Figure. 5.4: Ramachandran plots of modeled structure of a) β-glucosidase, b) endoglucanase and c) Exoglucanase showing the distribution of residue in most favoured regions (A, B & L), additional allowed regions (a, b, l & p), generously allowed regions (~a, ~b, ~l, ~p) and disallowed regions.

5.3.4. Binding site evaluation

Active site/catalytic site residues of β-glucosidase, endoglucanase, and exoglucanase enzyme of *R. flavipes* were located by two methods; one via literature (Scharf *et al*., 2010; Sethi *et al*., 2013; Tokuda *et al*., 2007b; Zhou *et al*., 2007) and second via conservation of active residues among 13 wood-feeding subterranean termites, protozoa and cockroaches

species; *Reticulitermes flavipes, Coptotermes gestroi, Coptotermes formosanus, Nasutitermes takasagoensis, Macrotermes barneyi, Microtermes sp., Neotermes koshunensis , Periplanata Americana, Cryptotermes scundus, Mastotermes darwiniensis, Macrotermes gulvis, Hodotermopsis sjoestedti, Cryptocercus punctulatus, and Pseudotrichonympha grassii* (Figure 5.5).

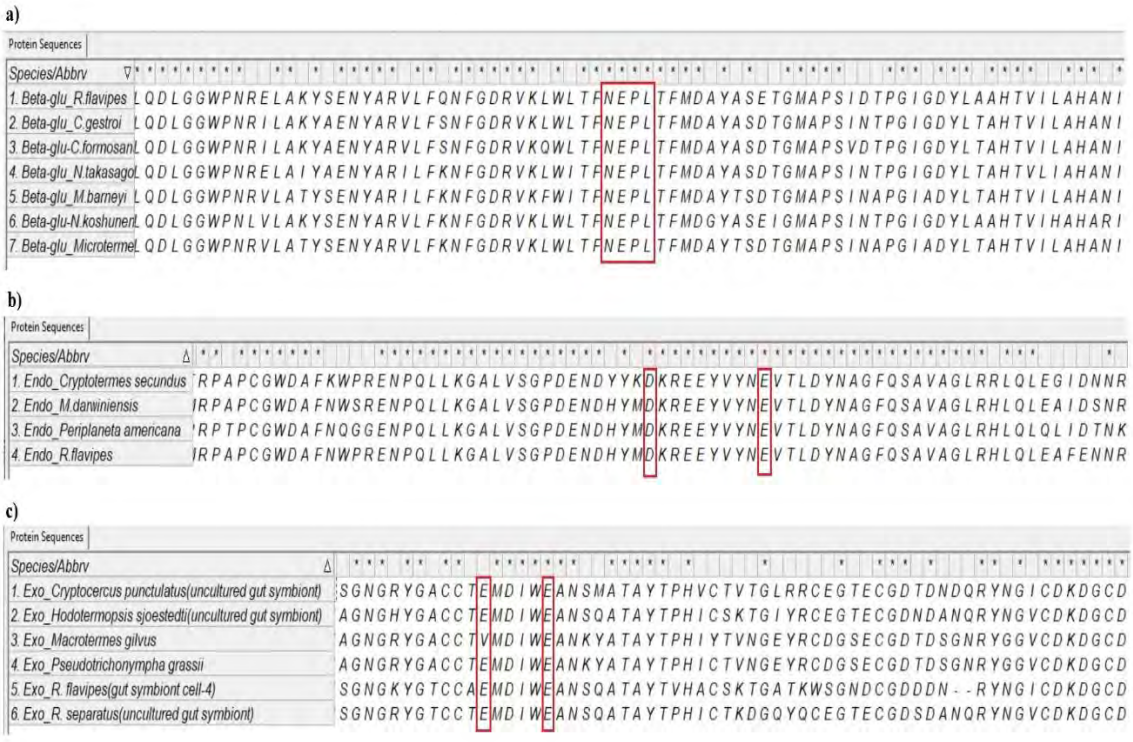


Figure. 5.5. Multiple sequence alignment of different termites, protozoa, and cockroach's species, where active site residues of a) β-glucosidase, b) endoglucanase, and c) exoglucanase were found conserved indicating by red boxes among the orthologs.

5.3.5. Molecular docking

Inhibitors were docked with target enzymes using GOLD. Primarily, 10 positions of each inhibitor were created and classified based on GOLD fitness score by setting criteria, higher the fitness score infers better binding of ligands with protein structures. The GOLD fitness score ranged from 34.48 to 78.14, while two inhibitors cellobiomidazole (CBI) and cellobiose (CB) attained the best fitness score of 78.14 and 62.17 against β-glucosidase, 72.40 and 52.02 against endoglucanase, and 77.20 and 64.03 against exoglucanase respectively. GOLD score of all inhibitors against each protein is shown in Table 4.2. An

inclusive imagining was carried out using USCF Chimera 1.14, LIGPLOT, and Molecular Operating Environment (MOE) software showed the detailed conformational and favored positioning of the binding of the inhibitor.

Table 5.2. GOLD fitted model score of carbohydrate-based inhibitors against β-glucosidase, endoglucanase, and exoglucanase

Compounds	Cellulase Enzymes		
	β-glucosidase	**Endoglucanase**	**Exoglucanase**
Cellobiomidazole (CBI)	78.14	72.40	77.20
Cellobiose (CB)	62.17	52.05	64.03
Fluoromethyl cellobiose (FMCB)	56.00	36.43	52.32
Glucose	41.88	34.48	39.67
Fluoromethyl glucose (FMG)	37.49	30.41	36.37

5.3.6. Binding site analysis of β-glucosidase

The docked inhibitors (CBI, CB, FMCB, Glucose, and FMG) in β-glucosidase active site cavity along with the interaction of the most active residues of binding pockets are indicated in Figure. 5.6. Cellobiomidazole (CBI) and Cellobiose (CB) showed a high binding score with β-glucosidase as compared to other inhibitors. Multiple hydrogen bonds were pragmatic between the active residues of β-glucosidase and inhibitors, along with some hydrophobic and ionic interactions (Figure 5.6). In the case of CBI, close binding was observed with active residues Glu448, Trp441, Phe457, Trp449, Glu399, Asn189, Tyr334, Glu190, Trp371, His145, Asn250, Thr193, Trp146 and His252 of β-glucosidase after visualizing the binding orientation (Figure 5.6 v and e). Hydrogen bonding was observed with Glu448, Trp441, Glu399, Asn250, and Tyr334 with a distance of 2.52 Å, 2.62 Å, 2.74 Å, 2.60 Å, and 2.86 Å respectively. Likewise, cellobiose also showed a close affinity with active residues Glu448, Trp441, Phe457, Glu399, Trp371, Glu190, Trp146, Asn250, Trp449, Thr193, and Trp449 of β-glucosidase after visualizing the binding orientation in LIGPLOT (Figure 5.6 iii and d). Hydrogen bonding was pragmatic with Trp371, Glu448, and His145 with a distance of 2.87 Å, 2.97 Å, and 3.15 Å respectively. Fluoromethyl cellobiose (FMCB) showed moderate attraction with active residues

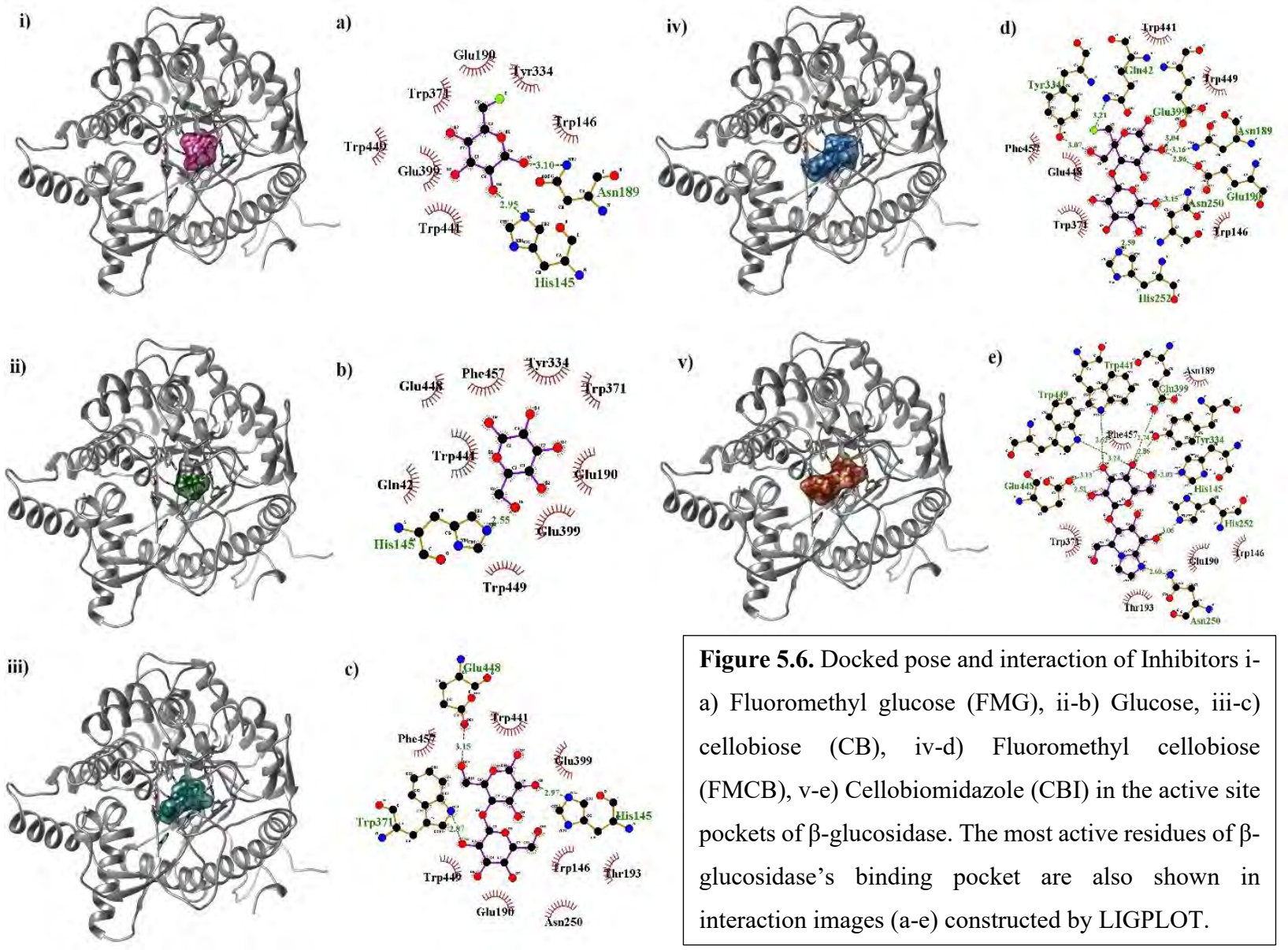


Figure 5.6. Docked pose and interaction of Inhibitors i-a) Fluoromethyl glucose (FMG), ii-b) Glucose, iii-c) cellobiose (CB), iv-d) Fluoromethyl cellobiose (FMCB), v-e) Cellobiomidazole (CBI) in the active site pockets of β-glucosidase. The most active residues of β-glucosidase's binding pocket are also shown in interaction images (a-e) constructed by LIGPLOT.

including Gln42, Glu448, Trp441, Phe457, Trp449, Glu399, Asn189, Tyr334, Glu190, Trp371, Asn250, Trp146 and His252 of β-glucosidase after visualizing the binding orientation (Figure 5.6 iii and d). Hydrogen bonding was observed with His252, Glu190, Glu399, and Tyr334 with a distance of 2.59 Å, 2.96 Å, 3.04 Å, and 3.15 Å respectively. In case of fluoromethyl glucose (FMG) showed a low affinity with active residues including Glu399, Tyr334, Trp371, Asn189, Trp441, His145, Trp449 and Glu190 of β-glucosidase and hydrogen bonding was only pragmatic with His145 and Asn189 of 2.95 and 3.10 Å distance respectively (Figure 5.6i and a). Whereas glucose showed a minimum affinity with Gln42, Trp441, Glu399, Trp449, Glu448, Phe457, Tyr334, Trp371, Glu190 and His145 active residues of β-glucosidase and hydrogen bonding was only pragmatic with His145 of 3.10 Å distance (Figure 5.6 ii and b).

5.3.7. Binding site analysis of endoglucanase

In the case of endoglucanase, the approximately same trend was shown by the docked inhibitors (CBI, CB, FMCB, Glucose, and FMG) in the active site cavity of protein structure along with the interaction of the most active residues of binding pockets (Figure. 5.7). However, all inhibitors showed a low affinity with endoglucanase than β-glucosidase. Cellobiomidazole (CBI) and Cellobiose (CB) indicated high binding GOLD scores 72.40 and 52.05 respectively with endoglucanase structure as compared to other inhibitors. Multiple hydrogen bonds were observed between the active residues of endoglucanase and inhibitors, along with some hydrophobic and ionic interactions (Figure 5.7). Cellobiomidazole (CBI) showed a close binding with active residues Glu431, Tyr224, Phe320, Tyr427, Thr433, Asp73, Ala74, Phe80, Asp76, His143, Tyr225, and His376 of endoglucanase that was developed after visualizing the binding orientation by LIGPLOT (Figure 5.7 v and e). Hydrogen bonding was observed with Tyr225, Asp73, Asp76, and His378 with a distance of 2.29 Å, 2.33 Å, 2.99 Å, and 3.05 Å respectively. The second most binding compound was cellobiose also which showed a close affinity with active residues Tyr225, Tyr427, His378, Glu431, Tyr224, Phe320, Asp73, and Ala74 of endoglucanase but less than β-glucosidase after visualizing the binding orientation in LIGPLOT (Figure 5.7 iii and d). Hydrogen bonding was pragmatic with Tyr225, His378, Glu431, and Ap73 with a distance of 2.74 Å, 2.87 Å, 2.97 Å, and 2.99 Å respectively. A

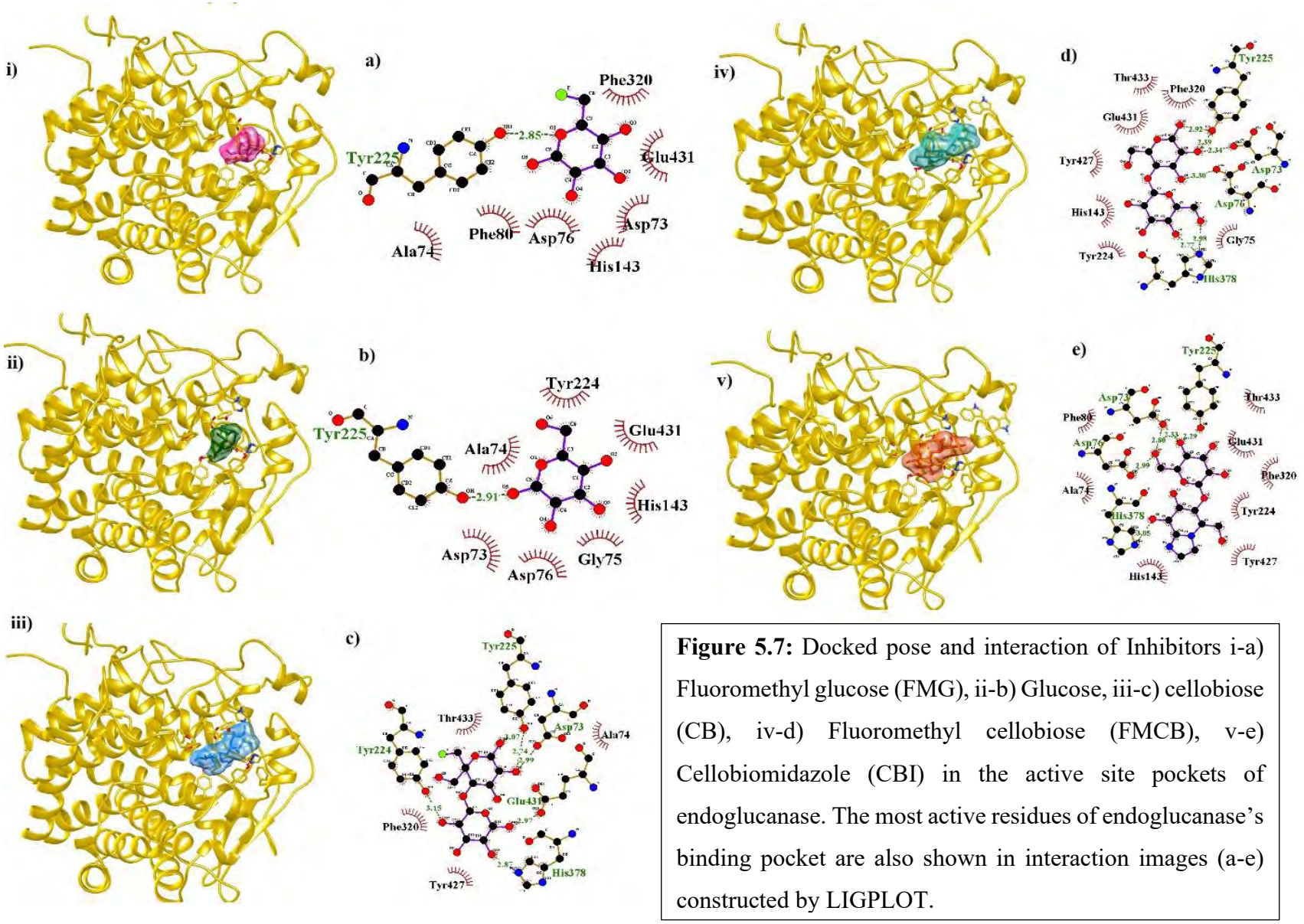


Figure 5.7: Docked pose and interaction of Inhibitors i-a) Fluoromethyl glucose (FMG), ii-b) Glucose, iii-c) cellobiose (CB), iv-d) Fluoromethyl cellobiose (FMCB), v-e) Cellobiomidazole (CBI) in the active site pockets of endoglucanase. The most active residues of endoglucanase's binding pocket are also shown in interaction images (a-e) constructed by LIGPLOT.

a similar trend was observed in the case of fluoromethyl cellobiose (FMCB) which showed moderate attraction with active residues including Tyr225, Gly75, His378, Tyr427, His143, Thr433, Phe320, Asp73, Tyr224, Glu431, and Asp76 from endoglucanase after visualizing the binding orientation (Figure 5.7 iii and d). However, fluoromethyl glucose (FMG) showed a low affinity with active residues including Tyr225, Ala74, Phe80, Asp80, His143, Asp73, and Glu431 and Phe320 of endoglucanase and indicated hydrogen bonding with the only Tyr225 along with 3.10 Å distance (Figure 5.7 i and a). Similarly, glucose showed minimum very low interaction with active residues of Tyr224, Ala74, Glu431, Gly75, Asp76, Asp73, His143, and Tyr225 of endoglucanase and hydrogen bonding was only pragmatic with Tyr225 of 2.99 Å distance (Figure 5.7 ii and b).

5.3.8. Binding site analysis of exoglucanase

Exoglucanase/cellobiohydrolase is a crucial enzyme for lignocellulose degradation in termite gut contributed by flagellates reside in the hindgut of *R. flavipes* (Rajarapu and Scharf, 2017). The docked inhibitors (CBI, CB, FMCB, Glucose, and FMG) in exoglucanase active site cavity along with the interaction of the most active residues of enzyme binding pockets are developed using GOLD and LIGPLOT (Figure. 5.8). All inhibitors showed very similar results to exoglucanase structure as in endoglucanase and, but the docking score was high in Table 5.2. than endoglucanase. Among all inhibitors, only cellobiomidazole (CBI) and cellobiose (CB) showed strong binding with exoglucanase as compared to other inhibitors. Multiple hydrogen bonds were pragmatic between the active residues of exoglucanase and inhibitors, along with some hydrophobic and ionic interactions (Figure 5.8). LIGPLOT binding visualization showed cellobiomidazole (CBI), as a more attractive inhibitor to exoglucanase with active residues Glu99, Trp255, Asp1, Trp264, Arg131, Asp137, Arg280, Gln57, Lys138, His110, Asp142, Asp139, Arg280, and Asp96 of than other inhibitors (Figure 5.8 v and e). Hydrogen bonding was observed with Asp142, Asp96, Glu99, and Arg131 with a distance of 2.70 Å, 2.90 Å, 3.22 Å, and 3.32 Å respectively. Similarly, cellobiose also showed a close affinity with active residues Glu94, Trp255, Glu99, Trp264, Val260, Tyr269, Asp137, Arg280, Gln57, Lys138, His110, Arg131, Asp139, and Asp96 of endoglucanase after visualizing the binding orientation in LIGPLOT (Figure 5.8 iii and d). Hydrogen bonding was pragmatic

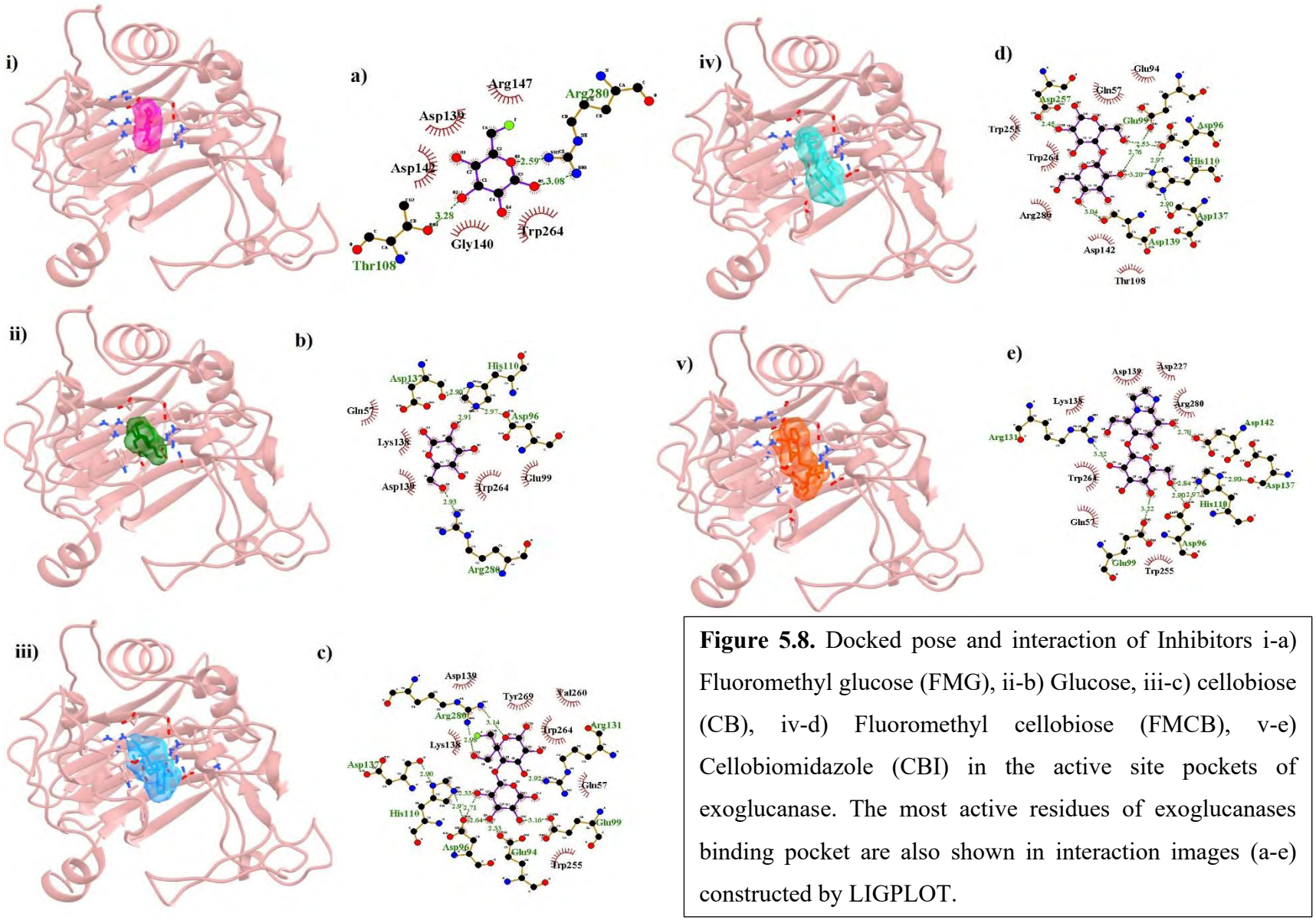


Figure 5.8. Docked pose and interaction of Inhibitors i-a) Fluoromethyl glucose (FMG), ii-b) Glucose, iii-c) cellobiose (CB), iv-d) Fluoromethyl cellobiose (FMCB), v-e) Cellobiomidazole (CBI) in the active site pockets of exoglucanase. The most active residues of exoglucanases binding pocket are also shown in interaction images (a-e) constructed by LIGPLOT.

with Glu94, His110, Asp96, Arg280, and Glu 99 with a distance of 2.33 Å, 2.33 Å, 2.71 Å, 2.98 Å, and 3.16 Å respectively. Fluoromethyl cellobiose (FMCB) showed moderate attraction (50%) with active residues including Gln57, Glu94, Asp96, Trp255, Arg280, Asp 257, Trp264, Glu99, Asp142, Thr108, Asp139, His110, and Asp137 of exoglucanase and indicated hydrogen bonding with Asp257, Asp96, Glu99, and Asp139 at a distance of 2.45 Å, 2.53 Å, 2.76 Å, and 3.04 Å respectively (Figure 5.8 iii and d). However, fluoromethyl glucose (FMG) showed a low affinity with a few active residues including Asp139, Thr108, Asp142, Arg147, Gly140, Trp264 and Arg280 of exoglucanase and indicated hydrogen bonding with Arg280 and Thr108 along at 2.59 and 3.28 Å distance (Figure 5.8 i and a). Similarly, glucose showed minimum interaction with active residues of Asp137, Trp264, Asp139, Arg280, Lys138, Gln57, Glu99 and His110 of exoglucanase and hydrogen bonding was pragmatic with only two residues His110 and Arg280 at 2.91 and 293 Å distance (Figure 5.8 ii and b).

Wet lab approach

5.3.9. *In vitro* cellulase inhibition by carbohydrate-based inhibitors

Percent enzyme activities in the presence of carbohydrate-based inhibitors in each gut fraction of *H. indicola* and *R. flavipes* have been summarized in Figures. 4.9 and 4.10. among all tested via *in vitro*; carbohydrate-based inhibitors, the glucose and its analog fluoromethyl glucose did not show the considerable reticence of any of the 03 activities (CMCase, pNPC, pNPG) in all fractions of *H. indicola* and *R. flavipes* guts (Figure 5.9 and 5.10). Whereas the cellobiose and its analog FMCB indicated the moderate to high range reduction in exoglucanase and β-glucosidase activities in the midgut and hindgut sections of *H. indicola* and *R. flavipes.* However, the percentage inhibition of activities in the salivary and foregut section (endogenous) was slightly lower than hindgut (symbiotic) in both wood-feeding termites. The effects of cellobiose (CB) and cellobiomidazole (CBI) were most pronounced on the exoglucanase and β-glucosidase activities in each gut section of *H. indicola* (Figure 5.9).

In the end, as it was observed that cellobiose (CB), Cellobiomidazole (CBI), and Fluoromethyl cellobiose (FMCB) were efficient exoglucanases and inhibitors of β-

glucosidase but these were unable to reduce the CMCase activity (endoglucanase) in both termite species.

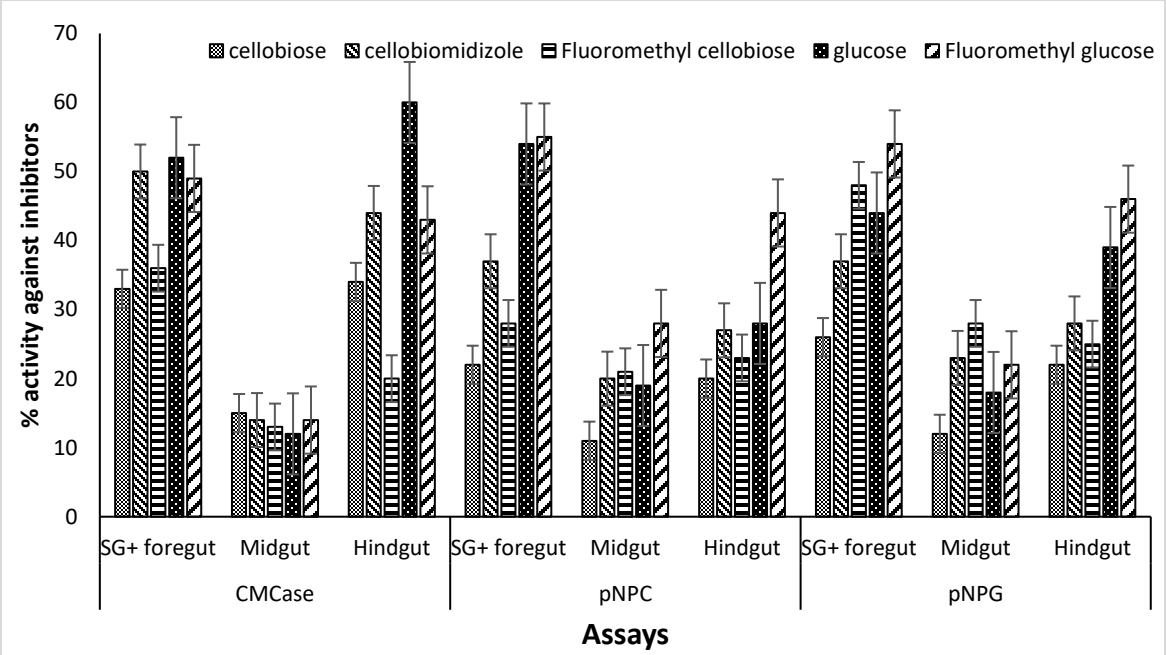


Figure 5.9. Efficacy of five carbohydrate-based inhibitors glucose, cellobiose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose CMCase inhibitors (endoglucanase activity), pNPG (β-glucosidase activity) and pNPC (exoglucanase activity) activities in section of each gut (salivary gland + foregut, hindgut and midgut) of *Heterotermes indicola*.

Correspondingly, in *R. flavipes* the CBI induced the highest inhibition of CMCase, (endoglucanase activity), pNPC (exoglucanase activity), and pNPG (β-glucosidase activity) activities in section of each gut (salivary gland + foregut, hindgut and midgut) as compared to other carbohydrate-based inhibitors as shown in Figure 5.10. From these experiments, it was concluded that the inhibitory effect of carbohydrate-based inhibitors on exoglucanases and β-glucosidase showed greater specificity against the endogenous cellulases of both termites since salivary gland+ foregut and midgut are free from symbiotic microbiota. It was also observed that the midgut fraction of crude cellulases was significantly suppressed by the inhibitors under *in vitro* conditions. Whereas hindgut fraction was still performing CMCase activity in the presence of all inhibitors. It may be due to the presence of flagellates with rigid pellicle covering that is performing at the cost of some remaining cellulase residues. The results of high suppression of

cellulase activities in endogenous (foregut, salivary gland, and midgut) indicating the inhibitors showed significantly ($p < 0.05$) more interaction to endogenous directly exposed cellulase source as compared to symbiotic cellulase.

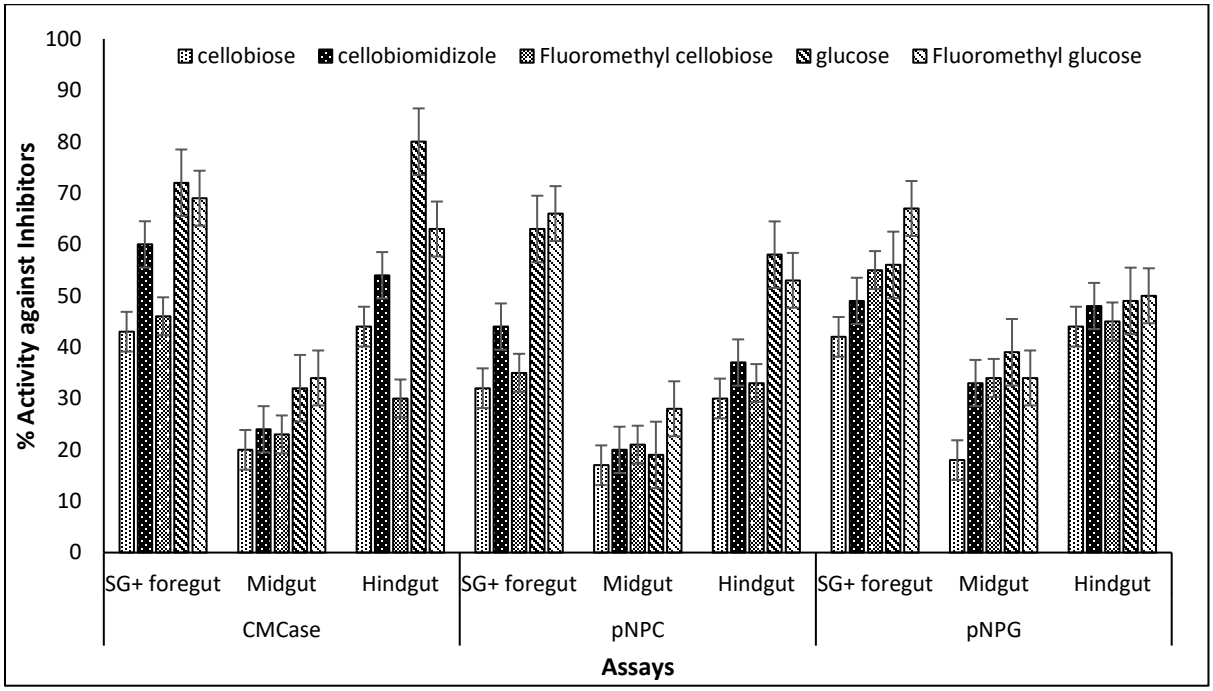


Figure 5.10: Efficacy of five carbohydrate-based inhibitors glucose, cellobiose, cellobiomidazole, fluoromethyl cellobiose, and fluoromethyl glucose CMCase inhibitors (endoglucanase activity), pNPG (β-glucosidase activity) and pNPC (exoglucanase activity) activities in section of each gut (salivary gland + foregut, hindgut and midgut) of *R. flavipes*.

5.3.10. Feeding Bioassays and *In vivo* effect of cellulase inhibitors

Feeding bioassays were conducted with each carbohydrate-based inhibitor to assess their *in vivo* impacts on termites feeding capabilities, survivorship, and cellulase activities. The carbohydrate-based inhibitors showed dose-response trends in form of more inhibitory and stimulating effects on both termite species *R. flavipes* and *H. indicola* (Figure 5.11 and 5.12). This inhibitory and stimulating effect was determined in terms of weight loss of filter paper and the number of dead termites (% mortality). The inhibitory effect for CBI increased with an increasing concentration on *R. flavipes* and *H. indicola* and overall, it caused significant termite mortality and reduced the filter paper consumption as indicated in Figure 5.11 and 5.12 respectively. Cellobiomidazole (CBI)

showed approximately 90% termite mortality and <50 mg of filter paper mass loss at the highest 25 mM in *R. flavipes* (Figure 4.11e). whereas FMG showed the least effects with >50 mg filter paper feeding and <50% termite mortality in *R. flavipes*.

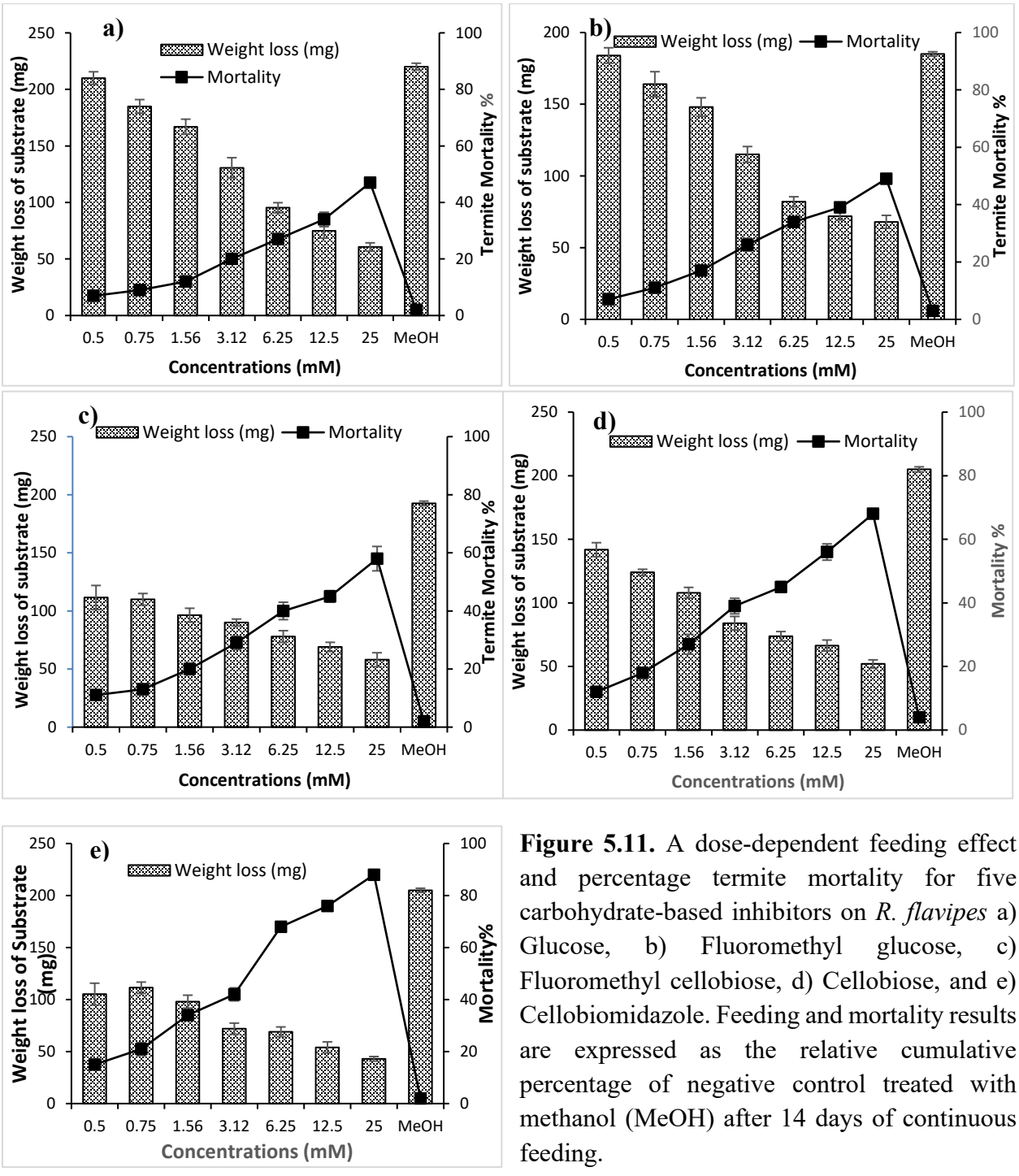


Figure 5.11. A dose-dependent feeding effect and percentage termite mortality for five carbohydrate-based inhibitors on *R. flavipes* a) Glucose, b) Fluoromethyl glucose, c) Fluoromethyl cellobiose, d) Cellobiose, and e) Cellobiomidazole. Feeding and mortality results are expressed as the relative cumulative percentage of negative control treated with methanol (MeOH) after 14 days of continuous feeding.

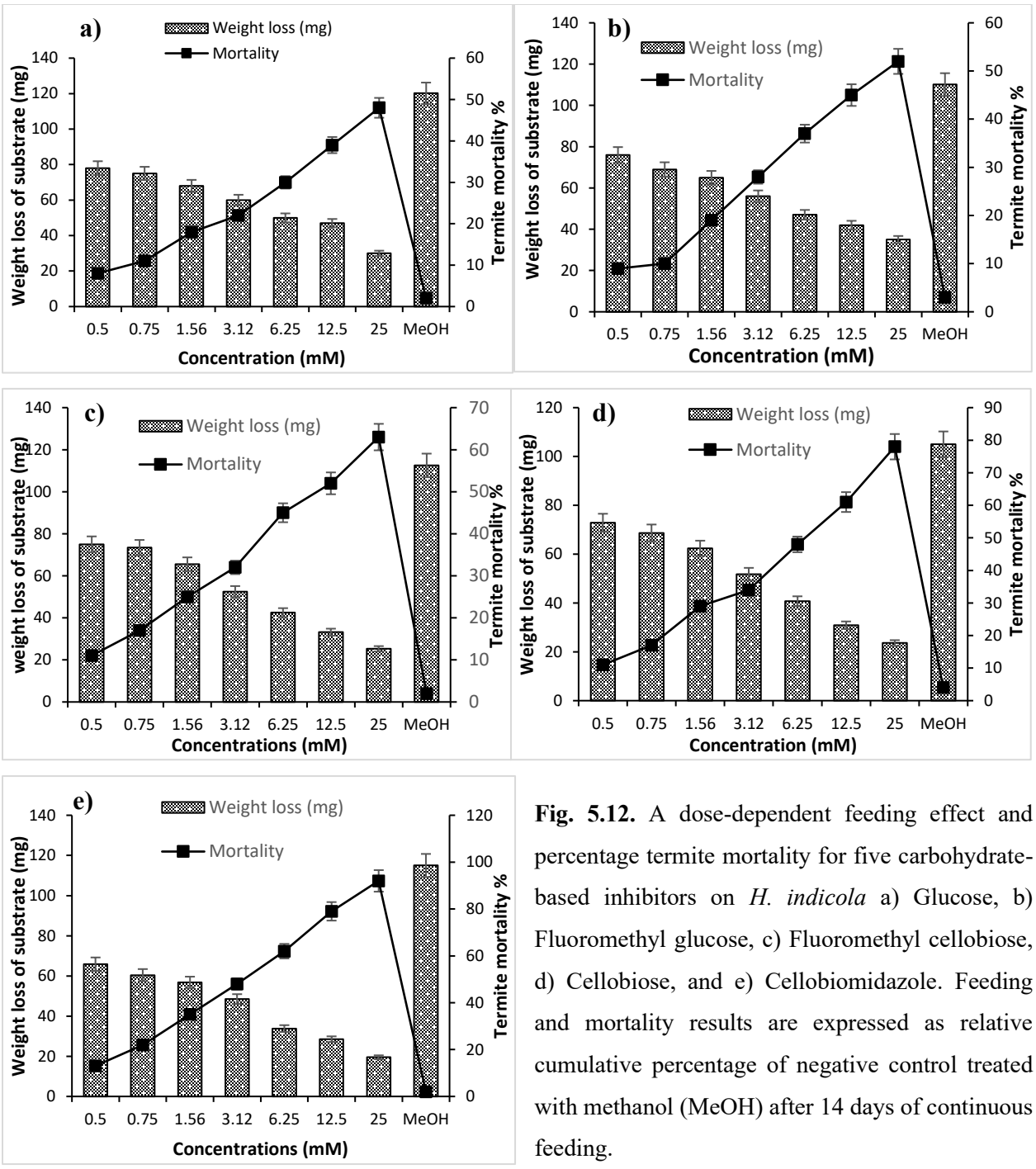


Fig. 5.12. A dose-dependent feeding effect and percentage termite mortality for five carbohydrate-based inhibitors on *H. indicola* a) Glucose, b) Fluoromethyl glucose, c) Fluoromethyl cellobiose, d) Cellobiose, and e) Cellobiomidazole. Feeding and mortality results are expressed as relative cumulative percentage of negative control treated with methanol (MeOH) after 14 days of continuous feeding.

Although cellobiomidazole (CBI) and fluoromethyl cellobiose (FMCB) induced significant mortality at the highest concentration (12.5, 25 mM), however, medium concentrations (3.12 to 6.25 mM) also induced significant mortality in *H. indicola* (Figure 5.12). The mortality observed in glucose and fluoromethyl glucose (FMG) assays, was not much pronounced, for example, the highest glucose and FMG concentrations induced <50% mortality in termites (Figure

5.12 a and b). Whereas cellobiose and its analogs (CBI and FMCB) induced significant mortality (>90%) and reduced the weight loss (<20 mg) of filter paper significantly ($p<0.05$) than control in *H. indicola*.

Table 5.3. Linear regression model of factors associated with termite mortality, general linear regressions of termite mortality (x-variable) with each of the tested predicted variables (Y-variables) with respective t-statistic, p-Value, and partial correlation values are shown in the table. Variables before the line are significantly associated with termite mortality after 14-days of in-vivo feeding bioassays with carbohydrate-based treatments.

X-variable [a]	Y-variables [b]	Beta In	t	p-Value	Partial Correlation
Termite Mortality (%)	(Constant)	98.414	42.817	.000	
	Protozoa Count	-.050[c]	-11.301	.000	-.894
	Relative % activity of Endo	-.272[c]	-4.965	.000	-.660
	Gene Expression Exo	-.533[c]	-2.749	.010	-.437
	Gene Expression _β-glu	-.154[d]	-.993	.328	-.176
	Gene Expression _Endo	.059[d]	.262	.795	.047
	Termite Weight (mg)	.033[d]	.379	.707	.068
	Relative % activity of β-glu	.194[d]	.990	.330	.175
	Relative % activity of CBH	-.050[d]	-.347	.731	-.062
	Feeding activity	-.074[d]	-.626	.536	-.112

Dependent Variable (X): Termite mortality (%) [a]

Predictors (Y): Protozoa Count, Relative % activity of Endo, Gene Expression Exo, Gene Expression _β-glu, Gene Expression _Endo, Termite Weight (mg), Relative % activity of β-glu. Relative % activity of CBH, Feeding activity [b];

Predictors in the Model: (Constant), Protozoa Count, Relative % Endo activity, Gene Expression _Exo [c]

Excluded Estimates: Gene Expression _β-glu, Gene Expression _Endo, Termite Weight (mg), Relative % activity of β-glu. Relative % activity of CBH, Feeding activity [d]

In an exertion, to recognize the factors involved significantly in termite mortality after 14-days of continuous feeding on filter paper treated with inhibitors, a multilinear regression model was applied (Table 5.3). Results indicated three tested variables (protozoa count, relative % activity of endoglucanase, and gene expression of exoglucanase) were significantly ($p < 0.05$) associated with termite mortality that reveals the importance of endosymbiotic protozoa, endoglucanase activity and expression of exoglucanase (GHF7 cellulase of endosymbionts) in

termite survival. A high negative strong correlation indicates an inverse relationship between the dependent variable and regressor which means if protozoa count decreases termite mortality increases (r = -0.894). whereas gene expression of exoglucanase showed moderate association (r = -0.437) with termite mortality.

5.3.11. Cellulase activity in survived individuals

Impacts of inhibitors (FMCB, CB, CBI, and EDTA) on three cellulolytic activities (β-glucosidase, exoglucanase and endoglucanase activities) were highly productive and in strongly in line with the outcome of in vitro inhibition experiments. Especially, carbohydrate-based analogs of cellobiose CBI and FMCB showed a pronounced dose-dependent trend of β-glucosidase and exoglucanase activities in *R. flavipes* and *H. indicola.* However, EDTA and PMSF non-carbohydrate inhibitors also induced mortality up to 50% in termites, contrary to some of the in vitro inhibition results (Fig. 5.9 a-c).

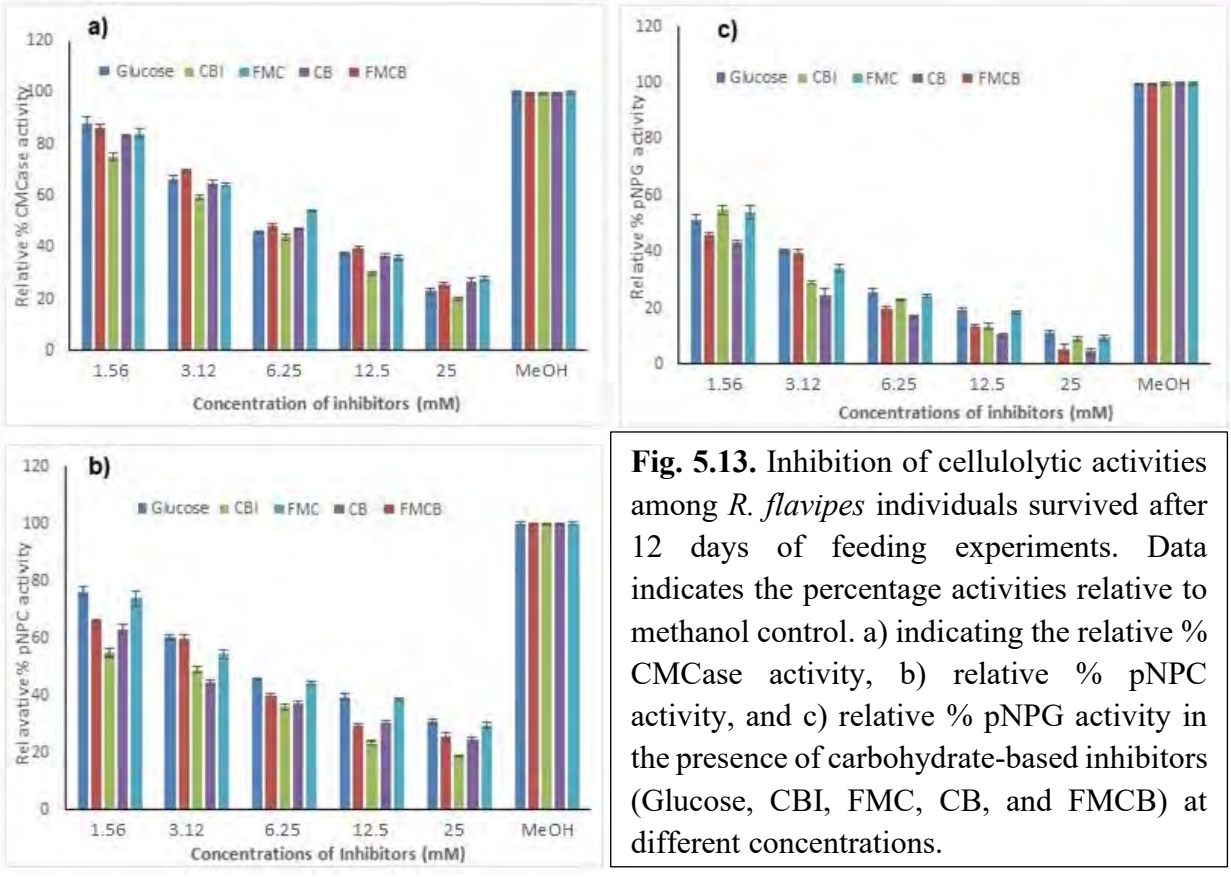


Fig. 5.13. Inhibition of cellulolytic activities among *R. flavipes* individuals survived after 12 days of feeding experiments. Data indicates the percentage activities relative to methanol control. a) indicating the relative % CMCase activity, b) relative % pNPC activity, and c) relative % pNPG activity in the presence of carbohydrate-based inhibitors (Glucose, CBI, FMC, CB, and FMCB) at different concentrations.

Exoglucanase activity was maximumly reduced by approximately 70-80 % in both termite species by CBI at a concentration of 50 mM. while β-glucosidase inhibited a maximum up to 80% at a dose level of 75 mM of CBI. Under *in-vitro* circumstances the endoglucanase activity was not inhibited significantly, however, it was significantly impacted by disaccharides inhibitors (CB and CBI) after feeding bioassays.

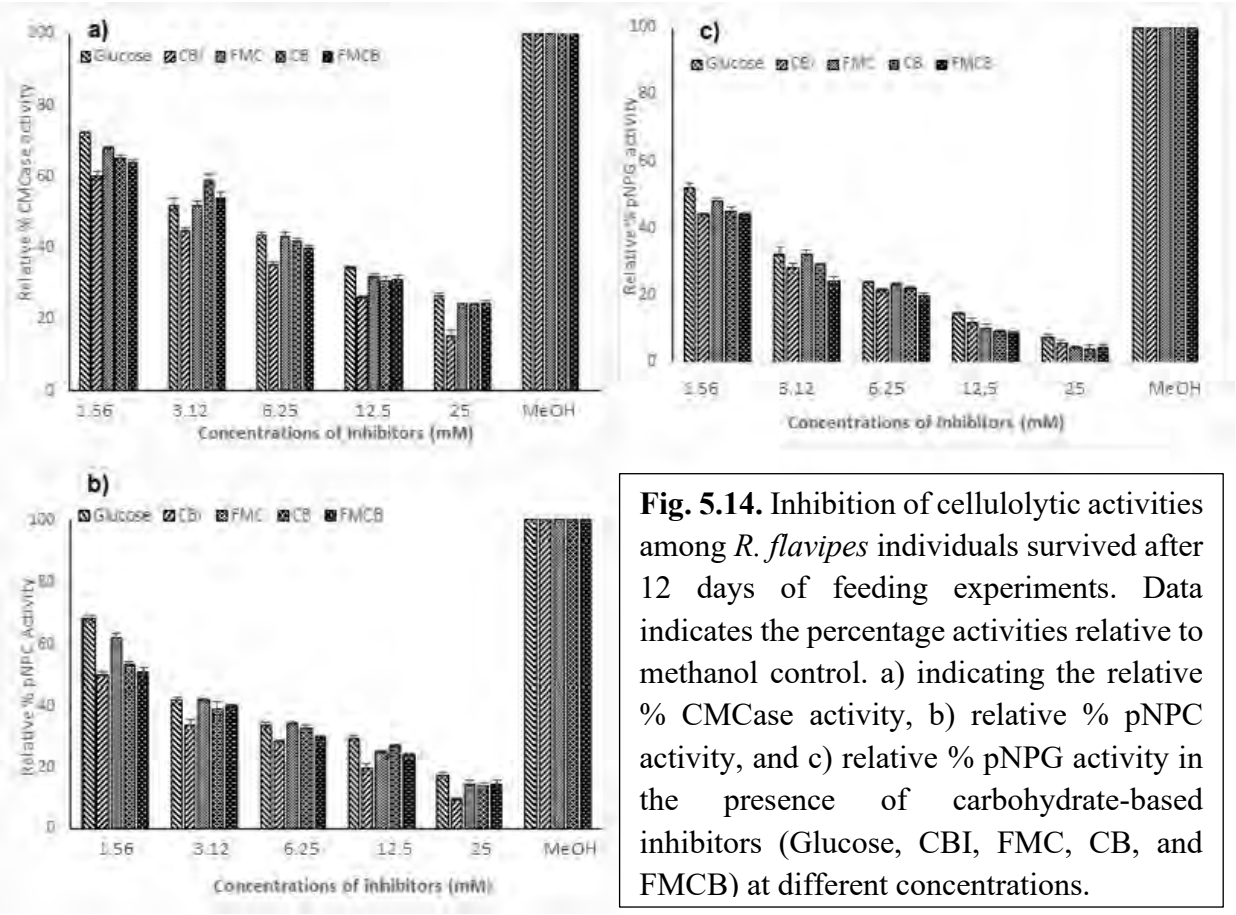


Fig. 5.14. Inhibition of cellulolytic activities among *R. flavipes* individuals survived after 12 days of feeding experiments. Data indicates the percentage activities relative to methanol control. a) indicating the relative % CMCase activity, b) relative % pNPC activity, and c) relative % pNPG activity in the presence of carbohydrate-based inhibitors (Glucose, CBI, FMC, CB, and FMCB) at different concentrations.

5.3.12. Impact on cellulase gene expressions

In addition to cellulase activity measurements, gene expression of three main cellulases i.e. endoglucanase, exoglucanase, and β-glucosidase were evaluated following carbohydrate-based feeding assays. The transcript level of two enzymes one exoglucanase (GH7-3) from symbiotic fraction and second β-glucosidase (GH-1) of endogenous origin significantly decreased in both termite species with carbohydrate-based inhibitors treatments (Figure 5.15). whereas the endoglucanase (GH9) did not significantly ($p>0.05$) changed it expression level in any treatment. The treatment of cellobiomidazole (CBI) showed a

higher reduction in all cellulase gene expression as compared to other inhibitors' treatments. Each cellulase gene showed a unique expression pattern depending on the *in vivo* feeding inhibitors treatment and it is evident to have impacts on the cellulolytic potential of termites.

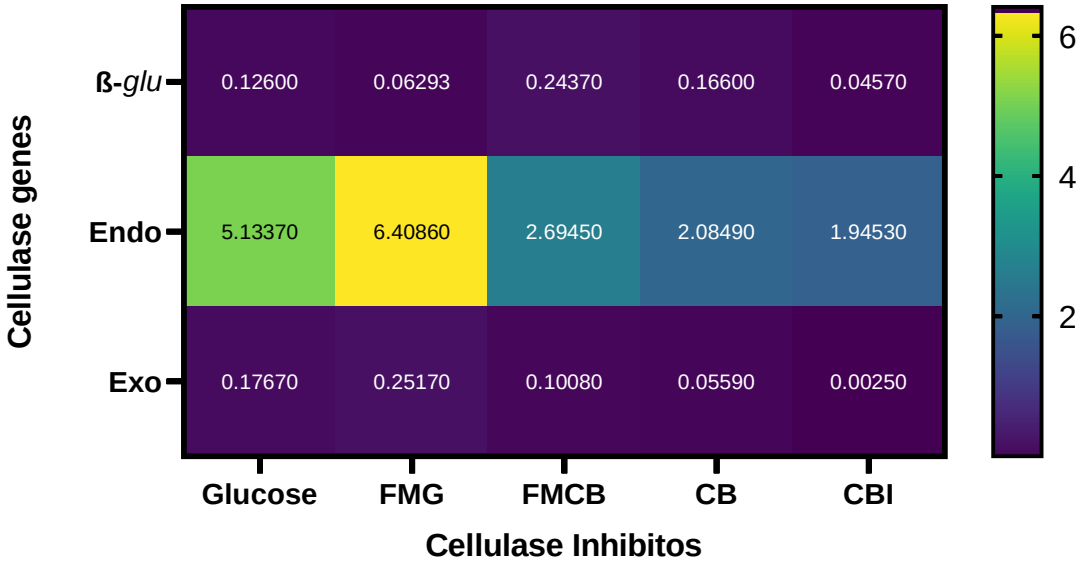


Figure 5.15. Heatmap chart showing the gene expression analysis of three main previously characterized cellulase genes from termites' endogenous fraction endoglucanase (GHF-9) and β-glucosidase (GHF-1) and symbiotic fraction exoglucanase (GHF7) in termites after feeding treatments using carbohydrate-based inhibitors (Glucose, cellobiomidazole (CBI), fluoromethyl glucose (FMG), cellobiose (CB) and fluoromethyl cellobiose (FMCB). Transcript levels are indicated as fold change relative to the reference gene ($NADH^{+}$) and the control treatment group (MeOH) for each cellulase gene (see 4.2.13).

5.3.13. Antiprotozoal effect of carbohydrate-based inhibitors

After 14-days of no-choice feeding on treated filter paper disks, the dose-dependent *in-vivo* effect of carbohydrate-based inhibitors (glucose, FMG, FMCB, CB, CBI) was observed in the number of gut protozoa and weight loss of *R. flavipes* (Figure 5.16) and *H. indicola* (Figure 5.17). The reduction of termite gut protozoa population was significantly and strongly positively correlated to a weight loss of termite ($r = 0.9525$; $p = 0.001$) against each inhibitor treatment suggesting that

the reduction of protozoal communities in termite gut causing the weight loss of termite workers. The maximum percentage loss of protozoa (95%) in *R flavipes* was observed after the exposure to the highest concentration (25mM) of CBI where the termite weight was > 2 mg and it was followed by CB (77.5%) and FMCB (76%) (Figure 4.16).

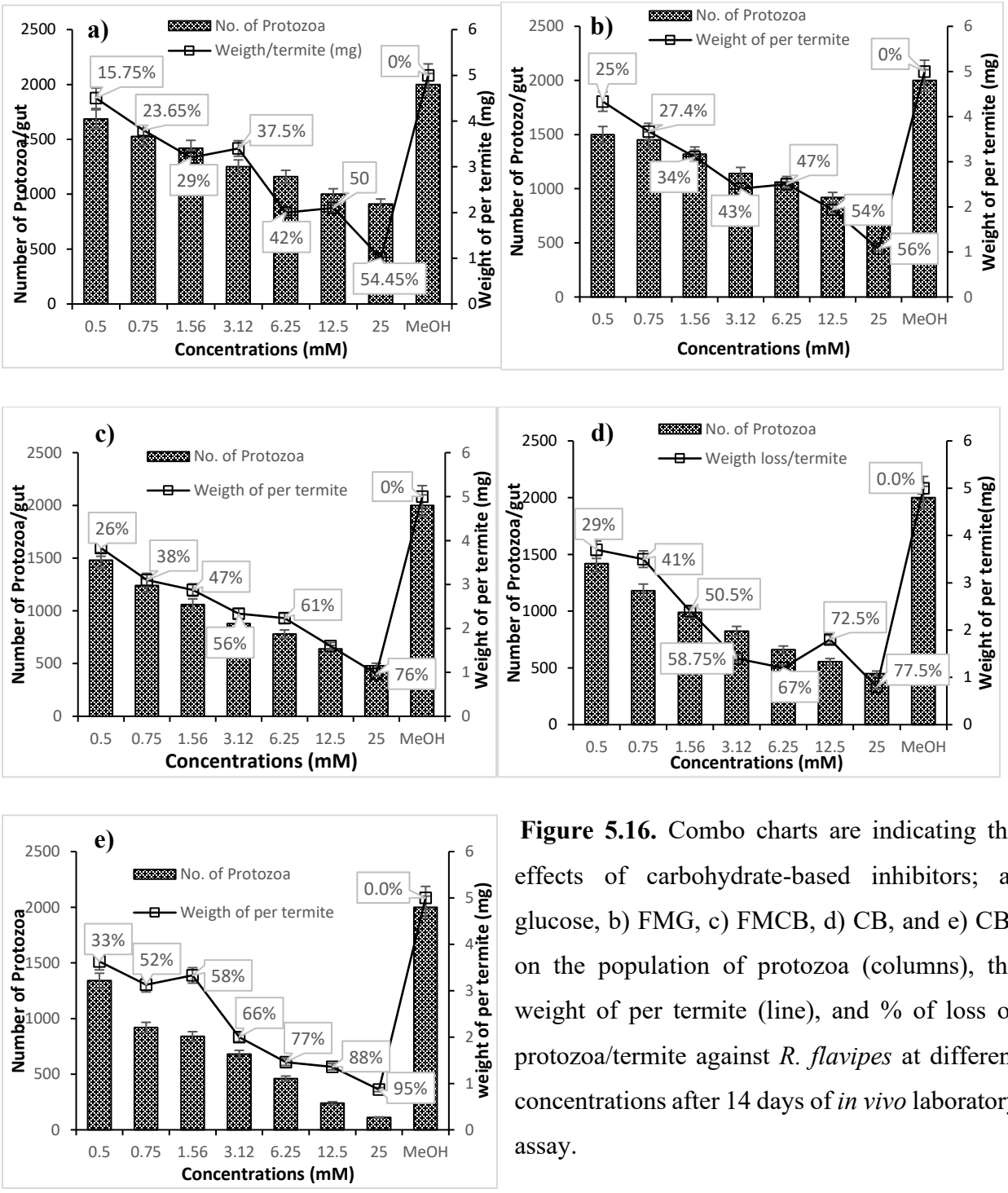


Figure 5.16. Combo charts are indicating the effects of carbohydrate-based inhibitors; a) glucose, b) FMG, c) FMCB, d) CB, and e) CBI on the population of protozoa (columns), the weight of per termite (line), and % of loss of protozoa/termite against *R. flavipes* at different concentrations after 14 days of *in vivo* laboratory assay.

Protozoa numbers and weight of termite were significantly different from each other ($p < 0.05$) as well as from the control at each treatment. However, in the case of glucose and FMG, an insignificant difference (p>0.05) was observed in protozoa counts and weight loss among different concentrations.

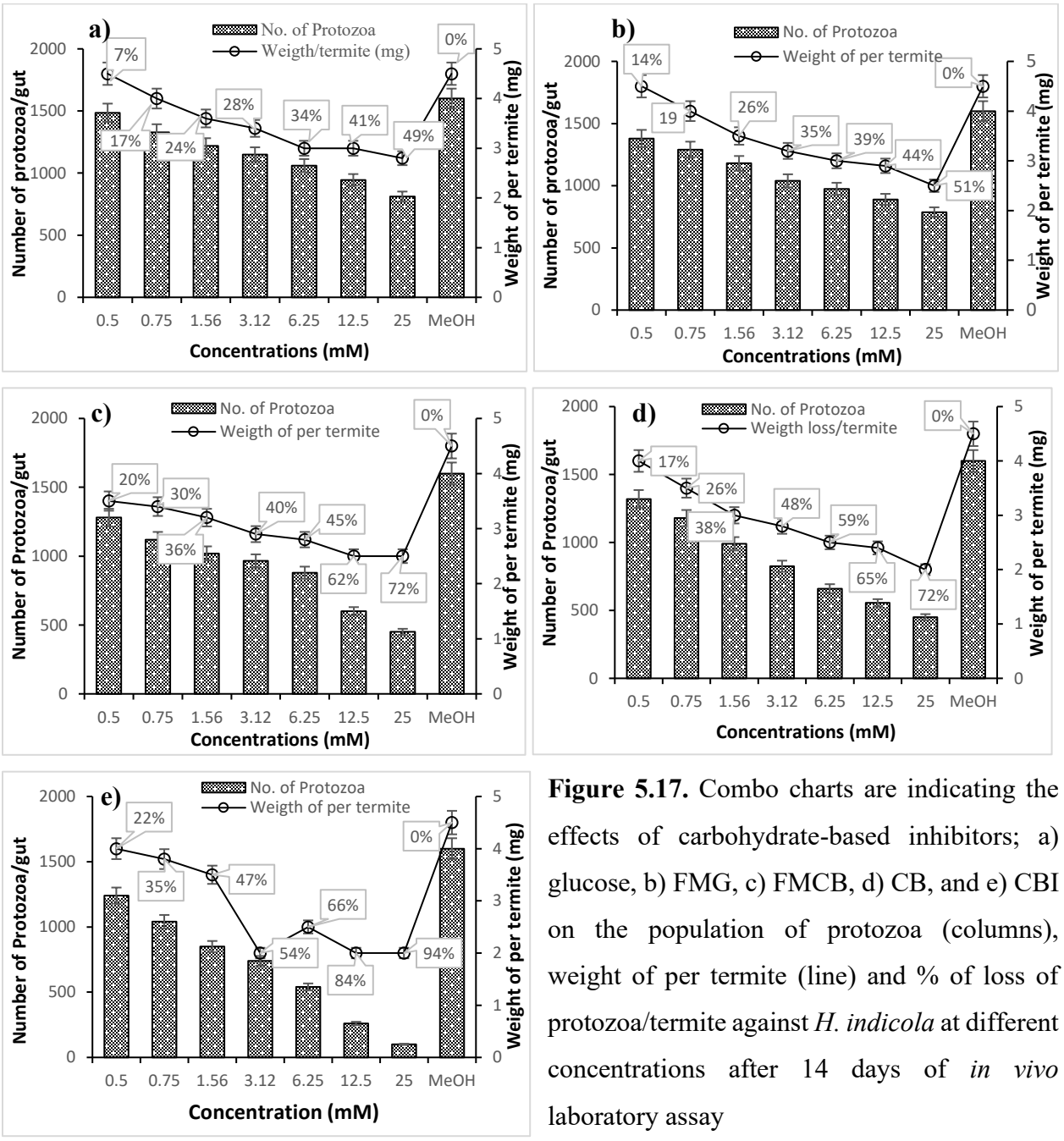


Figure 5.17. Combo charts are indicating the effects of carbohydrate-based inhibitors; a) glucose, b) FMG, c) FMCB, d) CB, and e) CBI on the population of protozoa (columns), weight of per termite (line) and % of loss of protozoa/termite against *H. indicola* at different concentrations after 14 days of *in vivo* laboratory assay

In case of *H. indicola*, a similar trend in the impacts of carbohydrate-based inhibitors was observed in the form of protozoal population reduction and termite weight loss (Figure 5.17). In

H. indicola the reduction of termite gut protozoa population was also significantly and positively correlated to a weight loss of termite ($r = 0.9265; p = 0.001$) against each inhibitor treatment which is revealing that weight loss of termites was significantly associated with loss of protozoa. Among inhibitors, CBI showed >90% reduction of protozoa population in the hindgut of *H. indicola* termite workers after 14-days of continuous feeding on treated filter paper treated with 25mM concentrations which are significantly higher than other concentrations as well as controls. However, there was no significant difference was noticed in termite's response to inhibitors between *R. flavipes* and *H. indicola.*

5.4. Discussion

5.4.1. Dry lab approach

In this study, dry and wet lab approaches were applied to inhibit the cellulase activities using carbohydrate-based inhibitors in wood-feeding termites. Incomplete hydrolysis of wood digestion in termites depends on three major cellulases; i) endoglucanase, ii) exoglucanase, and iii) β-glucosidase (Sethi *et al*., 2013). In the present study, these enzyme activities were inhibited using ecofriendly carbohydrate-based inhibitor via *in vitro* and *in vivo* wet laboratory bioassays and subsequently validated by *in silco* molecular docking tools. Among the carbohydrate-based inhibitors used in this study, some (CBI, FMBI, and FMG) were patented by Prof. Dr. Michael E Scharf (2008) for the control of *R. flavipes* in Florida, USA, while cellobiose and glucose (CB and Glucose) were reported by Tokuda *et al*., (1997) as cellulose inhibitors while their accumulation in termite digestive tract has been inhibited the further catalytic activities of exoglucanase and β-glucosidase in this study.

Homology modeling of protein/enzymes is considered a strong approach to attain structural image into those proteins having no experimentally predicted structures (Rodriguez *et al*., 1998). The essential cellulase enzymes (endoglucanase, exoglucanase, and β-glucosidase) in the lignocellulose hydrolysis from *R. flavipes* were modeled in this study and verified by PROCHECK, Z score, and Ramachandran plot. The active sites of endoglucanase, exoglucanase, and β-glucosidase structures were localized by searching in reported literature (Scharf *et al*., 2010; Sethi *et al*., 2013; Tokuda *et al*., 2007b; Zhou *et al*., 2007) and aligning the target sequences with different termite species to find out conserve motifs of active sites. The binding affinities of inhibitors CBI, FMCB, FMG, Glucose, and CB were determined respective docking scores against

each enzyme. All three cellulase including endoglucanase, exoglucanase, and β-glucosidase showed the highest interaction with cellobiomidazole (CBI) by showing 72.40, 77.20, and 78.14 docking score. Cellobiose (CB) was the second most interactive ligand followed by moderated binding affinity of fluoromethyl cellobiose (FMCB) to the catalytic sites of all three cellulases, whereas fluoromethyl glucose (FMG) and glucose showed minimum interaction to binding sites of cellulases. It was observed that binding of the inhibitors was emphasized by active amino acids varied with each enzyme structure in the formation of hydrogen bonding along with hydrophobic and ionic interactions as shown in Figures 5.5, 5.6, and 5.7. These results of Insilco studies validated the experimental outcomes which were further carried out in this study under lab conditions.

5.4.2. Wet lab practices

In the wet lab approach, the same cellulase inhibitors (CBI, FMG, FMCB, CB, and Glucose) with diverse modes of action were used and target the termite cellulases to garner as much information about cellulase importance in termite control strategies (Peterson *et al*., 2015). Impacts of these cellulase inhibitors were measured via *in-vitro* and *in-vivo* cellulase activities, symbiotic population shifts, cellulase gene-expression, termite feeding behavior, and mortality. In another effort to explore the variables, which were most significantly associated with termite mortality, multiple regression analysis (MRA) was applied (see Method 5.12, Table 5.3). In these results, three tested variables (protozoa count, relative % activity of endoglucanase, and gene expression of exoglucanase) were significantly associated with termite mortality after the 14-days of continuously treated filter paper bioassays with cellulase inhibitors. Furthermore, by this information, it is also revealed that which was variable more affected by cellulase and caused termite mortality. Most notably, inhibitors of cellulase revealed different levels of potential ranging from ineffective (FMG and Glucose) to moderately effective (FMCB and CB) and highly effective (CBI). Under *in vitro* conditions, CBI efficiently inhibited both β-glucosidase and exoglucanase activities during pNPG and pNPC assays. However, endoglucanase activity was not significantly inhibited by an inhibitor that was tested. Feeding bioassays using treated filter papers with inhibitors' different concentrations showed random effects on termite feeding behavior, enzyme activities, cellulase gene expression, termite physiology, protozoa communities, and termite percent mortality. Cellobiose and its analogs FMCB and CBI caused significant termite

mortality and feeding inhibition throughout lower to midrange tested concentrations (1-25 mM). Cellulase activities on whole-body homogenates of termites survived from feeding bioassays have revealed significant but dispersal inhibition of CMCase, pNPC, and pNPG activities. After 12 days of feeding experiments, carbohydrate-based inhibitor CBI showed dose-dependent β-glucosidase and exoglucanase activity substantial inhibition and besides CBI also has a moderate impact on endoglucanase activity in survivors (termites). Glucose and FMG have not shown greater impact, while the cellobiose, FMCB and showed moderately high inhibition influence on cellulase activities after feeding bioassays than in vitro assay results. These findings were strengthened by the cellulase gene expression results in survived termite workers, in which exoglucanase (GH-7) and β-glucosidase (GH-1) gene expression was suppressed after the FMCB, CBI, and CB treatment. Furthermore, FMCB, CBI, and CB were also found an active agent to reduce the protozoa counts in the termite hindgut at higher concentrations in both termite species. Therefore, it can be concluded that FMCB, CBI, and CB may have detrimental effects on the protozoa population in the hindgut region of the termites and resulted in compromised β-glucosidase, exo-, and endoglucanase activities. Such findings also suggest that the impact of five carbohydrate-based inhibitors on two wood-feeding termite *H. indicola* and *R. flavipes* inform of minimum feeding activity and high mortality at 25 mM concentration are the consequences of cellulase inhibition, symbionts mortality, and interaction of inhibitors with the digestive mechanism.

5.4.2.1. Comparison of findings with previous termite control strategies

In termites and other wood-feeding insects, cellulases are the main source of energy production and potential target sites for more eco-friendly and target specific pesticides (Zhu *et al*., 2005). The results of inhibition of cellulases activities in *R. flavipes* and *H. indicola* by glucose, cellobiose, and their analogs CBI, FMG, and FMCB in the present studies quietly resemble to study reported by (Zhou *et al*., 2008). They used three carbohydrate-based inhibitors (CBI, FMG, and FMCB) to control termites of Florida *Reticulitermes flavipes,* and CBI was considered the most active inhibitor to induce feeding stimulus and mortality in termites for control purposes. CBI, FMCB, and FMG showed I_{50} in nM, µM, and mM respectively. Likewise, cellobiomidazole (CBI) inhibited the endoglucanase activity by the cellulase enzyme (Cel5A) of *Bacillus agaradhaerens* with I_{50} of 88 µM. Non-carbohydrate-based inhibitors (SDS, EDTA, PMSF, and urea) have been used to inhibit the activity of bacteria cellulases by Gaur and Tiwari, (2015)

against the alkali stable *Bacillus vallismortis* RG-07 isolate. However, in Pakistan, there is no such study has been conducted in terms of termites' control strategies. The I_{50} concentration of inhibitors based on carbohydrate in this study is 2 times higher than Zhu *et al*., (2008) measured for CBI, FMCB, and FMG' under *in-vitro* conditions, against termites β-glucosidase and endogenous and symbiotic exoglucanase activity. These results screened and identified a high level of effectiveness by CB, CBI, and FMCB against termites' endoglucanase, exoglucanase activities. It suggests that these compounds and their derivatives can be used in termite control strategies via baiting technology.

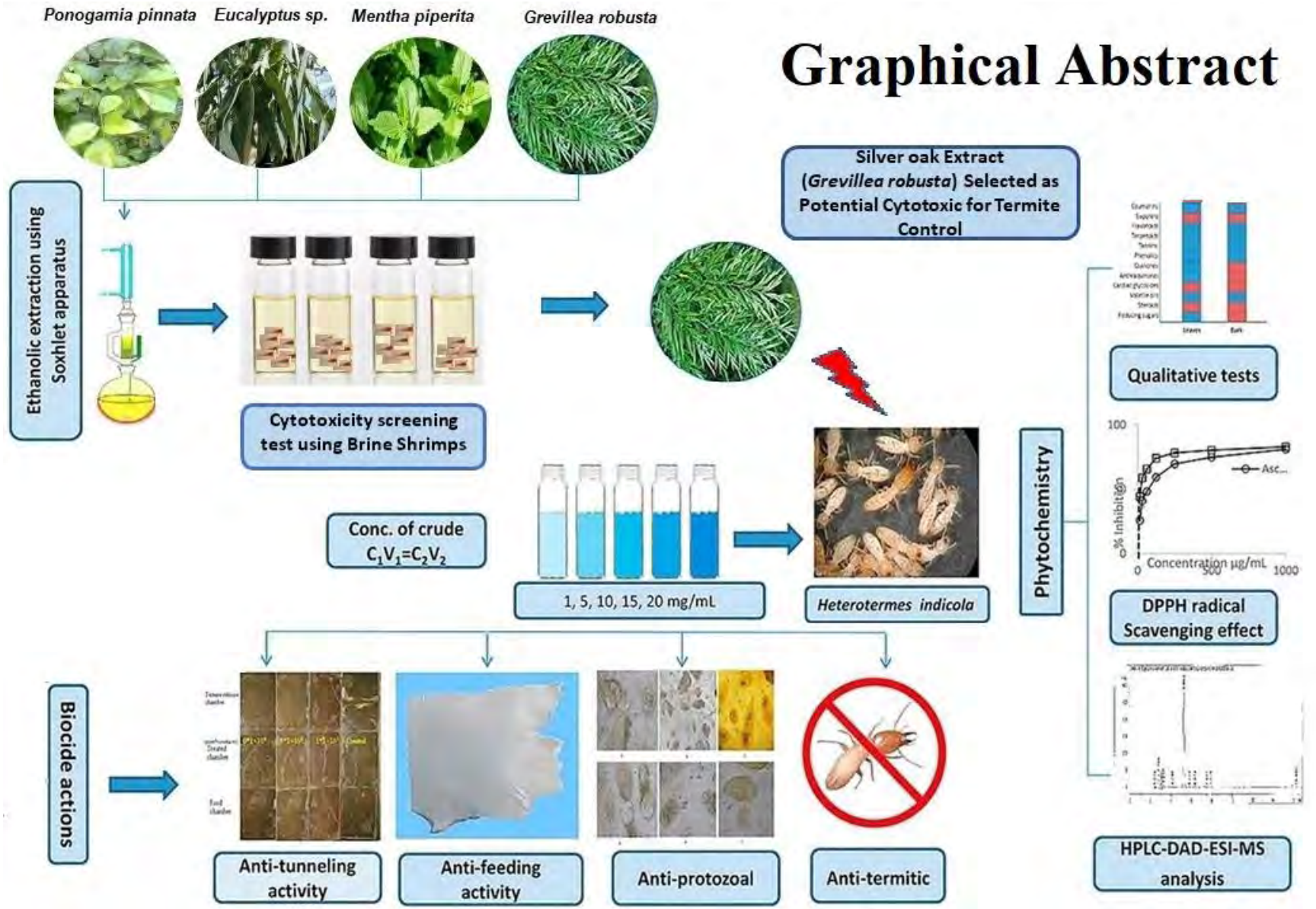
Zhu *et al*., (2005) already have examined the effect of five different prototype inhibitors of β-glucosidase activity after feeding bioassays. They found only one inhibitor (sinapinic acid) that did not show inhibitory effect, while the other four inhibitors (conduritol-β-epoxide, ferulic acid, gluconolactone, and 1-deoxynojirimycin) showed 25-65% cellulase inhibition after feeding experiments. However, our results showed 30-70% inhibition of cellulase activities in *H. indicola* and *R. flavipes* after feeding experiments. In this study, we also observed an important trend that the workers of *R. flavipes* showed different susceptible and inhibition rates as compared to *H. indicola. Heterotermes indicola* was found more tolerant and resistant to inhibitory and feeding stimulus effects and a more starvation period is required to induce feeding stimulus than *R. flavipes*. Such observations indicated a possibly important balance among the feeding behavior and susceptibility of termites to inhibitors of cellulases and hopefully, this relationship can be studied in detail under laboratory conditions in future.

Carbohydrate-based inhibitors have the advantage that they can attract the termites due to phagostimulant nature and can induce the whole termite colony. Such as Swoboda *et al*., (2004), reported that *Reticulitermes* utilized high sugar-coated substrate (filter paper) than untreated paper substrates under choice assay. While under no-choice assay rates of utilization were similar among treated and untreated filter paper. Similarly, in our feeding test, no significant attraction was observed by both termite species. The most reasonable explanation for the feeding deterioration is that only insecure feeding has occurred in response to nutritional capabilities deprivation from these cellulase inhibitors. In addition, low feeding activities were due to the mortality of symbiotic protozoa in termites that is associated with nutritional deprivation with the response of insecure

feeding. Another, possible explanation for feeding deterioration could be the pharmacological interaction by inhibitors with termite chemical sensation.

5.5. Conclusions

- The relative rank of five inhibitors of cellulase is CBI > CB > FMCB go after by FMG and glucose.
- In particularly, cellobiose and its analogs (CBI and FMCB) indicated β-glucosidase and exoglucanase activities strong inhibition at low and mid-range concentrations (6.25-25 mM) as well as they also showed *in-vivo* efficacy by revealing low cellulase gene expression, low consumption rates, reducing the termite weight loss and protozoa population, which resulting in high termite mortality.
- This study has triggered the relevant methods for use in additional evaluation of each carbohydrate under field condition trials.
- Based on our findings that *H. indicola* and *R. flavipes* showed varying degrees of susceptibility to cellobiose and its analogs, that could be tested for feeding and mortality in different biogeographic termite species with varying nutritional backgrounds.
- Different hypotheses, such as termites' colonies that are stressed nutritionally will be more susceptible to the effects of cellulase inhibition.
- Additionally, the current study did not assess the field efficacy bioassays and choice bioassays; therefore, we could not estimate that at which rate these inhibitors will feed preferentially by termites in the presence of other food sources under field conditions.


Graphical Abstract
Ponogamia pinnata
Eucalyptus sp.
Mentha piperita
Grevillea robusta
Ethanolic extraction using Soxhlet apparatus
Cytotoxicity screening test using Brine Shrimps
Silver oak Extract (Grevillea robusta) Selected as Potential Cytotoxic for Termite Control
Conc. of crude $C_1V_1=C_2V_2$
1, 5, 10, 15, 20 mg/mL
Heterotermes indicola
Phytochemistry
Qualitative tests
% Inhibition
Concentration µg/mL
DPPH radical Scavenging effect
HPLC-DAD-ESI-MS analysis
Biocide actions
Anti-tunneling activity
Anti-feeding activity
Anti-protozoal
Anti-termitic

Antitermitic activity of leaves extract of silver oak (*Grevillea robusta* Cunn.) (Proteaceae) on building infesting subterranean termite species in Pakistan

Abstract

The subterranean termite, *Heterotermes indicola* (Blattodea: Rhinotermitidae) is a serious pest to the wood in service causing a significant annual economic loss in Southeast Asia. In the present study, silver oak (*Grevillea robusta* Cunn.) (Proteaceae) leaf extract was investigated for its termiticidal properties against the termite *H. indicola* in laboratory tests after the screening of the cytotoxicity of four crude leaves extractives of plants *Ponogamia pinnata, Eucalyptus camaldulensis, Grevillea robusta* and *Mentha piperita* were using Brine Shrimp cytotoxicity screening test. An ethanol-solvent system was used to remove the extract from dry leaf powder via the Soxhlet apparatus. A dose-dependent foraging response and mortality were observed after continuous exposure to a dose range of 1 to 20 mg/mL of extract based on dry extract weight. Results indicated that maximum (> 90%) termite mortality occurred at 20 mg/mL along with a significant reduction in termite tunneling activity. The LC_{50} was calculated at 5.47 mg/mL using the concentration series data. In filter paper antifeeding and repellency bioassays, a significant reduction in the total population of gut protozoa compared to untreated and negative controls (solvent) was observed. After 2 weeks of no-choice continuous feeding on filter paper treated at 20 mg/mL, the hindgut protozoan population was reduced by 62.90%. The extract conveyed resistance to the non-durable cottonwood and pinewood after vacuum pressure treatment. At the highest concentration tested, 100% mortality was recorded after exposing termites to cottonwood and pinewood treated with silver oakleaf extract. HPLC-DAD-ESI-MS results of the extract showed a high level of phenolic compound, alkyl-resorcinol. Results indicated that silver oakleaf extract has termiticidal properties and could be of value in the development of botanical insecticides for termite control in wood products.

6.1. Introduction

Grevillea robusta A. Cunn. (Silver oak) is a member of the plant family Proteaceae and is native to Australia. It has spread invasively upon introduction in parts of Asia, Africa, Southern Europe, and the United States. It is a deciduous tree that grows up to 15-30 m tall and is used in landscaping and agroforestry applications in South Asia and East Africa to conserve soil (Harwood, 1992). The leaves and roots of the silver oak contain allelopathic compounds that inhibit the growth of other

plants in the surrounding soil (Smith *et al*., 1998). Its timber is of medium strength and is used for furniture, flooring, paneling, packing cases, window frames for aluminum-clad windows, musical instruments, pencils, and baskets (Skolmen, 1974). In Kenya and Sri Lanka, it is used as perimeter shade species in tea plantations (Baggio *et al*., 1997).

In previous studies, *G. robusta* leaf extracts have exhibited toxicity against the stored grain pests, *Callosbruchus chinesis* L. and *Sitophilus oryzae* (Waqas *et al*., 2011; Bhuvaneswari *et al*., 2014). Other studies showed antioxidant, antibacterial, antifungal, larvicidal, and anti-leishmanial properties associated with *G. robusta* bark extracts (Takahashi *et al*., 2004, Samarth and Krishna, 2007Cock, 2008; Cock and Ruebhart, 2008; Udayaprakash *et al*., 2014; Ullah *et al*., 2014).
Termites are considered major economic pests in some regions causing tremendous loss to wood structures as well as crops and tree plantations in tropical and subtropical areas. *Heterotermes indicola* Wasmann (Blattodea; Rhinotermitidae) occurs predominantly in tropical and subtropical regions of Pakistan, India, Afghanistan, and China (Maiti, 2006). Besides, infesting wooden structures, *H. indicola* can damage other cellulosic materials. *H. indicola* colonies attack wood in service where their colonies are cryptically located. The termites build mud tubes to move from the soil towards the wood that is in contact or above the soil. This process results in an increase in moisture transferred from soil to wood and thus in greater termite attack ¹⁷¹(Manzoor and Mir, 2010).

Global termite damage has been estimated at the US $ 22-40 billion annually [1–3]. In Southeast Asia, damage attributed to termites is estimated at approximately US $ 400 million/year [4]. *Coptotermes gestroi* Wasmann and *C. formosanus* Shiraki subterranean termites worldwide cause 90% of total economic loss and 70% of constrSuction damage [172]. Although chemical insecticides can effectively reduce termite attack, reliance on synthetic pesticides has produced concerns regarding human exposure and environmental pollution [173]. Synthetic insecticides, especially persistent organochlorines can lose their effectiveness on target pests as the pests have been shown to develop resistance to these insecticides [54,174]. In developing countries, structural timber is generally treated with commonly used wood preservative chemicals such as chromate copper arsenate (CCA) (Barnes *et al*., 2001; Bull, 2001). One of these compounds, CCA, has been banned for residential use in the United States.

Thus, some wood protection chemicals such as organochlorine, phosphorus, carbamates, and synthetic pyrethroids are no longer acceptable for certain uses due to health concerns e.g.,

metabolic and central nervous system disorders, hyperglycemia, and oxidative stress where, there is the potential for increased human exposure (Ward *et al*., 2009; Karam-Mohajeri and Abdollahi, 2010; Tascioglu *et al*., 2012). There has been increased interest in naturally occurring botanical compounds for use as insecticides and wood preservatives (Fatima and Morello, 2015; Gonzalez-Laredo *et al*., 2015; Hassan *et al*., 2017). Several studies have examined the extracts of *Tectona grandis* L. (Teak), *Azadirachta indica* A. Juss. (Neem), *Eucalyptus camaldulensis* Dehnh (River red gum), *Pinus roxburghii* Sarg. (Chir pine), *Dalbergia sissoo* Roxb. (Indian rosewood), *Chamaecyparis nootkatensis* D. Don (Alaskan yellow cedar), *Thuja plicata* D. Don (Western red cedar), *Sequoia sempervirens* D. Don (Red wood), *Juniperus occidentalis* (Western juniper), *Morus alba* L. (White mulberry), *Targetes erecta* L. (Marigold), *Jatropha curacus* L. and neotropical *Solanum* sp. as possible alternative biocides (Carter *et al*., 1983; Grace and Yamamoto, 1994; Maistrello *et al*., 2003; Acda, 2009; Kirker *et al*., 2013; Hassan *et al*., 2017).

Botanical biocides occur as naturally sequestered compounds in leaves, bark, and heartwood of plants and trees, where they often confer resistance to xylophagous insects (Adedeji *et al*., 2017). These bioactive compounds can induce contact repellency and reduce feeding in termites. Hassan *et al*. (2017) reported squalene, 2-methyl-9, 10-anthracenedione, and 1-methyl-3, 4-dihydroisoquinoline as termiticidal agents isolated from Teak heartwood (*Tectona grandis* L.) extract. In a similar study, Hassan *et al*. (2018) found a high level of resorcinol in white mulberry that was toxic to *Reticulitermes flavipes* (Kollar). Rasib *et al*. (2017) showed that coniferyl alcohol from *Saraca asoca* Roxb. (Ashoka) and carboxylic acid from *Magnolia grandiflora* L. (Southern Magnolia) were feeding deterrents to *Odontotermes obesus*. The compounds chamaecynone from *Chamaecyparis pisfera* (Swara cypress), limonoids from *Phellodendron amurense* (Amur cork tree), Azidiractin from *Azidirachta indica* (Neem), loganin from *Guettarda speciosa* (Zebrawood), 7-methyljuglone from *Diospyros virginiana* (Eastern persimmon), ferruginol and nezukol isolated from *Taxodium distichum* (Bald cypress) were found toxic to termites (Saeki *et al*., 1973; Carter *et al*., 1978; Yaga and Kinjo, 1985; Scheffrahn *et al*., 1988; Kawaguchi *et al*., 1989; Ishida *et al*., 1992). Different solvents and aqueous extractives of *Eucalyptus camaldulensis* Dehnh, *Dalbergia sissoo* Roxb, *Melia azedarach* Linn, *Pinus roxburghii* Sarag, *Morus alba* L., *Diospyros sylvatica* Roxb, and *Azadirachata indica* Juss. have been exhibited the antisemitic activities (Qureshi *et al*., 2015; Hassan *et al*., 2016; Mankowski *et al*., 2016; Afzal *et al*., 2017).

It is estimated that 40-84% of wood digestion in lower termites is attributable due to symbiotic phagocytic and cellulolytic actions as these organisms release different cellobiohydrolases [27]. *Heterotermes indicola* also relies on symbiotic hindgut protozoa for complete hydrolysis of ingested cellulosic materials (Maistrello *et al*., 2003; Duarte *et al*., 2016; Hassan *et al*., 2017). Increasing interest in studying lignocellulose degradation by termites and their endosymbionts has resulted in metagenomic and transcriptomic studies probing the effects of varying diet on these symbionts (Scharf, 2015). Other studies suggest protozoacidal bioactive ingredients present in plants in addition to low and high carbon sources can induce changes in protozoan composition and affect the termites themselves (Breznak, 1982; Mannesmann, 1972a; Adams, 2004; Tanaka *et al*., 2006).

The current investigation was carried out to examine the biocidal effects of silver oak leaf extract on tunneling activity, mortality, consumption rates, and protozoan population in *Heterotermes indicola* after the cytotoxicity screening test using brine shrimp letality assay. In addition, the repellency and preservative effects of transferring the *G. robusta* extract to non-durable wood was determined. The crude extract was analyzed by quantitative tests, and High-Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry (MS) to identify the bioactive components in terms of wood preservation.

6.2. Materials and Methods

6.2.1. Termite collection

Heterotermes indicola was collected from the infested basements in the Biological Science Department at Quaid-i-Azam University, Islamabad, Pakistan. Baits using plastic bottles containing wet toilet tissue rolls and molasses were used to attract and collect termites. The bottles were buried to half-length in a dark storeroom followed by regular inspection every15 days. After each inspection, baits were brought to the laboratory where healthy and active workers of *H. indicola* were separated in petri dishes and kept at 28 ± 1°C and RH 70% using damp cardboard until needed for testing.

6.2.2. Plant materials and extract preparation

Leaves of *Ponogamia pinnata, Eucalyptus camaldulensis, Grevillea robusta* and *Mentha piperita* were collected from forests at Margalla Hills (33.7439°N, 73.0228°E), Islamabad Capital of

Pakistan in August 2017. Leaves were dried outside in shaded conditions for 2 weeks at temperatures ranging from 27-37°C. After this period the leaves were powdered mechanically using an electric blender (Daigger Scientific®, USA). The dried powder (15-g) was wrapped in filter paper and extracted using 300 mL of ethanol solvent in a Soxhlet apparatus (Shanghai Heqi®, China) according to the protocol reported by the American Society for Testing Materials (ASTM-D6405-99, 2014). The crude extract was obtained by evaporating the solvent aliquot with a rotary evaporator (R-300, Rotavapor®, Germany). The resultant aliquots were dried and weighed to calculate the yield/gram (Ordonez *et al*., 2006). A stock solution of 100 mg/mL was prepared by re-dissolving the dried residue in ethanol based on the weight of the residue. Five concentrations (1, 5, 10, 15, and 20 mg/mL) were prepared by using the formula:

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

Where C_1: stock solution concentration; C_2: required concentration; V_2: required volume and V_1: volume to be removed.

Cytotoxicity assay

6.2.3. Brine Shrimp lethality assay (BSLA)

Brine shrimp lethality assay is commonly used to determine the cytotoxicity of bioactive constituents from the natural sources. Current experiment was conducted according to the method described by McLaughlin *et al*., (1991) with slight modification. A glass Petri dish containing sea water (4 g salt/L) was taken, added 75 mg of brine shrimp eggs and allowed to hatch eggs at room temperature (25±1°C) for 24 h under constant conditions of illumination and aeration to ensure the maturity and the survival of shrimp nauplii. For the screening of the toxicity of EtOH plant extractives (*Ponogamia pinnata, Eucalyptus camaldulensis, Grevillea robusta,* and *Mentha piperita*) stock solutions were prepared and transferred to clean vials with selected concentration of tested extractives (1, 5, 10, 15, and 20 mg/mL), assay was carried out in triplicate with the help of pipet 25 nauplii shrimps were put in each vial and final volume up to 10 ml by using sea water. Etoposide was used as positive control while shrimps nauplii in sea water without plant exact were considered as negative control. The vials were incubated at 25±1°C for 24 hours and number of dead larvae were counted using magnifying lens. Percentage mortality was determined as following formula;

$$\% \, Mortality = \frac{Number \, of \, Dead \, Nauplii}{Total \, Number \, of \, Nauplii} \times 100$$

6.2.4. Sand barrier bioassay

A transparent plastic tray (30×15×3.5 cm^3 W×L×H) was used as a tunneling apparatus to evaluate silver oak extract on the tunneling ability of *H. indicola* (Figure 6.1) (Acda, 2009). Each bioassay unit consisted of 6×3 W×L chambers (5×5×3.5 cm^3 W×L×H of each chamber), with small openings (2×0.5 cm^2) at the bottom of each of two walls connecting chambers lengthwise. Middle chambers were treated with different treatments (1, 5, 10, 15, and 20 mg/mL) of silver oak leaf extract and the control was treated with ethanol for 14 days. One hundred workers and 25 soldiers (5:1) of *H. indicola* were initially placed in the termite chamber of each treatment since soldiers' exhibit arresting activity to workers and keep fungistatic conditions (Mitaka *et al*., 2017).

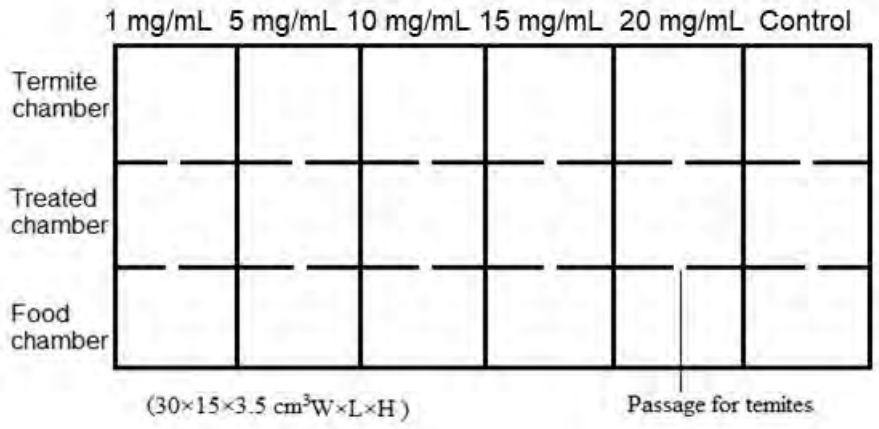


Figure 6.1. Tunneling apparatus to evaluate the toxic potential of silver oakleaf extract against *H. indicola*.

Autoclaved #4 fine blasting sand (50 g) was moistened with 3 mL of distilled H_2O and placed in each chamber. Every 48 hours, 100 µL of distilled water was pipetted into each chamber to maintain moisture [177]. In the food chamber, molasses (sugarcane) was used as the food source. Test termites had to tunnel through the sand in the middle chamber (treated chamber) to reach the food chamber.

6.2.5. Filter paper bioassay

The toxic potential of silver oak leaf extract against *H. indicola* was assessed by conducting a no-choice assay using filter paper (Whatman No. 1, 9.0 cm diameter) as a substrate (Cheng *et al*., 2007; Elango *et al*., 2012). The filter paper was pre-weighed and treated with 1.0 mL of each

concentration of silver oak leaf extract, air-dried at ambient temperature, and kept in Petri dishes (3×3 inches, China). Three replicates were employed for each concentration as well as control paper treated with ethanol only. Fifty workers and 10 soldiers were released in each replicate. Distilled H_2O (0.5 mL) was periodically added at the bottom edge of each petri dish to maintain humidity. The Petri dishes were kept in the dark at 28 ± 1°C and 70 ± 5% RH. Percent mortality was calculated for each treatment after 2 weeks.

$$Mortality = ODP \div TP \times 100$$

Where: ODP = observed the dead population of workers; TP = total population of workers

After the 2 weeks, the filter paper was cleaned and re-weighed after oven drying at 60°C for 12 h. The percent weight loss for filter paper exposed to termites was calculated using the formula [126].

$$\%\, Weight\ loss = \frac{W_b - W_a}{W_a} \times 100$$

Where: W_b= weight of filter paper before experiment; Wa= weight of filter paper after the experiment. After equilibrating the filter paper using a vacuum desiccator Image J software was used to calculate the area of filter paper consumed by termites according to Hassan *et al*., (2018). The concentration of extract on per unit area of filter paper was determined using the following formula (Rech-Cainelli *et al*., 2015);

$$Concentration\ per\ unit\ area = C\,\frac{(mg/mL)}{\pi r^2}$$

Where the values for filter bioassay were C= 1, 5, 10, 15 and 20 mg/mL/3.14159× $(4.5)^2$.

6.2.6. Protozoa counts

The effect of extractives on hindgut protozoa was determined according to the method described by Hassan *et al*., 2017. The termite workers were fed on filter paper treated with different extract concentrations and control with ethanol as described in the filter paper bioassay. A starvation control was also established by depriving the food source (filter paper) of termites. After 14 days, five termites from each treatment were collected and removed their hindguts using sterilized needle and forceps. The contents of the five hindguts were pooled for one treatment as a single sample

against each treatment of extract and homogenized in 250 µL of 0.2% saline solution using sterilized micro-pestle.

Twenty-five microliters of the resulting gut contents were loaded onto a Neubauer hemocytometer (Bright-Line™ Sigma, USA) and protozoa numbers were calculated by determining the mean numbers of protozoa in four squares of each chamber under a digital biological trinocular microscope (Optika® B-500, Ponteranica, Italia). Counts were made for all flagellate species and the number of protozoa was calculated according to Lewis and Forschler (2014);

$$X_{=} \frac{\left(No.\ of\ cell\ counted \times Volume\ of\ saline \frac{solution}{sample}\right)}{\left(Volume\ of\ hemocytometer \times No. \frac{termites}{sample}\right)}$$

The reduction in the protozoa population was determined by comparing the resulting population with the protozoa population in the control treatments.

6.2.7. Repellency and antifeedant bioassays

Repellent effects of silver oak leaf extract against *H. indicola* were determined according to the methodology used by Hassan *et al*. (2018). A filter paper 9.0 cm in diameter was cut into two halves, weighed individually, and then one half treated with 1.0 mL of each treatment of extract and the second half considered a control treated with ethanol only. After air-drying under a fume hood, both halves were rejoined by adhesive tape and placed in the same 3.5×3.5 diameter petri dish. Fifty termite workers and 10 soldiers were released into each petri dish and the number of termites present on each half of the filter paper was calculated after different time intervals (2, 4, 6, 8, and 12 h). The repellency (%) was calculated using the following formula (Kadir *et al*., 2014).

$$Repellency\ (\%)_{=} 100 \times \frac{(N_C - N_T)}{(N_C + N_T)}$$

Where N_C = number of termites present on the control area; N_T = number of termites present on a treated area of the filter paper.

Antifeedant activities of extracts were estimated by determining the antifeedant indices based on the weight loss of the control and treated filter paper. The following formula was used to calculate the antifeedant indices (Dungani *et al*., 2012; Hassan *et al*., 2018);

$$Antifeedancy\ indices\ (A)_{=}\ 100 \times \frac{(CC - TT)}{(CC + TT)}$$

Where CC= weight losses of the control; TT= weight losses of treated filter paper pre. According to Hassan *et al*., 2018, the antifeedant potential of extracts classified into four groups based on their A value as shown in Table 5.1.

6.2.8. Toxicity bioassay using non-durable commercial wood species treated with leaf extract

The no-choice AWPA E1-17 (AWPA, 2017) laboratory termite method was performed to determine the toxic potential of silver oakleaf extract against *H. indicola*. Sapwood blocks of cottonwood and Chir Pine (4 × 4 × 2 cm) were conditioned at 33°C and 65±5 RH and weighed (Hassan *et al*., 2018). Blocks were treated with different concentrations (1, 5, 10, 15, 20 mg/mL) of extract and using a vacuum pressure process based on the permeability of the non-durable wood. In the vacuum pressure process, three blocks of each non-durable wood were placed in a beaker with 400 mL in a sealed chamber of each treatment solution and subjected to a vacuum pressure of 80 KPa for 30 min to remove air from the cell pores of the wood and 250 KPa of pressure was applied for 1 h to impregnate the solution into the wood. All pressure treated woodblocks were dried with blotting paper, reweighed, and placed in a petri dish. For controls, a subset of blocks was treated with solvent (ethanol) or water. Fifty termite workers and 10 soldiers were released in each treatment.

The retention level of each treatment absorbed by wooden blocks was determined as followed by Sarker (2006):

$$Retention\ level\ (g/cm^3)_{=}\ 10 \times \frac{\mathrm{GC}}{V}$$

Where, G= (T_2-T_1) = grams of treating solution absorbed by block (weight of block after treatment – weight before treatment), C= gram of extract in 100 mL treating solution, V= volume of the block in a cubic centimeter.

Mortality, weight loss, and a visual consumption rate were determined after 14 d of exposure to the termites according to the AWPA E1-17 rating descriptions (AWPA, 2017).

6.2.9. DPPH radical scavenging effect (%)

The crude extract of silver oak may contain potential radical scavengers, that can enhance the toxic effect on termites via synergy with other compounds (Hassan *et al*., 2018). To evaluate DPPH (1,1-diphenyl-2-picryl-hydrazyl) free radical scavenging ability of the extract, the protocol described by Brand-Williams (1995) was followed. 0.25 g of DPPH was dissolved in 90 mL of methanol and the optical density (0.900 ± 0.04) was adjusted at 517 nm by diluting the solution with methanol. An aliquot of 100 µL of each concentration (1, 5, 10, 15, and 20 mg/mL) of the extract was dissolved in 900 µL of DPPH solution in a test tube and incubated at 37°C for 15 min after which it was shaken vigorously for 30 seconds. The optical density of samples and the standard (ascorbic acid) was recorded at 517 nm using a spectrophotometer (BioRad, USA).

$$\text{DPPH radical scavenging effect (\%)} = 100 \times \frac{(\text{OD Control} - \text{OD Sample})}{(\text{OD Control})}$$

Where OD control is the optical density of DPPH radical + methanol and the OD sample is the optical density of DPPH + extract concentration.

6.2.10. HPLC-DAD-ESI-MS analysis

Crude silver oak leaves extract was chromatographed using Liquid Chromatography with a 1200 series HPLC system equipped with an autosampler (GI329A), quaternary pump (GI311A), degasser (GI322A), thermos column compartment (GI316A) and coupled with Chem Station 4.0 V (Agilent Technologies, Santa Clara, USA). The LC separation of bioactive constituents was recovered by using a reverse-phase symmetry HPLC column (4.6 mm × 150, Water®, Milford, MA, USA) packed with 5 µm spherical silica C-18. Crude extract samples were prepared with a hydrodistillation process under 200°C and filtered through 0.2 µm syringe filters (Minisart® NML, Sartorius, Goettingen, Germany). The mobile phase composition and conditions were followed as by Muazzam and Farman, (2018); Solvent A (dH_2O containing 0.1% formic acid), Solvent B (Acetonitrile) with a linear gradient change from 10% at 0-15 min, 10-40% at 15-40 min, 40-80% at 40-50 min and 10% at 60 min in Solvent B and total run time was 60 min. The column

temperature was maintained at 35°C and the injection volume was 5 µL at the flow of 0.5 mL/min. Diode-Array Detector (DAD) spectra were obtained at 254, 320, and 380 nm.

Mass spectrometry was performed by using Ion Trap mass detector MS 6310 (Agilent Technologies) equipped with an Electro-Spray Ionization system as an ion source. The following operating parameters were used; capillary voltage-3500 V, capillary temperature 325°C, sheath gas, nitrogen generator (N-118LA), Edward EIM18 pump (A22304199), and ionization mode was positive. Detection was performed in splitless scan mode (0-1000 *amu*).

6.2.11. Statistical analysis

For each bioassay, a One-way ANOVA was applied to determine the significance of the effects of different concentrations on the response (termites' mortality), Tukey' analysis was also applied to evaluate significance among groups. LC_{50} was determined by applying probit analysis for each extract against termites and their gut protozoa using the software SPSS 19 version.

6.3. Results

6.3.1. Screening for cytotoxic plant extract (BSLA test)

The results of brine shrimp lethality assay of ethanolic extractives of the four plants for the screening of most cytotoxic plant extract are shown in Table 6.1. A dose-dependent trend was observed with the maximum percentage mortality of brine shrimps nauplii at the highest concentration 20 mg/mL. Experimental results indicating that leaf extract of *Grevillea robusta* induced maximum toxicity and caused 80% mortality of shrimp larvae after 24 hours. Whereas, other three plant extracts (*Ponogamia pinnata*, *Eucalyptus camaldulensis*, *Mentha piperita*) did not show significant toxicity and they were unable to induce > 60% mortality, even at the highest concentration (20 mg/mL). It reveals that *G. robusta* has potential to induce significant cytotoxicity in the living cells and lead to death. By keeping in view these finding, *G. robusta* crude leaf extract was selected as potential natural agent in further termite control bioassays.

Table 6.1. The screening of plant extractives toxicity using brine shrimp lethality assay (n = 3) indicated number of larvae survival and % mortality at different concentration (1, 5, 10, 15, and 20 mg/mL).

Plant Extract	Concentrations (mg/mL)	No. of Shrimps	No of Survival	% Mortality
Ponogamia pinnata	1	50	50	0
	5	50	44	12
	10	50	36	28
	15	50	29	42
	20	50	20	60
Eucalyptus camaldulensis	1	50	50	0
	5	50	46	8
	10	50	38	24
	15	50	30	40
	20	50	25	50
Grevillea robusta	1	50	45	10
	5	50	40	20
	10	50	28	44
	15	50	22	56
	20	50	10	80
Mentha piperita	1	50	50	0
	5	50	44	12
	10	50	40	20
	15	50	35	30
	20	50	28	44

6.3.2. Sand barrier bioassay

The *G. robusta* leaf extract exhibited a dose-dependent effect on the tunneling activity of *H. indicola*. All treatments showed significantly different effects on tunneling activity compared to the controls ($F = 38.01$; $P < 0.05$; $df = 5$). Untreated and treated sand barriers with 1, 5, and 10 mg/mL silver oak extract were successfully breached by *H. indicola* up to 5.00, 4.50, 3.80, and 2.50 cm respectively after 48 to 72 h of exposure (Figure 6.2). However, sand treated with high concentrations (15 and 20 mg/mL) of crude extract of silver oak restricted the tunneling activity of *H. indicola* and termites crawled over the treated chamber to get access for food instead of breaching the sand barrier.

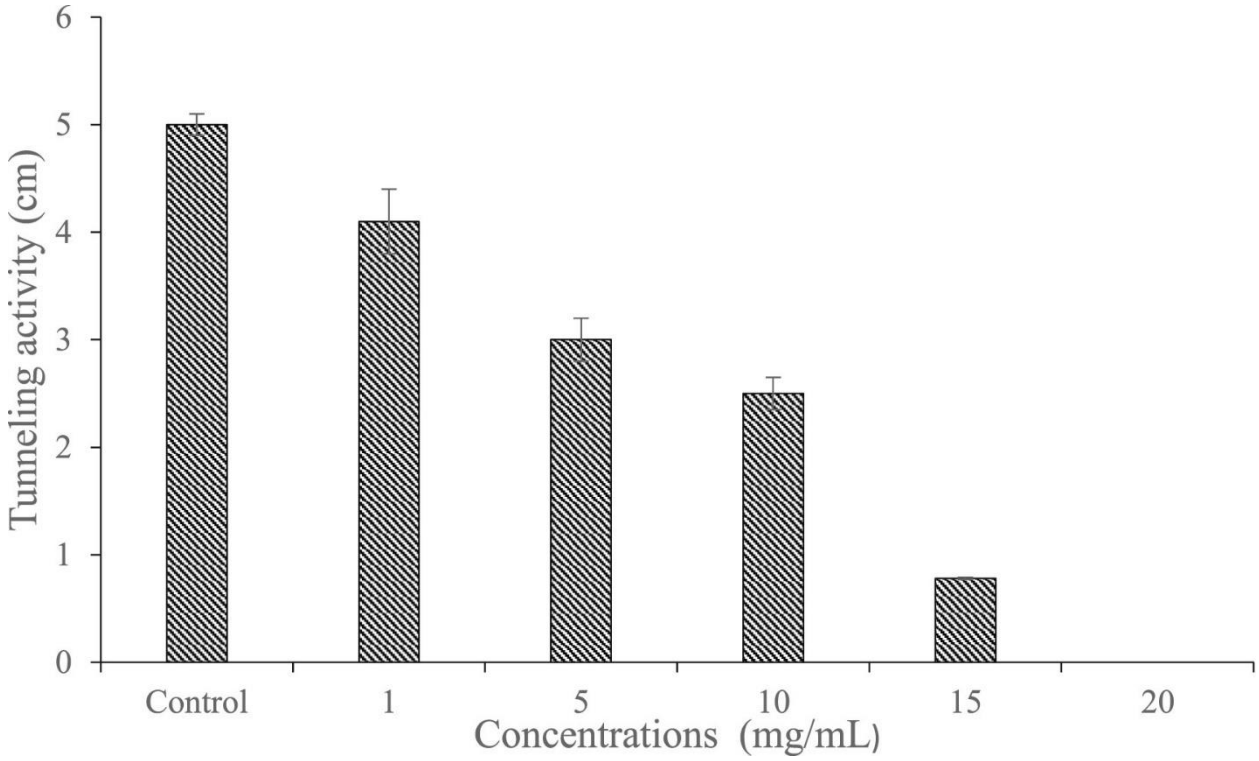


Figure 6.2. Tunneling activity of *H. indicola* after 14-days exposure to sand treated with silver oak leaf extract.

6.3.3. Filter paper bioassay

In no-choice feeding tests, silver oak leaf extracts adversely affected termite behavior and induced significant mortality. All treatments were found to show significant mortality compared to the control treatments (F (df) = 168.20 (5); $P < 0.05$). The silver oak extract exhibited termiticidal and antiprotozoal effects with an LC_{50} of 5.47 and 10.80 mg/mL. Rapid termite mortality (> 50%) was recorded in all filter paper replicates treated with 5,

10, 15, and 20 mg/mL of extract after 14 days of exposure (Figure 6.3), except in 1 mg/mL treatment.

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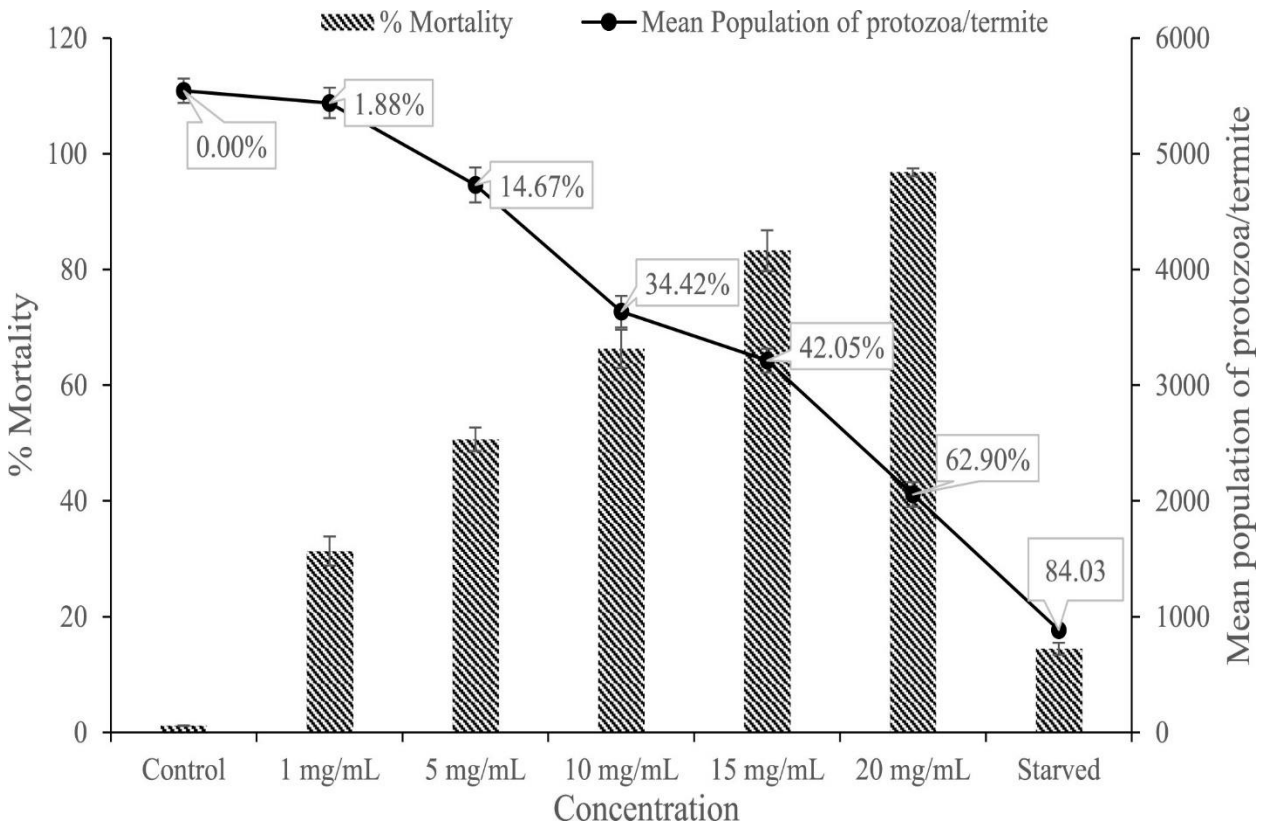

Figure 6.3. Mean termite mortality (%) and number of protozoa per termite after a 14-day filter paper bioassay and the percentages in boxes indicating the percentage loss of protozoa.

Termites consumed a significantly lower percentage area of filter paper treated with the high concentration of extract compared to the controls All treated groups were significantly different compared to control treatments (F (df) = 198.44 (5); $P < 0.05$). Results indicated an inverse association between filter paper percentage area consumption and termite mortality, which was statistically significant (r = 0.997; $P < 0.001$). A corresponding trend was recorded between filter paper weight loss and termite mortality. Minimum filter paper weight loss (11.60%) was observed at the maximum concentration of silver oak extract where termite mortality was more than 50% (Figure 6.4). The weight loss (%) of filter paper and percentage mortality in termites were positively correlated (r = 0.906; P = 0.004) and the filter paper consumption rate of *H. indicola* at high treatments was significantly different from control treatments (F (df) = 159.46 (5); $P < 0.05$).

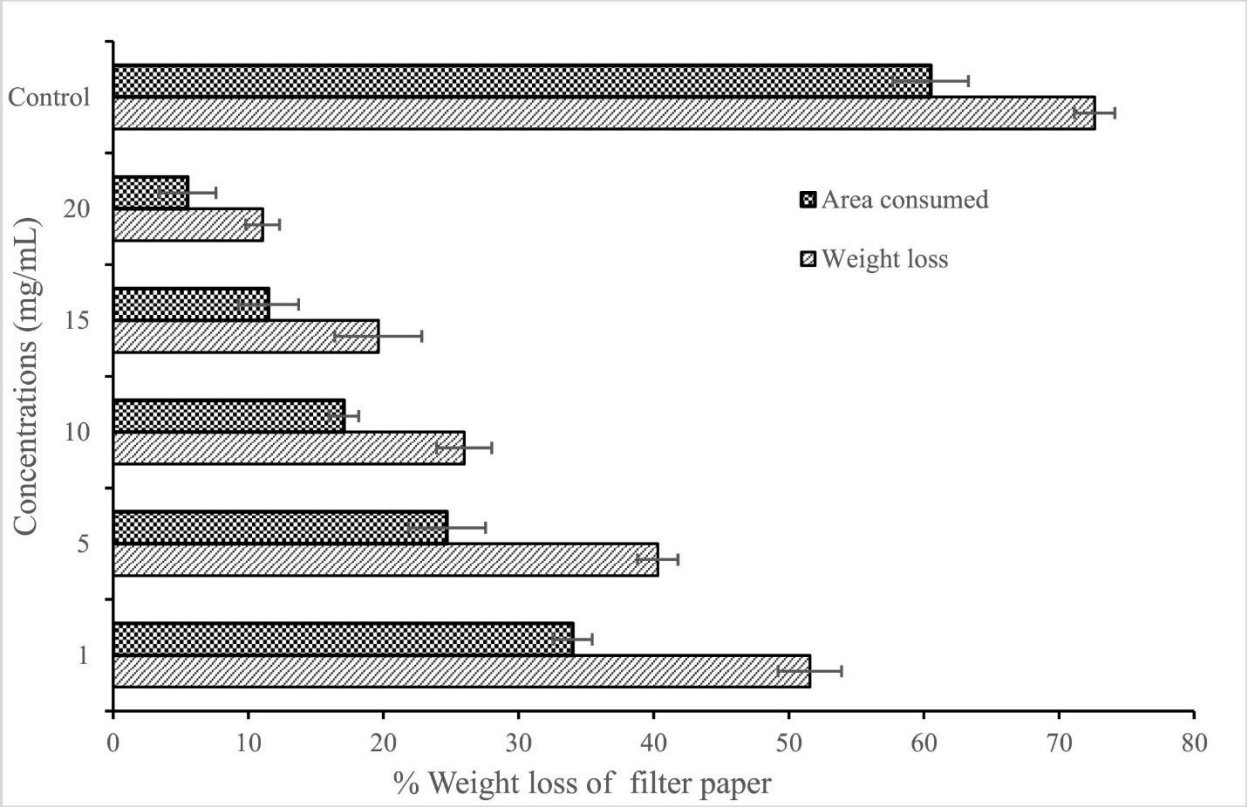

Figure 6.4. Weight loss and area loss of filter paper consumed (%±SE) by *H. indicola*.

6.3.4. Antiprotozoal effect

After 14 days of no-choice feeding on treated filter paper, the dose-dependent in-vivo effect of silver oak leaf extract was observed in the number of gut protozoa in *H. indicola* workers (Figure 6.3). The reduced number of gut protozoa was significantly correlated to termite mortality (r = 0.966; P = 0.002) suggesting that the reduction in gut protozoa was causing high termite mortality. The maximum percentage loss of protozoa (62.90%) was recorded after exposure to the highest concentration (20 mg/mL) of silver oak leaf extract, where the percentage mortality was > 90% (Figure 6.3).

Protozoa numbers at each concentration were significantly different from each other (F (df) = 96389.26 (6); P < 0.05) as well as from the control and starvation groups. In the starved treatment, the maximum reduction in gut protozoa was 84.03%, but the mortality in *H. indicola* was less than 20%.

6.3.5. Repellent and antifeedant activities

Repellency and antifeedant indices for silver oak leaf extract are shown in Table 6.1. The repellent activity on *H. indicola* was dose-dependent, shown from minimum to strong activity for the concentration series. A significant difference was observed in termite numbers on filter paper treated with extract compared to the solvent (control) indicating minimal % repellency in the control unit (71.40 vs 3.40%). The repellent activities on the termites at 1 and 5 mg/mL were not significantly different, however all other treatments were significantly different compared to the 1 mg/mL treatment (F (df) = 43.59 (5); $P < 0.05$). The antifeedant effect of extract attained from silver oak ranged from12.80 to 66.60% from the lowest to the highest concentrations. Antifeedant activities of all treatments were significantly different from each other and control units (F (df) = 595.5 (5); $P < 0.05$).

Table 6.1. Antifeedant and repellent effects (±SE) of silver oak leaf extract on termites at different concentrations.

Concentrations	Repellency (%)	Antifeedant indices (A)	Activity level [a]
Control (ethanol)	3.40 ± 0.34^{d}	NA^{f}	Minimum activity
1 mg/mL	15.60 ± 1.26^{de}	12.80 ± 1.92^{e}	Minimum activity
5 mg/mL	20.80 ± 1.45^{d}	18.40 ± 1.14^{d}	Minimum activity
10 mg/mL	27.60 ± 1.84^{c}	31.80 ± 1.64^{c}	Moderate activity
15 mg/mL	40.40 ± 1.13^{b}	43.60 ± 1.05^{b}	Moderate activity
20 mg/mL	71.40 ± 1.30^{a}	66.60 ± 2.07^{a}	Strong activity

[a] Mini = $0 \leq A < 25$; Moderate = $25 \leq A < 50$; Strong = $50 \leq A < 75$; Very strong = $75 \leq A < 100$ (Hassan *et al*. 2018). Tukey, HSD tests indicating the mean followed by different letters within column indicated the significantly different.

6.3.6. Toxicity Bioassay using Non-durable commercial woods treated with the extract

Results of mean percent termite mortality and weight loss of the extract-treated and non-treated pinewood or cottonwood exposed to *H. indicola* are shown in Table 6.2. The chir pine controls treated with water or solvent (ethanol) lost 40.09 and 37.28% mass, respectively. Termites consumed 53.66 and 47.63% cottonwood treated with water or solvent, respectively. Tukey's test indicated no significant difference between water or ethanol control treatments. An inverse relationship between weight loss % and extract concentration was noted. We observed minimum weight losses of 3.89 and 6.83% for chir pine and cottonwood, respectively when these woodblocks species were treated with the highest concentration of extract.

Table 6.2. Mean termite mortality (%±SE), wood mass loss (%±SE and g/termite/day), and visual wood damage rating of non-durable commercial wood species treated with silver oak leaf extract, exposed to *Heterotermes indicola* (0 = failure; 10 = sound).

	Eastern Cottonwood			Chir pine		
Concentrations	Mortality (%)	Mass loss % (g/termite/day)	Rating	Mortality (%)	Mass loss % (g/termite/day)	Rating*
Water	8.70 ± 0.44^{f}	53.66±1.06^{a} (0.021)	0	11.28±0.48^{e}	40.09±1.30^{a} (0.023)	0
Solvent	9.12 ± 0.47^{f}	47.63±1.25^{a} (0.018)	4	13.45±1.17^{e}	37.28±1.06^{a} (0.019)	0
1 mg/mL	31.14 ± 1.50^{e}	31.56±2.56^{b} (0.016)	7.0	35.81±1.35^{d}	24.64±2.45^{b} (0.013)	7.5
5 mg/mL	46.57 ± 2.56^{d}	21.00±1.20^{c} (0.009)	7.5	56.90±3.80^{c}	19.00±1.40^{b} (0.007)	8.0
10 mg/mL	60.43 ± 0.84^{c}	12.80±1.52^{d} (0.005)	9.0	85.43±1.18^{b}	11.31±1.26^{c} (0.003)	8.0
15 mg/mL	85.55 ± 0.82^{b}	8.16±0.54^{e} (0.003)	9.5	100.00±0.00^{a}	06.36±1.79^{d} (0.002)	9.5
20 mg/mL	100 ± 0.00^{a}	6.83±0.21^{e} (0.002)	9.7	100.00±0.00^{a}	03.89±1.34^{e} (0.001)	9.8
F (df)	456.12 (6)	74.94(6)		1219.0 (6)	78.09 (6)	
P	0.001	0.001		0.000	0.001	

* 0 = No repellency; 2-4 = very severe attack; 4-6 = severe attack; 6-8 = moderate attack; 9-10 = slight attack or trace (Hassan *et al*., 2018). Tukey, HSD tests indicating the mean followed by different letters within column indicated the significantly different.

The weight loss (%) of chir pine treated with silver oak extract was significantly less as compared to control units (F *(df)* = 78.09 (6); $P < 0.05$). A similar trend was noticed for cottonwood (F (*df*) = 74.94 (6); $P < 0.05$). The AWPA E1-17 visual ratings results indicated minimum feeding activity by *H. indicola* on treated chir pine and cottonwood with ratings of 9.8 and 9.7, respectively at the 20 mg/mL.

6.3.7. DPPH radical scavenging effect %

In this experiment, the scavenging effect of silver oak leaf extract on DPPH radicals varied considerably for all extract concentrations. Our results showed that the scavenging effect of the silver oak extract on DPPH radicals increased with increasing extract concentration (Figure 6.5). The IC_{50} is the inhibition concentration at which 50% free radicals of DPPH are foraged by different extract concentrations. In our results, the IC_{50} of the silver oak extract was significantly lower (18.50 µg/mL) than the IC_{50} of the positive control standard, ascorbic acid (63.64 µg/mL).

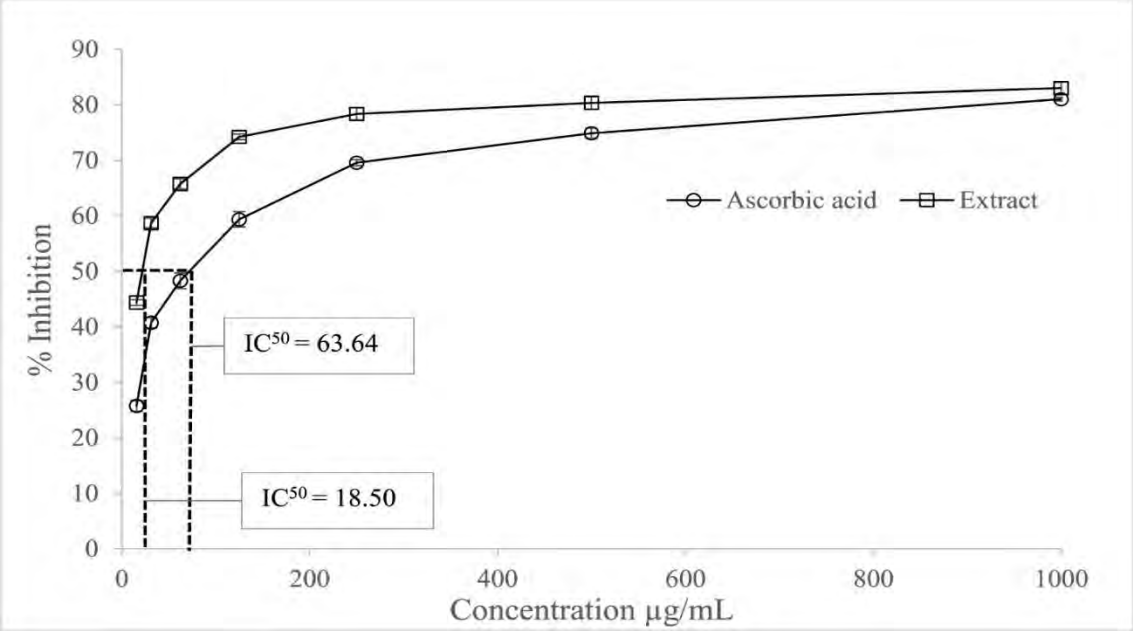

Figure 6. 5. Scavenging effect (%) for different concentrations of silver oak leaf extract on DPPH free radicals along with standard ascorbic acid with IC_{50}.

The DPPH scavenging activity was augmented with increasing concentrations of silver oakleaf extract. The maximum and minimum scavenging activity at the highest and lowest extract concentrations were 83.04% and 44.37%, respectively. A significant difference (T (*df*) = 3.40 (37); p = 0.002) was observed in scavenging activity at all concentrations (15.6–1000 µg/mL) of extract and ascorbic acid.

6.3.8. HPLC-DAD-ESI-MS analysis

Table 6.3 and Figure 6.6 indicate the major compounds along with retention time, wavelength, ESI-MS fragmentation ions, and identification of compounds from the extract. The most abundant compounds identified were graviquinone, methyle 5-ethoxy-2-hydroxycinnamate, and grevillol (alky-resorcinol). Grevillol was the most abundant and major component of the silver oakleaf extract.

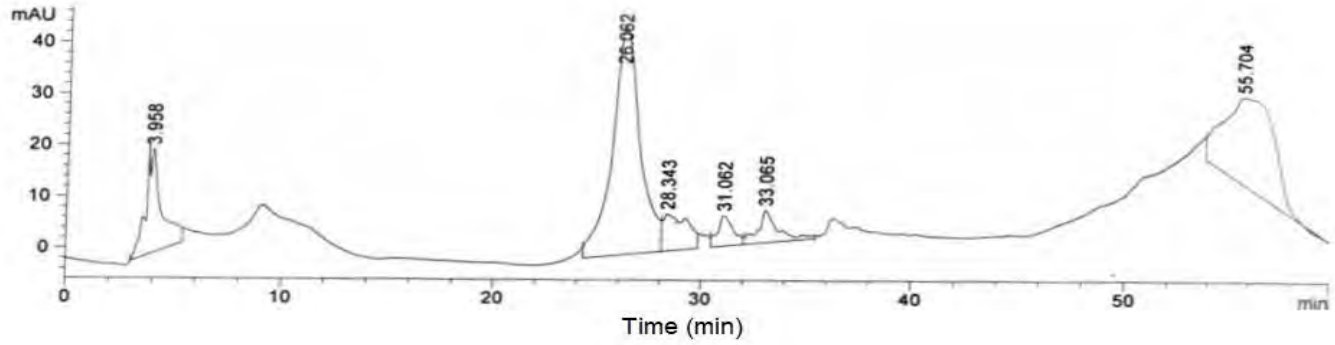

Figure 6.6. The representative HPLC-DAD chromatogram indicating the peaks at different retention time.

Table 6.3. The HPLC-DAD UV absorption data and ESI-MS fragmentation (+ ion mode) of silver oak leaf extract.

No.	Retention time (min)	UV (λ = nm)	ESI-MS	Fragment ion (*m/z*)	Identification
1	3.958	220, 225	915$[M+H]^+$	825, **410**, 360	Cinnamic acid, coumaric acids
2	26.062	260, 360	903$[M+CH_3OH+H]^+$	800, 680, **610**, 430, 300	Alkyl-resorcinols (grevillol, bis-gravillol, robustol, gravibustol)
3	28.343	219, 225, 235	970$[M+CH_3CN+H]^+$	**810**, 710, 560, 450, 280, 230	4-methoxy-5,7-dihydroxyflavones, methyle 5--ethoxy-2-hydroxycinnamate, quercetin, rhamnocitrin
4	31.062	225, 230, 240	920$[2M+H_2O]^+$	830, **790**, 680, 614, 480	Graviquinones
5	33.065	230, 255, 350	900$[M+CH_3OH+H]^+$	820, 710, **625**, 512, 350	*Cis*-*5*-*n*-pentadic-8-enylresorcinol, gravicycles

6.4. Discussion

Extract of silver oak (*Grevillea robusta*) is of interest in wood protection technology because it possesses toxic chemical constituents that may have the capacity to protect non-durable wood, against peculiar wood degradation agents. The crude leaf extracts of silver oak limited tunneling activity in *Heterotermes indicola* and showed other termiticidal properties. The results of our anti-tunneling assay were comparable to Acda, (2009) who reported that the milk termite *Coptotermes gestroi* (Wasmann) of the Philippines was tolerant to a sand barrier treated with 2-5% *Jatropha curcas* L. oil, but 10-20% oil-induced high deterrence in termites during contact with treated sand. In our anti-tunneling assay, *H. indicola* was able to cross a sand barrier treated with 1-5 mg/mL of *G. robusta* extract, however, the higher concentrations of extract tested exhibited toxic properties and significantly repelled termites. The mechanism of repellency and avoidance was involved in reducing the penetration of *H. indicola*. Another explanation for these results is the continuous exposure of termites to high concentrations of the extract could be a definite reason for termite lethargy. The compounds present in *G. robusta*, such as grevillol, graviphane, and other phenols (rutin) may affect *H. indicola*, since phenols are known to hinder foraging activities and normal behavior in insects [179]. Termites accessed the food source by crawling over the sand barrier treated with high concentrations instead of breaching. This crawling along the edge of the treated chamber is also known as the edge effect that is common in termites' behavior and can be noticed in laboratory and field experiments [44,180,181].

The results of the filter paper bioassay in the present study were comparable with Hassan *et al*., (2018) who reported that percent termite mortality and foraging rates were concentration-dependent, as workers of *Reticulitermes flavipes* were exposed to mulberry (*Morus alba*) heartwood extract-treated filter paper. In our feeding tests, a fivefold decrease in the consumption rate of filter paper was observed at the highest treatments compared to control treatments. The silver oak extract also caused significant termite mortality as the extract concentration increased. There was no significant mortality observed in a starvation control unit. Weight loss, termite nibbling, and reduction in surface area of the filter paper treated with a low concentration of silver oak extract indicated the feeding activity of *H. indicola*. Termite exposed to filter paper treated with high concentrations of leaf extract became lethargic and showed abdominal shrinkage during the 14 days of exposure. These observations suggest that a high amount of biocide components was found in higher concentrations of extract that induced delayed toxicity and maximum

mortality as compared to lower concentrations of the extract. Besides, allelochemicals such as terpenoids, flavonoids, quinones, and phenolic components in the leaf extracts might be responsible for digestive toxicity in chewing insects [36,43,182,183]. Yamashita-Higuchi *et al*. (2014) reported six different grevillosides from the leave extract of *G. robusta* with high cytotoxicity and melanogenesis inhibitory activity.

In previous studies, it was reported that flux in protozoa's population might be due to environmental factors such as temperature and humidity (Mannesmann, 1972a). However, in another study, toxic compounds introduced to gut symbionts via host feeding preferences caused the total loss of protozoa in the hindgut leading to increased termite mortality (Mannesmann, 1972b). In our tests, the average population of protozoa in control treatments of *H. indicola* was 5545.0 ± 102.15 that is comparable to the protozoa count results of control groups (3530 ± 62.4) reported by Hassan *et al*. (2017) for *H. indicola*. The protozoa population in the hindgut of *H. indicola* is significantly lower than *Reticulitermes* species such as reported by Lewis and Forschler (2014) for *R. flavipes* (59000), *R. hageni* (21000), and *R. virginicus* (14000). This disparity in the protozoa population in *H. indicola* (Asian) and *Reticulitermes sp*. (Western) indicates that the size of the hindgut, wood-feeding preferences, and ecological habitat may play an important role in the composition of protozoan communities in lower termites. Our results agreed with Jones *et al*., 1983, who reported a reduction in percent weight loss and filter paper area consumption was positively correlated with a reduction in protozoa as compared to control treatments. In our tests, a 62.90% reduction was observed in the protozoa population at the highest concentration of silver oak leaf extract tested with 96.82% termite mortality. However, Lewis and Forschler (2010) reported a 30% reduction in termite gut protozoa after treatments using chitin synthesis inhibitors. In a recent study, Hassan *et al*., (2017) reported 45.7, 39.2, 36.4, and 15% declines in protozoa in *H. indicola* after force-feeding on filter paper treated 10 mg/mL *Dalbergia sissoo* (Roxb), *Tectona grandis* (Linn), *Pinus roxburgii* (Sarg) and *Cedrus deodara* (Roxb) heartwood extracts, respectively. Our protozoacidal results also agreed with Hassan *et al*., (2018) who documented a 55.2% reduction in protozoa number for *R. flavipes* exposed to 10 mg/mL white mulberry extract, where termite mortality was 93%.

The variance among total protozoa population and termite mortality rate may suggest some other physiological phenomenon involved due to the presence of antioxidants in the extract. Since, these antioxidants can interfere with different single electron redox reactions in the hindgut of

termite where cellulose converts into acetate via the shikimate acid pathways (Raje *et al*., 2015; Hassan *et al*., 2017). In the present protozoacidal assays, we observed the highest decline in protozoa in the starvation treatment where termite survival was > 80%, indicating another mode of silver oak extract toxicity (Figure 5.3). Raje *et al*. (2015) reported that turmerone from turmeric extract induced high mortality in termites by attacking the nervous respiratory systems. Cannibalism may also be the factor contributing to termite survival in the starvation controls (no food and no extract exposure) as suggested by Hu *et al*. (2011). In wood-feeding lower termites, wood digestion is not possible without cellulases produced by symbiotic protozoa (Cleveland, 1925). The removal of even a single species of protozoa from the hindgut can cause significant mortality in termite (Mannesmann,1972b). In the present study, the decline in protozoa after continuous no-choice feeding on leaf extract could be an effect of digestive toxicity to the gut microenvironment at the cellular level by toxic constituents present in the silver oak extract. These components induced slow-acting toxicity in protozoa communities that could be efficient in termite control baiting technology since termites return to the colony after foraging with these toxic compounds and transfer them to other members of the colony via trophallaxis (Mauldin et 1981; Hassan *et al*., 2017).

The simple filter paper bioassays can be suitable to screen and explore the insect antifeeding and repellent properties of natural crude extracts in laboratory conditions (Sharma *et al*., 1994). The results of our repellency and antifeedant bioassays are comparable with Hassan *et al*., (2018) who reported that white paper mulberry heartwood extract showed repellent and antifeedant activities against *R. flavipes*. In our experiments, high concentrations (10-20 mg/mL) of silver oak leaf extract also exhibited repellent effects. We saw antifeedant indices like Hassan *et al*., 2017, who reported that teak heartwood extract induced high mortality and antifeedant in *R. flavipes* and *H. indicola*. This may be due to the presence of phenols and quinones that are repellent, antifeedant, and have antioxidant properties (Ates *et al*., 2015; Abbas *et al*., 2013; Rasib *et al*., 2014).

In our experiments, the pressure treatment of non-durable cottonwood and pinewood blocks with silver oak extract showed 100% termite mortality at the highest concentrations (15-20 mg/mL) tested. This mortality confirms the successful penetration of extract into non-durable woods and termiticidal properties of silver oak that interfered with the digestion of cellulosic material and endosymbiotic cellulases in termite gut (Ragon *et al*., 2008; Abe *et al*., 2000). These

results agree with Hassan *et al*., 2018 who observed 100% mortality and minimum foraging activity in *R. flavipes*, when exposed to non-durable wood treated with different concentrations (1-10 mg/mL) of white mulberry heartwood extract for two weeks. Our results suggest that silver oak leaf extract can induce repellent and wood resistant properties in non-durable wood species. Similar results were also observed in previous studies using plant extracts (Hassan *et al*., 2016, 2017 and 2018; Kirker *et al*., 2013; Ahmed *et al*., 2007; Yamaguchi *et al*., 2002; Qureshi *et al*., 2015; Rasib *et al*., 2017).

In previous studies, major phytocompounds in plant extracts were tannins, phenolic, flavonoids, alkaloids, and quinones that were found to possess both termiticidal and antioxidant properties (Torres *et al*., 2003; Fava *et al*., 2006; Little *et al*., 2010; Abbas *et al*., 2013; Qureshi *et al*., 2015). Our DPPH and hydroxyl radical scavenging assay results of the silver oak extract are comparable with Das *et al*., (1984) and Hassan *et al*., (2018), who reported that free radical scavengers act as synergists by interfering with Glutathione S-transferase (GST) arbitrated detoxification phenomenon and could serve as a substitute substrate for the antioxidant enzymes. The authors also reported that antioxidants can disturb the function of antioxidant enzyme systems by serving as competitive and non-competitive inhibitors against GST and esterase substrates. These antioxidants are also lethal to insects causing the decomposition of DNA strand bonding (Appel and Schultz, 1992). Poly and monocyclic phenolic compounds have been characterized by the Silver oak (Ahmed *et al*., 2000). Our HPLC-MS results indicated that silver oakleaf extract contains high levels of resorcinol (83%). These compounds were also reported by the previous studies (Chuang *et al*., 2011; Chuang and Wu, 2007; Yamashita-Higuchi *et al*., 2014). In previous studies, the alkylresorcinols and monocyclic phenolic components such as grevillol, grevirobustol, and robustaside were identified from silver oakleaf extract that has been reported as toxic ingredients to different human cancer cell lines [187,188]. Menz *et al*. (1986) also reported that silver oakleaf extract contains a high amount of 5-tridecylresorcinols that are responsible for contact dermatitis in organisms. In another study, resorcinols have also been involved in the inhibition of enzymes such as tryptophan peroxidase [190]. Recently, 4-n-butylresorcinols were found as strong inhibitors of melanogenesis and enhanced the proteolytic degradation of tyrosinase by competitive binding [191]. It was reported that resorcinols are hydrophilic phenolic compounds and can interact with other cellular digestive enzymes by forming covalent, ionic, and hydrogen bonding in the intestinal tract of insects to affect nutrient digestion (Appel and Schultz, 1992; Sadeghifar *et al*.

2011). Hassan *et al*., (2018) also reported the heartwood durability of white mulberry might be due to the presence of resorcinols in the heartwood that acts synergistically with other compounds to disturb insect gut metabolism. Auto-oxidation of these hydrophilic alkyl resorcinols due to the alkalic environment in the termite gut can interact with gut proteins and form quinones that reduce the protein digestibility (Bhonwong *et al*., 2009). Veliká and Kron, (2013) reported the antioxidant and pro-oxidant activities of resorcinols isolated from plant sources. Likewise, the antioxidants present in silver oakleaf extract can be used as wood preservatives, since they induced the highest mortality rate in wood-feeding termite *H. indicola* and minimum loss of treated cellulosic material.

6.5. Conclusion

The results of this study illustrate that *G. robusta* leaf extract had a deterrent and contact bioactivity in addition to digestive toxicity against wood-feeding termites. The extract showed repellent, antifeedant and termiticidal properties along with significantly reducing hindgut protozoa populations during no-choice bioassays. These are basic findings being used to assess the biological relevance and use of silver oakleaf extract against pest termites. Future investigations will examine the isolation of bioactive compounds such as resorcinol in the extract in hopes of finding useful and renewable substitutes for some currently used chemical biocides.

General Discussion

Subterranean lower termites are the most important wood-feeding social insects that convert the complex lignocellulosic material into simple sugars by digesting complex polymers of plant cell walls. Lower termites possess a dual cellulose digestion system comprising of their endogenous cellulases (secreted from labial glands and midgut epithelium) and endosymbiotic fauna of protists and prokaryotes living in the hindgut of lower termites [8]. Both systems possess novel kinds of cellulases that digest the organic lignocellulosic materials and play a significant role in nutrients cycling of the habitat [108]. Over millions of years, lower termites developed specific adaptations including the efficiency of wood degradation and many other known natural micro-bioreactor systems [192], making them attractive and optimized model for bioprocessing such as industrial biomass conversion for the production of biofuels and many other biochemical commodities. To further elucidate the strategies of endogenous and symbiotic cellulase fractions in lignocellulose decomposition by the two lower termite species belonging to the two different geographic regions, the present study explored the comparative distribution of cellulolytic activities in the gut of termites and their correlation with the cellulase gene expression. This study also explored the comparative saccharification potential of endogenous and symbiotic cellulase systems from the subterranean termites against agriculture biomass. Furthermore, the role of bacterial symbionts from subterranean termites was characterized through isolating, producing, and purifying the cellulases by termite endosymbiotic bacterium. All above-mentioned objectives were correlated with the biomass degradation capabilities and nutritional functions of termites which are important for the sustainability of the natural habitats

However, an economically important problematic aspect of lower subterranean termite is also existing, which is the infestation of lower subterranean termites to wooden structures that damage the wood structures severely and cause biodeterioration of cellulose products. Up to date, termite constitutes approximately 6.1% of total recorded species which are economically important termite species in urban, agriculture, and forest ecosystems [193]. Global termite damage has been estimated at the US $ 22-40 billion annually [1–3]. These losses were conventionally estimated at 11 billion USD in United States, 04 billion USD in Australia, and approximately 35 million USD/year in India (Ghaly, 2011; Rust and Su, 2012; Su, 2002). In Southeast Asia, damage attributed to termites is estimated at approximately US $ 400 million/year [4]. In Japan, Indonesia and Malaysia

have been estimated at 0.8 to 1 billion USD, 6 to 7 million USD, and 10 to 12 million USD per annum respectively (Tsunoda, 2005; Yeoh and Lee, 2007; Nandika, 2014). In subterranean termite showed high wood damage cost of 289 million USD/annum and caused 80% building infestation in Guangdong province [195]. In Pakistan, subterranean causes tremendous loss to wooden structures in urban and rural settings, but no annual cost is determined or estimated yet. *Coptotermes gestroi* Wasmann and *C. formosanus* Shiraki subterranean termites worldwide cause 90% of total economic loss and 70% of construction damage [172].

By keeping in view such huge economic losses, this study also tried to explore innovative methods to constrain the termite infestation using ecofriendly and non-toxic methodologies. The present study used environment-friendly termite control strategies by target the main constituent of the wood digestion system in termite (cellulases) using carbohydrate-based inhibitors and naturally occurring toxic compounds in plants' leaves extract. Hence the present study tried to achieve two main targets with one approach that means cellulases of lower termite could be a possible source for biomass conversion into energy and these enzymes can easily be targeted by inhibitors to inhibit the termite infestation. Eastern subterranean lower termite *Reticulitermes flavipes* from United States and Southeast Asian subterranean termite *Heterotermes indicola* were used for all study objectives. Both termites are declared as an active and vigorous pest of an urban setting with high damage costs.

An overview of major outputs

In the first three objectives/chapters of this study, distribution and the role of endogenous and symbiotic cellulases from the subterranean termites in the biomass saccharification was evaluated. Whereas the last two objectives/chapter of the present study revealed the role of cellulases in the termite control strategies using nontoxic carbohydrate-based inhibitors and naturally occurring toxic plant leaves extract. This study suggested that lower termite *R. flavipes* has higher endogenous and symbiotic cellulases activity than *H. indicola*. The salivary gland was the main site to produce endogenous cellulases (β-glucosidase and endoglucanases) in both lower termites. The gene expression of different cellulases significantly correlated with their exclusive cellulolytic activities measured in all gut regions (head, salivary gland, foregut, midgut, and hindgut). The findings of the first objective also suggest that lower termites despite different zoogeographic

zones showed the same single unified cellulolytic system, whereby, endogenous and symbiotic origin cellulases act consecutively and collaboratively across the entire digestive tract of *H. indicola* and *R. flavipes*. The results of the distribution of endo- and exoglucanase activities support the previous studies reporting that endoglucanases are distributed in both salivary gland (endogenous) and hindgut symbionts (Breznak and Brune, 1994; Watanbe *et al*., 1998; Zhu *et al*., 2005; Zhu *et al*., 2007).

In the second objective, the results of the present study revealed that Sugarcane bagasse, pinewood, cottonwood, and corn stovers were saccharified at significantly high levels as compared to the control diet and rice husk by subterranean cellulase fractions. However, *R. flavipes* showed significantly ($p<0.05$) high saccharification potential than *H. indicola*. Consumption of the sugarcane bagasse, pinewood, and corn stover was maximum by *R. flavipes*, whereas *H. indicola* exposed cottonwood and sugarcane feedstocks as the most palatable food source with 100% survivability. This study hypothesized that feeding on various feedstocks showed varied impacts on enzyme activities since an adaptive mechanism enables the termites and their endosymbionts for the optimized consumption of variable food sources. Results supported the "diet adaptation" hypothesis by indicating β- glucosidase, exoglucanase, and xylanase activities significantly highest for sugarcane bagasse, pinewood, and cottonwood as compared to rice husk and control diets in both termite species. These results are important from the perspective of physiological changes in termites that lead to gut microbial environments changes and 100% survivability on exclusive sugarcane bagasse, pinewood, and cottonwood feedstocks.

In the third objective of this study a symbiotic cellulolytic strain was isolated from the gut of building infesting termite species *Heterotermes indicola* and *Reticulitermes flavipes* to produce ionic-solvent tolerant cellulase. After screening by Congo red, strain HI-08 was identified as *Bacillus licheniformis* using 16S rDNA sequence analysis. It was accounted for maximum cellulase production on 2% sugarcane bagasse (1156 U/mL). The crude cellulase was purified by $(NH_4)_2SO_4$ precipitation, affinity and size exclusion chromatography, with 12.02 purification fold and 32.33% overall recovery. Approximately 55 kDa molecular weight of gel-purified cellulase was revealed by SDS-PAGE analysis. The optimum temperature and pH for maximum cellulase activity were determined as 45°C and 7.0, respectively. Kinetic analysis of purified cellulase showed the K_m and V_{max} be 2.24 mg/mL and 454.05 µg/mL/min, respectively. Statistical

optimization using RSM indicated that sugarcane bagasse was the most useful agricultural waste for cellulase production, which proved its candidature in industrial bio ventures.

In the fourth objective of this project, dry lab and wet lab approaches were conducted to cutoff the lignocellulose activities of termites by targeting termite cellulases including endoglucanase, exoglucanase, and β-glucosidase using ecofriendly carbohydrate-based cellulase inhibitors. The results of *in silco* studies revealed Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose showed higher promising binding affinities with exoglucanase and β-glucosidase as compared endoglucanase based on GOLD docking scores. These results were supported by wet-lab studies, in which the efficacy of these inhibitors was investigated by conducting biochemical and feeding bioassays. Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose significantly ($p < 0.05$) inhibited the activities of exoglucanase and β-glucosidase during *in vitro* assays. Feeding bioassays revealed significant effects of Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose inhibitors on all three enzyme activities and subsequently reduced the gene expression levels, termite foraging activity, protozoal communities, and at end termite mortality. All these results suggest the efficacy of Cellobiomidazole, Cellobiose, and Fluoromethyl cellobiose as termite control agents and can be applied in field conditions using baiting technology.

In the last objective of the present study, silver oak (*Grevillea robusta* Cunn.) (Proteaceae) leaf extract was investigated for its termiticidal properties against the termite *H. indicola* in the laboratory tests. Results indicated that maximum (> 90%) termite mortality occurred at 20 mg/mL along with a significant reduction in termite tunneling activity. The LC_{50} was calculated at 5.47 mg/mL using the concentration series data. After 2 weeks of no-choice continuous feeding on filter paper treated at 20 mg/mL, the hindgut protozoan population was reduced by 62.90%. At the highest concentration tested, 100% mortality was recorded after exposing termites to cottonwood and pinewood treated with silver oak leaf extract. HPLC-DAD-ESI-MS results of the extract showed a high level of phenolic compound, alkyl-resorcinol.

General Conclusion

This study suggested that lower termite *R. flavipes* has higher endogenous and symbiotic cellulases activity than *H. indicola*. The salivary gland was the main site to produce endogenous cellulases (β-glucosidase and endoglucanases) in both lower termites. Assays for cellulase activities mapped the distribution patterns of endoglucanase, β-glucosidase, exoglucanases, and xylanase activity throughout the gut regions of *H. indicola* and *R. flavipes*. The gene expression of different cellulases significantly correlated with their exclusive cellulolytic activities measured in all gut regions (head, salivary gland, foregut, midgut, and hindgut). The finding of this chapter suggests that lower termites despite different zoogeographic zones showed the same single unified cellulolytic system, whereby, endogenous and symbiotic origin cellulases act consecutively and collaboratively across the entire digestive tract of *H. indicola* and *R. flavipes*.

This study assessed the simultaneous enzymatic pretreatment and saccharification potential of two lower termite species *R. flavipes* and *H. indicola* against agricultural feeding stocks under *in vitro* and *in vivo* conditions. The overall objective of this study chapter was to elucidate the potential of endogenous and symbiotic protein of termites in the degradation of agricultural residues. The results reveal the capability of lower termites' gut to saccharify lignocellulosic feedstocks sugarcane, cotton and pinewood, corn stovers, and rice husk, substantiating the efficacy of termite gut system as a bio-sourced of enzymes for enzymatic saccharification or pretreatment of agricultural wastes. Moreover, this chapter study identified certain lignocellulase activities that might be responsible for the complete digestion of all feeding stock by supporting the hypothesis "diet-adaptation" proposed by Karl and Scharf, (2015). Sugarcane bagasse was the most and highly palatable feeding stock for both termites' enzymatic system that likely would extend to microenvironments of fermentation chamber. The cellulase of *B. licheniformis* HI-08 is unique with respect to isolation sources that are wood-feeding termites. It confirmed its candidature in biomass conversion application by hydrolysis of agricultural wastes with optimal activity at temperature 45°C and pH 7.0. The retention of cellulase activity in presence of various metal ions indicated the organic ionic-tolerant nature of cellulose. The optimization model using RSM enhanced the cellulase activity by 1.8 folds with optimized conditions. HI-08 is a wild type strain with lucrative cellulase titers that can be further improved using mutations and other biotechnological techniques.

In the conclusion of this study, five carbohydrate-based inhibitors contributed as eco-friendly termite control agents by inhibiting the cellulase activities, feeding activities, fluctuating the protozoa count, causing termite weight loss, and eventually inducing termite mortality. In the observations of the present study, the relative rank of these 05 inhibitors is CBI > CB > FMCB followed by FMG and glucose. In particularly, cellobiose and its analogs (CBI and FMCB) indicated strong inhibition of exoglucanase and β-glucosidase activities at low and mid-range concentrations (6.25-25 mM) as well as they also showed *in-vivo* efficacy by revealing low cellulase gene expression, low consumption rates, reducing the termite weight loss and protozoa population, and high termite mortality.

This research has triggered the relevant approaches for use in further evaluation of each carbohydrate under field condition trials. Based on our findings that *H. indicola* and *R. flavipes* showed varying degrees of susceptibility to cellobiose and its analogs, that could be tested for feeding and mortality in different biogeographic termite species with varying nutritional backgrounds. Different hypotheses, such as termites' colonies that are stressed nutritionally will be more susceptible to the effects of cellulase inhibition. Additionally, the current study did not assess the field efficacy bioassays and choice bioassays; therefore, we could not estimate that at which rate these inhibitors will feed preferentially by termites in the presence of other food sources under field conditions.

The results of this study illustrate that *G. robusta* leaf extract had a deterrent and contact bioactivity in addition to digestive toxicity against wood-feeding termites. The extract showed repellent, antifeedant and termiticidal properties along with significantly reducing hindgut protozoa populations during no-choice bioassays. These are basic findings being used to assess the biological relevance and use of silver oakleaf extract against pest termites. Future investigations will examine the isolation of bioactive compounds such as resorcinol in the extract in hopes of finding useful and renewable substitutes for some currently used chemical biocides.

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