MOLECULAR PHYLOGENETIC CHARACTERIZATION OF SYMBIOTIC MICROBIOTA IN THE INTESTINAL TRACTS OF WILD LEPIDOPTERAN STEM BORER *ELDANA SACCHARINA* WALKER (PYRALIDAE)

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A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science (Biotechnology), in the School of Pure and Applied Sciences of Kenyatta University.

October, 2011

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DECLARATION

The experimental work described in this thesis is my own. The contents of this thesis have not been submitted for any other award.

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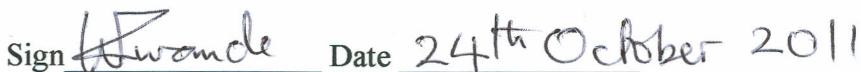
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DEDICATION

This thesis is dedicated to the memory of the late Professor Thomas R. Odhiambo (1931-2007). His vision of an African Centre of Excellence in Insect Science continues to inspire and transform the lives of many.

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ACRONYMS AND ABBREVIATIONS

ARCU	Animal Rearing and Containment Unit
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
CFU	Colony forming units
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
eDNA	environmental DNA
FISH	Fluorescence In -Situ Hybridization
AGSA	Arginine Glycerol salts agar
GAESS	Actinobacteria isolated from *E. saccharina* larval guts from sugarcane host plants using AGSA culture medium
GAESP	Actinobacteria isolated from *E. saccharina* larval guts from Papyrus host plants using AGSA culture medium
icipe	International Centre of Insect Physiology and Ecology
KMESC	Unicellular bacteria isolated from laboratory reared *E.saccharina* larvae using KMM1 medium
KMESP	Unicellular bacteria isolated from *E. saccharina* larvae from Papyrus host plants using KMM1 medium
KMESS	Unicellular bacteria isolated from *E. saccharina* larvae from sugarcane host plants using KMM1 medium
KMM1	Kenya Minimal Medium
MEGA	Molecular Evolutionary Genetics Analysis
mV	Milli volt
NCBI	National Council for Biotechnology Information
NEB	New England Biolabs
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDAESP	Culture isolates obtained from *E. saccharina* larvae from Papyrus host plants cultured on Potato Dextrose Agar medium
PDAESS	Culture isolates obtained from *E. saccharina* larvae from Sugarcane host plants cultured on Potato Dextrose Agar medium
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
Redox	Oxidation-Reduction
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions per Minute
S.O.C medium	Super Optimal Catabolite repressed broth
TAE	Tris-acetate Ethylene diaminetetraacetic acid

ABSTRACT

Intestinal tracts of plant feeding insects are potentially important sources of novel biocatalysts. However, the microbial populations in these guts, especially in the Lepidoptera, are not well characterized. In this study, thirty two (32) bacterial isolates from homogenized guts of late instar larvae of *Eldana saccharina* from papyrus rhizomes and infested sugarcane stalks were assayed for their ability to utilize alpha cellulose and sugarcane bagasse as sole carbon sources. All isolates were viable for 14 days in liquid fermentation cultures. The microbial isolates were also characterized by a combination of microbiological and 16S rRNA gene sequence analysis. In addition, microbial communities in the gut homogenates were investigated by extracting total genomic DNA from eviscerated larval guts, followed by PCR amplification of the 16S rRNA gene. The diversity of the gut symbiotic bacteria was characterized by genetic profiling of the partial bacterial 16S rRNA gene by the Small Sub Unit PCR-Restriction Fragment Length Polymorphism (SSU-RFLP) technique followed by clonal sequencing and phylogenetic analysis. A total of 48 cloned 16S rRNA gene sequences together with 16S rRNA gene sequences from 32 bacterial isolates obtained by two different culture methods were compared with sequences in the GenBank database. Seventeen (17) of the clonal sequences were closely affiliated to the alpha Proteobacteria subdivision of the Kingdom Bacteria, followed by gamma Proteobacteria (15), Firmicutes (9), Actinobacteria (5) and βeta Proteobacteria (2). In culture dependent studies, most of the isolates obtained were affiliated to the high G+C content, Gram positive bacteria in the Actinobacteria subdivision, represented by the *Streptomyces* operational taxonomic unit (18 isolates), *Microbacterium* (4 isolates) and the *Cellulosimicrobium* (1 isolate). In addition, five (5) isolates clustered closely with the γ- Proteobacteria, three (3) with α-Proteobacteria and one (1) isolate was affiliated to the Firmicutes group. Only three bacterial taxa were isolated from gut homogenates of laboratory reared control larvae. Furthermore, only five bacterial taxa were detected in common by both culture dependent and independent methods. The results show a high microbial diversity in the guts of wild *E. saccharina* larvae and demonstrate that multiple approaches are necessary to characterize microbial diversity in a complex micro-biome like the insect gut. The results further suggest mutual benefits between microbial isolates and the host *E. saccharina* larvae and infer their potential applications in biotechnology. Moreover, these results provide the first comprehensive description of microbial diversity in the guts of the African Sugarcane stem borer and demonstrate that insect diet and habitat influence the composition of the gut microbial communities.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Plants constitute the largest and most readily available source of food for terrestrial insect communities. Herbivorous insect species are very abundant and comprise perhaps half of all individuals found in nature (May, 1988). However, out of 32 insect orders, only nine feed on living plants, suggesting a formidable evolutionary barrier that most groups have not been able to overcome (Bernays and Chapman, 1994; Borror *et al.*, 1989). Among the nine orders of phytophagous insects is the Lepidoptera, the second largest order which comprises the moths, butterflies and skippers. Two pairs of membranous, scaled wings with much diversity in color and wing pattern characterize the adult members of this order. They undergo complete metamorphosis in their life cycle, with a developmental cycle that takes between 25 and 60 days depending on the species (Plate 1). *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is reported to develop up to six generations in one year without larval diapause (Overholt *et al.*, 2001). Lepidopteran larvae, commonly called caterpillars, are eruciform, having a well-developed head and a cylindrical body with 13 segments (3 thoracic and 10 abdominal). Each of the thoracic segments bears a pair of legs while abdominal segment numbers 3 to 6 and 10 usually bear a pair of prolegs. These prolegs differ from the thoracic legs by being fleshier, differently segmented and by possessing a number of tiny hooks, at the apex, which are called crochets.

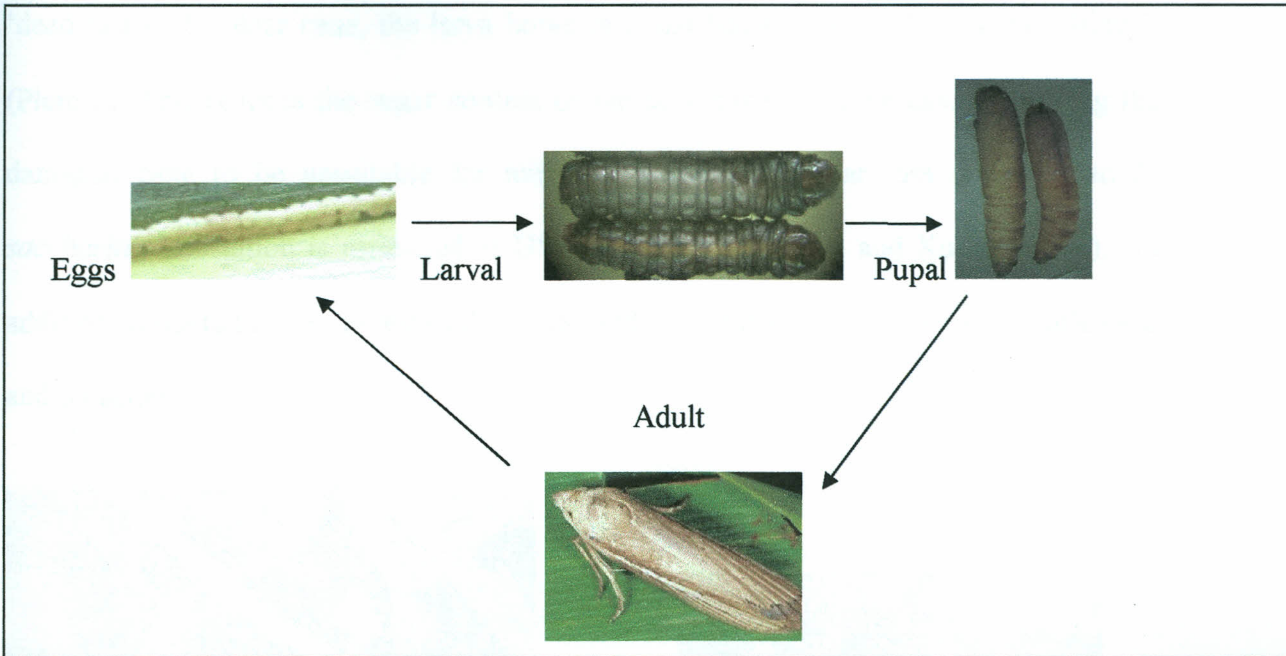

Plate 1: Life cycle of *Eldana saccharina*. The developmental cycle varies between 25 and 60 days.

1.2 Economic importance of *Eldana saccharina*

The larvae of most lepidopteran species are phytophagus, with many being serious pests of cultivated crops. Many cause damage to stored grain while some attack fiber in clothing. Lepidopteran caterpillars are endowed with strong mandibular surfaces for chewing and grinding plant tissues. Their chewing action on foliage results in leaves that are skeletonized, riddled with holes, eaten around the edges or entirely consumed (Borror *et al.*, 1989). The term stem borer refers to larvae of certain moths and beetles that bore into live plant stems as they feed. *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is a serious pest of sugarcane in Southern Africa (Atkinson, 1980), and of both maize and sugarcane in West Africa, with yield losses in maize being estimated at 20% (Bosque-Perez and Mareck, 1991). In sugarcane the stem borers can destroy the meristematic tissues of the shoots leading to the death of the shoot, in a condition called

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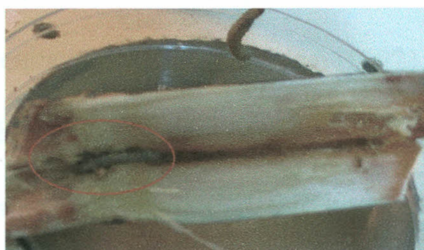
'dead heart'. In older cane, the larva bores into and tunnels the stalk without killing it (Plate 2). This reduces the sugar content of the sugarcane, in some cases rendering the damaged cane to be unsuitable for milling. Recoverable sugar loss attributed to *E. saccharina* infestation is estimated at US$ 332.10/ha (Simpson and Kumar, 1985). In addition, cane–tunneling increases the vulnerability of the crop to other pests, pathogens and parasites.

a)



b)

Plate 2: a) An infested sugarcane stalk showing deposition of flass between two nodes (circle); b) Dissected sugarcane stem showing tunneling by an infesting *E. saccharina* larva (circle) and red discoloration which results in cane spoilage through fungal fermentation and eventual decay.

1.3 Biological significance of symbiotic microbiota

Wetland cyperaceous plants and a variety of wild grasses like *Panicum maximum*, *Pennisetum purpuream*, and Sudan grass provide natural refugia for *E. saccharina* from where invasions to cultivated crops occur. Besides sugarcane and maize, *E. saccharina* also attacks sorghum and rice (Atkinson, 1980; Khan *et al.*, 1997). *Eldana saccharina* is widely distributed in most parts of sub-Saharan Africa, where it accounts for up to 65% of stem borer pest populations in some countries (Maes, 1998). This wide distribution and abundance of *E. saccharina* could be attributable to its ability to utilize recalcitrant structural plant components found in both cultivated and natural host plant species.

Hydrolysis of plant structural polymers like cellulose and hemi-cellulose is critical to the nutrition of phytophagous insects. These polysaccharides are resistant to degradation and the insect is not able to secrete all the digestive enzymes to hydrolyze β- linkages in the polymers (Brennan *et al.,* 2004). To overcome this, insects have evolved consortia of microbial symbionts like bacteria, protozoa and yeast which secrete enzymes that hydrolyze the polymers (Kaltenpoth, 2009; Scharf and Tartar, 2008; Brune and Friedrich, 2000; Martin, 1991). Microorganisms possess metabolic properties that are absent in insects and in this way act as 'metabolic brokers', enabling phytophagous insects to overcome biochemical barriers to herbivory (Berenbaum, 1988; Douglas, 1992). The consortia of gut bacteria are believed to adapt in the host insect by transfer of plasmids and trans-conjugation between bacterial strains within the insect gut (Watanabe and Sato, 1998). Some insect species provide ideal conditions for bacterial conjugation making the insect gut a 'hotspot' for gene transfer (Hoffmann *et al.*, 1998). Other possible roles that can be attributed to symbiotic microbiota in the insect gut include nitrogen fixation, synthesis of protein from nitrogenous waste materials and the provision of vitamins (B complex), sterols and amino acids (Campbell, 1990), and defense against pathogens and predators (Cardoza *et al.*, 2006).

A lot of scientific research has been done on insects' herbivory and their nutritional ecology (Bernays and Chapman, 1994; Hochuli, 1996; Martin, 1991; Slansky, 1992). However, little has been done to describe and characterize the microbial flora in the gut of the African graminivorous lepidopteran insects. Recent work in other insects supports the proposition that symbiotic microbiota might play an essential role in the stem borers' metabolic utilization of plant structural polymers among other metabolic

benefits (Gündüz & Douglas, 2009; Brennan *et al.*, 2004; Kaufman and Klug, 1991; Gijzen *et al.*, 1994).

This study investigated the diversity and role of in-*vivo* biotic factors that may be responsible for the fitness and survival of the sugarcane stem borer on wild and cultivated host plants.

1.4 Justification

The microbiology of the insect gut has been studied for many decades (Steinhaus, 1960; Breznak, 1982; Brune & Friedrich, 2000). Until recently, progress on the molecular characterization of insect gut symbionts has been slow, largely because many gut microbes are unculturable and are consequently intractable to the traditional methods of microbiology based on axenic cultivation. Studies of various types of environments estimate that more than 99% of microorganisms seen microscopically cannot be cultivated by routine techniques (Amann *et al.*, 1995). This represents a vast microbial metagenome that remains unexploited for biotechnological applications. However, nucleic acid sequence-based techniques are now available that can classify microbes without the need for culturing. As opposed to metabolic properties this sequence-based approach can be applied to the study of natural microbial ecosystems hither-to little known (Pace, 1997; Ohkuma, 2002). Using this approach, new possible genotypes and classes in the eubacteria and archaea domains may be identified from the intestinal tracts of wild strains of *E. saccharina* and other species. An understanding of the metabolic properties of the studied symbiotic microbes can provide insights for targeted industrial

applications. Unlike conventional microbiological methods this approach is faster, accurate and is increasingly being used in studying natural microbial communities in order to avoid the largely unrepresentative nature of microbial cultivation.

1.5 Hypothesis

The gut of *Eldana saccharina* is inhabited by diverse microbial communities which are influenced by colonized host plant species.

1.6 General objective

The overall objective of this study was to investigate and assess the potential biotechnological applications of symbiotic microorganisms from the intestinal tracts of wild strains of *Eldana saccharina* larvae.

1.7 Specific objectives:

1. To determine and compare the bacterial diversity in the guts of 5th – 6th instar larvae of *Eldana saccharina* from papyrus and sugarcane host plants using culture dependent and sequence based techniques, and to compare these to laboratory reared controls;
2. To determine the hydrolysis of plant structural polymeric components (cellulose, lignocellulose) by *in-vitro* bioassays using gut extracts and culture isolates from eviscerated *E. saccharina* larvae.

1.8 Significance and anticipated outputs

This study on the diversity and properties of microbial communities in the gut of *E. saccharina* is aimed at providing new taxonomic information about microbial associations in the guts of the stem borer, to infer their contributions to the stem borer's survival and resilience and to find their practical applications in biotechnology. The insect gut is increasingly becoming an important source of microbial secondary metabolites that may offer attractive characteristics for targeted industrial use (Kaltenpoth, 2009; Piel, 2009; Brennan *et al*, 2004; Ohkuma, 2003). Through use of direct cloning techniques, environmental DNA (eDNA) libraries from microbial genomes can be generated, that could provide a format for expression screening for targeted activities and discovery of unique enzymes and other metabolites (Short, 2001). The discovery of novel microbial enzymes from wild strains of *E. saccharina* can present useful agricultural and industrial applications. For instance, microbial cellulases and xylanases have the potential of being used as feed additives to enhance utilization of high crude fiber plant materials in livestock feeds through improved feed digestibility. Efficient utilization of cereals in feed formulations would minimize wastage and thus assure availability of more grain for the rising human population. Actinomycetes are well known for their ability to decompose complex molecules, particularly lignocellulose components, which makes them important agents in decomposition processes (Lacey, 1997; Abdulla & El-Shatoury, 2007). The anaerobic conversion of cellulose results in various fermentation end products including ethanol, organic acids, carbon dioxide and hydrogen (Lynd *et al.*, 2002). Novel microbial cellulolytic enzymes would find applications in the industrial production of bioethanol and polylactic acid from sugarcane

and other cereal crop biomass such as maize stover, barley, rice and wheat straw (Olsson *et al.*, 1999; Garde *et al.*, 2000; Zaldivar *et al.*, 2001). In addition to extracellular cellulase secretion, aerobic cellulolytic bacteria produce high cell yields typical of aerobic respiratory growth, a feature that could be an attractive source of microbial single cell protein from waste cellulosic biomass (El-Nawwi and El-Kader, 1996). Screening of symbiotic microbial isolates from *E. saccharina* larvae for anti-infective compounds would provide an important application in human health because the compounds involved, being produced for protection of the eukaryotic host insect would be less likely to have harmful side effects on humans (Kaltenpoth, 2009). Several novel compounds with antimicrobial activity have been isolated from mutualistic bacteria, particularly those that protect their insect host against pathogens (Kaltenpoth *et al.*, 2005; Piel, 2009). Knowledge on the diversity of indigenous microbial species in *E. saccharina* guts could also find useful applications in designing classical biological control strategies against the stem borer (Conlong, 2001).

CHAPTER TWO

LITERATURE REVIEW

2.1 Ecology and Host Range of *Eldana saccharina*

Eldana saccharina Walker (Lepidoptera: Pyralidae) is indigenous to Africa, where it has existed naturally in numerous wetland sedges and indigenous grasses (Conlong, 1994; Atkinson, *et al.*, 1981; Girling, 1972); (Plate 3). *E. saccharina* has become a pest of increasing proportions in South African sugarcane since 1971, and is spreading throughout the African continent (Carnegie, 1974; Way, 1994; Horton *et al.*, 2002). This shift by *E. saccharina* from its indigenous host plants to crop hosts is thought to have occurred due to a disturbance in the ecological balance arising from increased opening up of swampy areas for cultivation of graminaceous crops, thereby providing alternative host plants for the insects' colonization (Atkinson, 1980; Assefa *et al.*, 2008).

Plate 3: Dissected umbel rays of *Cyperus papyrus* showing *Eldana saccharina* larvae (green circle), with brown discoloration of the base indicating commencement of downward tunneling of the culm for pupation and eventual exit of the moth. On the right is an infested *papyrus* inflorescence with yellow discoloration of the pseudo leaves that is indicative of an infestation.

2.2 Insect metabolic micro-biomes

The diversity of the insecta is reflected in the large and varied microbial communities inhabiting the gut. Insect gut microbiota represents all aspects of microbial relationships from pathogenic to obligate mutualism. Indigenous gut bacteria also play an important role in withstanding the colonization of the gut by non-indigenous bacteria including pathogens (Dillon and Dillon, 2004). Like in the human gut (Kurokawa *et al.*, 2007; Turnbaugh *et al.*, 2007) many insect species are inhabited by large diverse communities of microorganisms that could out-number their own cells and with considerable metabolic activity (Brooks, 1963; Dasch *et al.*, 1984; Gündüz & Douglas, 2009). With the exception of the termite, few studies on the functional role of the insect gut microbiota have been done (Brune and Friedrich, 2000; Warnecke *et al.*, 2007; Scharf & Tarter, 2008). This may partly be due to the complexity of insect gut symbioses. There are also difficulties in recognizing beneficial relationships that are often intermittent or may completely be absent in laboratory-reared insects (Dillon and Dillon, 2004). Lederberg and McCray (2001) coined the term 'microbiome' to describe the collective genome of the indigenous microbiota, their genetic elements (genomes), and environmental interactions in a defined environment. The completion of a number of insect and microbial genome sequencing projects and introduction of other molecular techniques and bioinformatics now provide useful tools for detailed studies of insect microbiomes.

2.3 Intestinal symbioses.

A symbiotic relationship of a microbe with its insect host can be defined as the acquisition and maintenance of the microorganism that results in novel structures or metabolism (Klepzig *et al.*, 2009; Zook, 1998). This interaction can be positive or negative. Commensalism and mutualism characterize a positive association while parasitism and insect pathogens characterize negative symbioses (Daida *et al.*, 1996). A symbiotic relationship should be defined after careful consideration of the time range and resource conditions in which it is examined (Klepzig *et al.*, 2001b). Similarly, isolation and characterization of insect intestinal microbiota should be correlative either with the ability of the host to control its biota or the effect of the biota on the host's physiology (Brooks, 1963). In the insect an indigenous biota should be present in all individuals of the species and should maintain stable climax communities. Though many insect species are believed to derive their microbiota from the phylloplane of food plants, the degree of persistent strains of the ingested species is largely unknown (Dillon and Dillon, 2004). It is, therefore, necessary to show whether strains of these species occupy particular niches in the gut and colonize gut epithelia or whether they are present in all of the same insect species. In insects the most important distinction is the ability of a microbial species to colonize the gut habitat. Colonizing microbes should be able to multiply at a rate that equals or exceeds the rate of elimination from the gut.

2.4 The insect gut structure and chemical environment

The diversity of symbiotic microbiota relates in part to the variety of specialized structures present in the gut as well as the effect of pH and redox conditions, digestive

enzymes present and the type of food ingested. Insects with a simple, straight digestive tract are believed to possess a less diverse microbiota whereas those with more complex structures like paunches, diverticula and caeca have a wide variety of mutualistic microorganisms (Bignell, 1982; Tanada and Kaya, 1993). The digestive tracts of lepidopteran larvae have for long been thought not to have specialized structures that are usually associated with microorganisms. Some of these folivores are also characterized by rapid food throughputs. Gut microorganisms in such insects were assumed to play little part in nutrition and digestion (Appel, 1994). However, it is now known that herbivorous insects with a food throughput time as rapid as 1.5hr can harbor indigenous microbiota (Dillon and Charnley, 1991). For instance, despite the absence of specialized structures, there is a substantial population of bacteria residing on the locust hindgut cuticle (Hunt and Charnley 1981). Bacteria have also been found occupying the gut lumen of the tobacco hornworm *Manduca sexta* and colonizing the hindgut epithelia (Toth-Prestia and Hirshfield, 1988). Moreover, bacterial division in the insect gut can occur as often as every twenty minutes with viable bacterial mutations being generated during every cycle, allowing the indigenous microbiota to adapt rapidly to changes in the gut environment (Dillon and Dillon, 2004).

The pH and redox potential inside the gut lumen are important in selecting and enriching for certain species of bacteria. Most bacteria have a growth pH optimum of 6.0 -7.0 but numerous exceptions include lactic acid bacteria that can grow at acidic pH. Strictly aerobic microbes grow only at positive redox potentials whereas anaerobes grow at negative redox potentials. Multiple solutions are developed in the guts of insects to enable efficient digestion of the insects' diets. The great variation in pH and redox

potential in the insect gut are part of this adaptation (Plate 4). Lepidopterans and sawflies possess alkaline gut conditions of pH 8 to 10, with widely differing redox potentials ranging from + 400mV to – 200mV (Appel, 1994). Large microbial populations are sometimes evident in insects with extreme pH levels (Dillon and Charnley, 1991; Bignell and Eggleton, 1995).

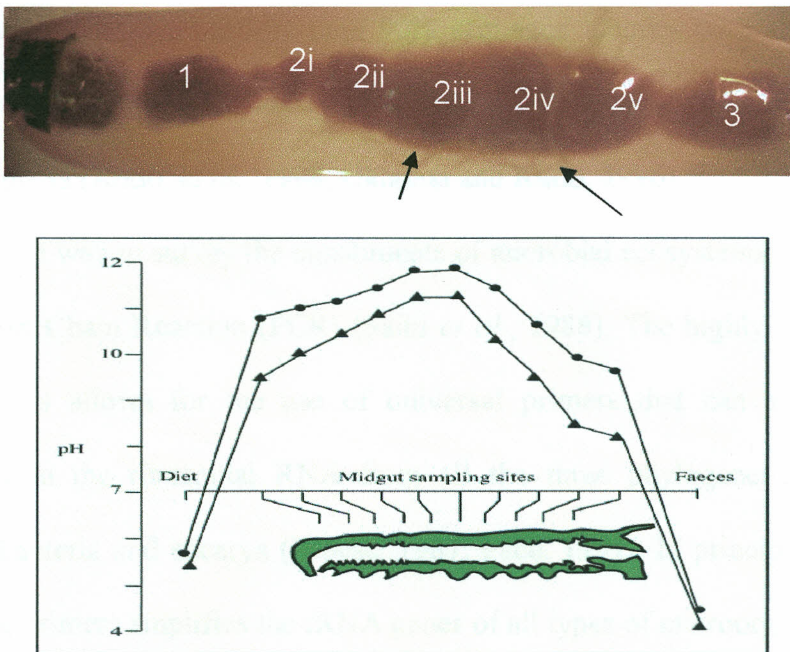


Plate 4: A digital image of a whole dissected gut from a late instar *Eldana saccharina* larva, showing the fore gut (1); an extensive mid gut with five partitions (2i-2v) and the hind gut (3). Arrows show a network of malpighian tubules mainly attaching to the mid gut. (X1000 Magnification). The graph below shows pH profiles along the gut lumens of two lepidoperan species, *Lichnoptera felina* (circles) and *Manduca sexta* (triangles).

Source:http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi.

2.5 Tools in Molecular Phylogeny

2.5.1 Small Sub Unit rRNA-PCR

Nucleic acid sequence based approaches particularly those using 16S rRNA genes enable definition of the microbial community of insects (Brauman *et al.*, 2001). In one study, two thirds of clonally isolated 16S rDNAs from the gut microbiota of the termite *Reculitermes speratus* had less than 90% sequence identity with any known bacterial species, with ten of the clones failing to show close similarity with any recognized bacterial phyla (Kudo, *et al.*, 1998; Okhuma and Kudo, 1996).

The quickest way to survey the constituents of microbial ecosystems is through the use of Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988). The highly conserved nature of rRNA genes allows for the use of universal primers that can anneal to sequences conserved in the ribosomal RNA from all the three phylogenetic domains namely, archaea, bacteria and eucarya (Woese, 1987; Pace, 1997). In principle, PCR carried out with these primers amplifies the rRNA genes of all types of microorganisms present in an environmental sample. Individual types of genes in the mixture are separated by a cloning step and then sequenced. A molecular phylogenetic assessment of uncultivated microorganisms can provide insights into many properties and functions of the microbe through comparison with its studied relatives.

2.5.2 Fluorescence In -Situ Hybridization (FISH)

Fluorescein or Digoxigenin labeled specific oligonucleotide probes can be used to visualize phylotypes, establish morphology and to determine the number and spatial arrangement of cells in the gut. In-situ localization of the bacterial community on cryosections of the hindgut of lower termites was achieved using domain-specific rRNA

Fluorescein probes (Berchtold *et al.*, 1999). Similarly, two intracellular symbiotic bacteria from the mulberry psyllid *Anomoeura mori* were characterized using digoxigenin labeled oligonucleotide probes (Fukatsu and Nikoh, 1998). Using fluorescently labeled probes in a survey of gut microbiota of five cricket species it was possible to identify unculturable *Bacteroides* and *Prevotella* species as well as detect changes in the profile of microbial community due to dietary changes (Santo Domingo *et al.*, 1998).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Insect larvae collection and dissections

A total of one hundred mature *E. saccharina* larvae were randomly sampled from rhizomes, culms and umbel rays of *Cyperus papyrus* host plants at Luanda-Nyamasare, Homa Bay County, along the shores of Lake Victoria (GPS: 00 ° 27.641'S; 034° 16.681' E; 7327ft ASL). An equal number was sampled from infested sugarcanes in smallholder farms at Kibisi swamp (GPS 0° 46.617' N; 34° 43.989' E; 5406ft ASL); Marofu swamp (GPS 0° 43.870' N; 34° 46.848' E; 5523ft ASL) and Sinoko river swamp (GPS: 0° 48.590'N; 34° 47.059' E; 5544ft ASL), all in the Bungoma County of Western Kenya). The larvae were maintained on pieces of the host plants while being transported to the laboratory. The larvae were surface sterilized by three washes in 20% (V/V) bleach, each lasting three minutes, followed by one wash in 70% ethanol. Whole guts from the two populations were separately eviscerated by aseptically pulling out the gut from the anal segment using a pair of sterile fine tip forceps while holding the head with the second pair (plate 5). To obtain a wide coverage of microbial species composition, ten pools of 10 individual guts were placed into sterile 1.5ml epitubes containing 500µl of chilled 1x Phosphate Buffered Saline (PBS) pH 7.4. Laboratory reared control *E. saccharina* 5th instar larvae, reared on artificial diet, were acquired from the Animal Rearing and Containment Unit (ARCU) at *icipe* and dissected the same way.

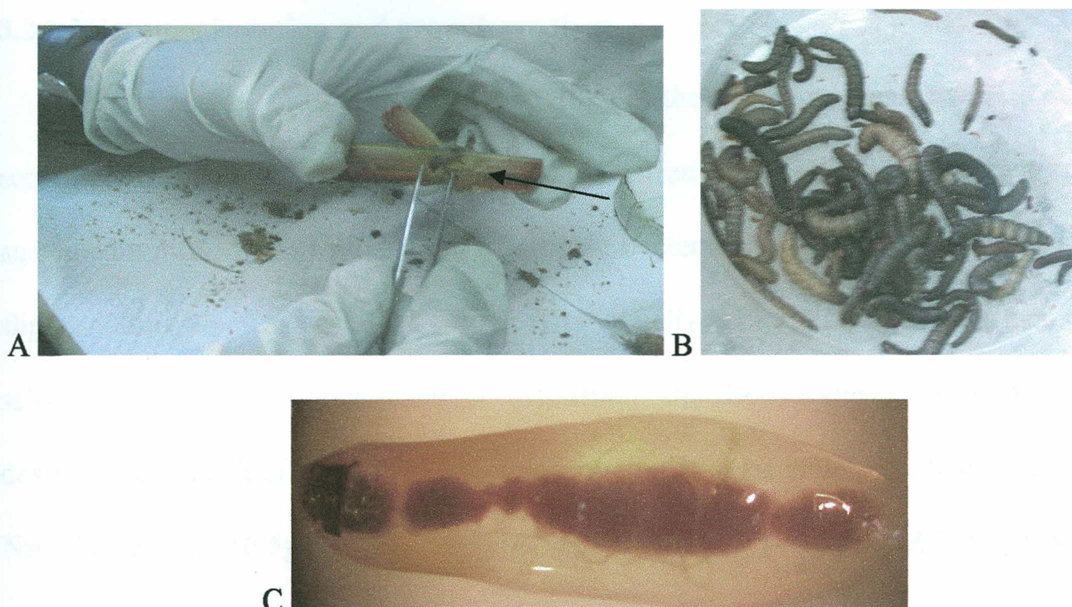


Plate 5: A. Laboratory dissection of infested sugarcane stalks to recover *Eldana saccharina* larvae. The arrow shows a late stage larva emerging from a split piece of infested sugarcane. B. Late stage *E.saccharina* larvae in a Petri plate; C. Eviscerated whole gut from late stage *E.saccharina* larva.

3.2 Culture Dependent Characterization

3.2.1 Gut homogenization and serial dilutions.

Ten pools of dissected *E. saccharina* larval whole guts from papyrus and sugarcane host plants as well as laboratory reared controls were placed in 1.5ml epitubes and homogenized in 0.5ml of sterile 1x PBS pH 7.4, using plastic pestles in the presence of fine glass beads. PBS was composed of NaCl [8.0g]; KCl [0.2g], Na_2HPO_4 [1.44g], KH_2PO_4 [0.24g] per litre of distilled water. The homogenates were further vortex mixed at high speed for five minutes in the presence of one larger glass bead (size 710-1180µm, Sigma Aldrich Inc, USA) in every tube. Larval gut homogenates from papyrus and sugarcane were code named ESP and ESS respectively while ESC was used for laboratory controls. The homogenates were initially diluted at 1/100 and then subjected to ten fold serial dilution steps up to a final dilution of 10^{-5}.

3.2.2 Microbial isolation and growth medium

Four dilutions [10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}] of the gut homogenates were inoculated separately in three different microbiological isolation media as follows: Kenya minimal agar medium (KMM1) for isolation of aerobic bacteria (Mackenzie, *et al.*, 2007), with slight modifications as described below; Arginine Glycerol Salts (AGS) agar medium for selective isolation of actinobacteria (Moustafa and Lechevalier, 1963), and Potato dextrose agar medium (PDA) for selective isolation of yeast and fungi. KMM1 contained NaCl [1.7g], KCl [6.5g], $MgCl_2.6H_2O$ [0.5g], $CaCl_2.2H_2O$ [0.1g], NH_4Cl [5.6g], KH_2PO_4 [1.0g], $NaSO_4$ [1.0] and Bacto agar [15g] per liter of distilled water. The medium was supplemented with 0.1% each of Gelatin, Glucose, Casamino acids and yeast extract [Difco, Detroit, MI, USA] and autoclaved at 121°C for 20 minutes. After cooling to 50°C the medium was further supplemented with 2ml each of the following 0.2 micron filter sterilized stock solutions: Biotin[10μg/ml], Folic acid [50 μg/ml], Riboflavin [62.5 μg/ml], Thiamin [50 μg/ml]; Isobutyric acid [25mM], Isovaleric acid [25mM] and Lipoic acid [1mM]. The medium was plated in 90mm disposable Petri dishes (Greiner Inc, Austria) under sterile conditions and allowed to solidify at room temperature. Arginine Glycerol Salts agar medium had the following composition per litre of distilled water: NaCl [1.0g], K_2HPO_4 [1.0g], $MgSO_4.7H_2O$ [0.5g], $FeSO_4.7H_2O$ [0.01g], $CuSO_4.5H_2O$ [0.001g], $ZnSO_4.7H_2O$ [0.001g], $MnSO_4.7H_2O$ [0.001g], Glycerol [12.5g; Fisher, UK] and L-(+)- Arginine monohydrochloride [1g; J.T Baker, Japan]. Agar (2% w/v) was added as solidifying agent. The medium was autoclaved for 20 minutes, allowed to cool to 40°C and then plated on 90mm Petri dishes as described above. PDA was prepared by dissolving 39g of Potato dextrose agar powder [DIFCO™, USA] in 1 liter of distilled

water and autoclaving at 121°C for 20 minutes. After cooling the medium to 40°C, filter sterilized Kanamycin sulfate [50mg/ml; Fluka Biochemika] was added to a final concentration of 100µg/ml. The medium was gently mixed on a roller mixer for 5 minutes and then plated as described above.

Triplicate inoculations were separately applied for the three isolation media by pour plating 0.1ml of the appropriate dilution in the middle of the agar plate and then spreading the inoculums evenly on the agar surface with a sterile glass spreader. Control plates were inoculated with sterile distilled water. Cultures were incubated at 30°C under aerobic conditions in the dark. Colony forming units were enumerated under dissecting microscope and colonies described according to their morphological features. Colonies with unique observable morphological characteristics in shape, color and elevation were selected, streak-plated onto fresh medium and isolated into pure cultures. Cell and Gram staining characteristics were observed under a compound microscope. Slide cultures were grown to observe hyphae and spore chain characteristics for the Actinobacteria. Data on colony forming unit counts was subjected to Poisson regression analysis to test for effect of host plants and isolation media on microbial abundance.

3.2.3 Bioassays for utilization of cellulose and lignocellulose as sole organic carbon source

GAESS and GAESP isolates were separately inoculated in sterile mineral medium containing alpha cellulose and sugarcane bagasse respectively, to test their abilities to utilize the two substrates as sole carbon source. The mineral medium contained the following salts per liter of distilled water: NaCl [1g], K_2HPO_4 [1g], $MgSO_4.7H_2O$ [0.5g],

$CuSO_4$ [1mg], $ZnSO_4.7H_2O$ [10mg], $FeSO_4.7H_2O$ [100mg], $CaCl_2$ [0.1g], $(NH4)_2SO_4$ [6g] and 2ml of trace elements. 100 ml volumes of the mineral medium were distributed in duplicates into appropriately labeled 250 ml baffled Erlenmeyer flasks. For cellulose degradation test, 0.5g of commercial alpha cellulose (Sigma Aldrich Inc, USA) was added to individual flasks with mineral medium, mixed and autoclaved for 30 minutes at 121℃ covered with cotton wool layered with aluminum foil. For bagasse testing, 0.1g of freshly milled sugarcane waste, collected from Muhoroni Sugar Factory, was added to individual flasks. The bagasse had previously been washed in 10 changes of hot distilled water to remove traces of soluble sugars, dried in an oven and then milled to powder with a kitchen mill (Sanyo Inc, Japan), and autoclaved as described for alpha cellulose. Control flasks were prepared wherein the organic carbon source was omitted.

After cooling to room temperature, the medium was separately inoculated with 10 % (v/v) of the individual bacterial isolates under aseptic conditions and incubated at 30℃ in a shaker incubator set at 150 rpm for up to 14 days (Ponce-Noyola & de la Torre, 2001; Ponce-Noyola & de la Torre, 1995). Evaluation of viability of the isolates was based on turbidity of the fermentation broth, gas formation (including Geosmin production), and recovery of viable isolates in fresh medium after 14 days by the pour plate technique. Microbial cell biomass was assessed by determining total protein by the Bicinchoninic acid (BCA) assay method (Smith *et al.,* 1985), using Bovine serum albumin as a standard.

3.3 Molecular Characterization of Bacterial Isolates

3.3.1 DNA extraction and amplification of bacterial 16S rRNA gene

Genomic DNA was extracted from the microbial isolates GAESP 1-25; GAESS 1-17; KMESP1-11, KESS1-7 and KMESC 1-5 respectively, using the Fast DNA® Spin Kit for Soil (Qbiogene, Inc. USA), according to instructions supplied. The DNA was resolved on a 1% agarose gel containing 1x Tris Acetic acid EDTA (TAE) and stained with ethidium bromide to assess the yield and quality (Sambrook *et al.*, 1989).

Genomic DNA from individual isolates was used as template for amplification of the bacterial 16S rRNA gene. Amplifications were performed using the HotStar Taq® Master Mix kit (Qiagen, Inc, USA) following the manufacturer's instructions. The PCR reaction was done in 25µl reaction volumes comprising of 1 template DNA, 12.5µl of 2x HotStar Taq Master mix, and 0.5µmol each of universal primers 27F 5'-TAG AGT TTGATC CTG GCT CAG-3' forward and 1392R 5'- GAC GGG CGG TGT GTA CAT CCT GGC TCA G- 3' reverse primer, based on the *E. coli* 16S rRNA gene sequence (Lane *et al.*, 1985; Wang & Qian, 2009). A further 10.5µl of DNase free water was added to the master mix to a final volume of 25µl. The PCR programme, performed using a PTC-100 thermal cycler (MJ Research Inc, USA) , comprised of an initial enzyme activation at 95°C for 15 minutes followed by 29 repeated cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 90 seconds, primer extension at 72°C for 60 seconds and a final extension step of 72°C for 10 minutes. The PCR products were resolved on 1% agarose TAE gel electrophoresis and visualized by ethidium bromide staining. The amplified fragment of approximately 1365bp from each set of samples was excised from the gel and purified in individual 1.5ml epitubes using the QIAquick® gel

extraction kit (Qiagen Inc, USA) according to the manufacturer's instructions. The purified PCR fragments were submitted to a commercial sequencing service provider for sequencing.

3.3.2 Phylogenetic analysis of isolates' 16S rRNA gene sequences

Sequencing of purified PCR products was done using Macrogen commercial sequencing services (http://dna.macrogen.com/eng/order/seq/order_std_step1.jsp). The Sequencing reactions were performed in both directions without cloning. Sequence trace viewing and sequence editing were performed with the BioEdit Sequence Alignment Editor Software (Hall, 1999). The CHECK-CHIMERA program (http://rdp.cme.msu.edu/) of the Ribosomal Database Project (Maidak *et al.*, 2001; Cole *et al.*, 2003) was used to check the sequences for presence of possible chimeric artifacts. Sequence data was analyzed with RDP online software package (Release 10.13; Cole *et al.*, 2009). The new sequences were added to the *my*RDP space of the database (Cole *et al.*, 2007), aligned in sync with the RDP Public database sequences (http://rdp.cme.msu.edu/), using the SeqMatch Tool and tested for anomalies with the Pintail online software trained on the infernal secondary structure aware alignment tool (Cole *et al.*, 2009; Ashelford *et al.*, 2005). The 16S rRNA gene sequences were compared to sequences in the public nucleotide database using Basic Local Alignment Search Tool (BLASTn) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) for similarities to sequences in the GenBank database (Altschul *et al.*, 1990; Shayne *et al.*, 2003). GenBank sequences with highest similarities to the query sequences in the study were retrieved and used in

the construction of phylogenetic trees. Phylogenetic analyses were conducted using MEGA Version 4 (Tamura *et al.*, 2007). The Maximum composite likelihood model was used for Neighbour joining tree constructions (Saitou & Nei, 1987) with bootstrap confidence of 1000 replicate samplings (Tamura *et al.*, 2004; Felsenstein, 1985).

3.4 Culture Independent Characterization

3.4.1 Total DNA extraction

Total microbial DNA was extracted from the eviscerated *E. saccharina* larval guts using the Fast DNA® Spin Kit for Soil (Qbiogene, Inc., USA), according to manufacturer's instructions. DNA concentration and purity were determined by absorbance ratio at 260/280 nm, and quality visualized by ethidium bromide stained agarose gel electrophoresis (Sambrook *et al.*, 1989). The DNA suspensions were stored at -20°C until they were required for PCR and further analysis.

3.4.2 PCR amplification of bacterial 16S rRNA

Purified total DNA from the two wild stem borer populations and a laboratory-reared control were separately used as templates for the amplification of the 16S rRNA gene, using universal primers 27F 5'-TAG AGT TTGATC CTG GCT CAG-3' forward and 1393R 5'- GAC GGG CGG TGT GTA CAT CCT GGC TCA G- 3' reverse primer, based on the *E. coli* 16SrDNA gene sequence (Lane *et al.*, 1985; Wang & Qian, 2009). The PCR was carried out using HotStar Taq Master Mix kit (Qiagen Inc, USA) according to the manufacturer's instructions. The PCR reaction was performed in 25µl reaction comprising of 1µl template DNA, 12.5µl of 2x HotStar Taq Master Mix, and 0.5µmol each of the primers. The amplification comprised of an initial enzyme activation at 95°C

for 15 minutes followed by 29 repeated cycles of denaturation at 94 °C for 30 seconds, annealing at 55°C for 90 seconds, primer extension at 72°C for 60 seconds and a final extension step of 72°C for 10 minutes. The PCR products were verified by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The bands of PCR products from each set of samples were excised from the gel and purified using the QIAquick® gel extraction kit (Qiagen Inc, USA) according to the manufacturer's instructions. The gel purified PCR products were resolved on a 1% agarose gel to check for purity. Products showing a single band of approximately 1365 bp were used for cloning and subsequent sequencing.

3.4.3 Cloning PCR products and Restriction Fragment Length Polymorphism (PCR-RFLP)

The purified PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, USA) following the manufacturer's instructions. The ligation mix for each *E. saccharina* population consisted of 3µl of gel purified PCR product, 1µl of salt solution, 1µl of pCR® 2.1 TOPO vector and 1µl of sterile water to make a total of reaction volume of 6µl. The cloning reactants were mixed gently and incubated at room temperature for 30 minutes. Chemically competent *E. coli* cells were transformed by adding 3µl of each TOPO cloning reaction to three separate vials of the DH5α™ -T1 strain of *E. coli* cells with gentle mixing. The transformation mix was incubated on ice for 30 minutes after which the cells were heat-shocked by placing the tubes for 30 seconds in a water bath set at 42°C. The tubes were immediately transferred to an ice bath after which 250µl of S.O.C medium was added and the tubes incubated for one hour at 37°C with horizontal shaking

at 200 rpm. The transformation mix was plated on pre-warmed Lauria-Bertani (LB) agar medium using Kanamycin sulfate (50μg/ml) as selection marker and incubated overnight at 37℃. Prior to plating, every LB agar plate was layered with 40μl of X-gal (40mg/ml in DMF). Individual white bacterial colonies bearing the PCR construct were picked with a sterile tooth pick and inoculated into tubes with 3ml of LB broth containing 50μg/ml Kanamycin sulfate. The liquid cultures were incubated for 16 hrs at 37℃ with shaking (150 rev / minute). Plasmid DNAs bearing the 16S rRNA gene constructs were purified using the Qiaquick miniprep kit (QIAGEN Inc, USA) following the Manufacturer's directions. The purified plasmids were digested with a cocktail of 5 restriction enzymes, namely *Ava*I, *BamH*I, *Hind* III, *Kpn*I and *Xba*I, (New England Biolabs, USA) all of which only cut the plasmid once. The digestion reaction contained 0.2μl of each restriction enzyme, 2μl of 10X NEB Buffer 2, 1μl of 1:100 diluted BSA, 8μl plasmid DNA and 8.8μl of sterile water to make a 20μl reaction volume. Incubation was done overnight at 37℃ in a water bath. Plasmids from a few blue colonies were also included in the digestions as controls. Clones were selected on the basis of their band polymorphism on a 1.2% agarose gel. Sequencing of clones with unique band profiles was outsourced from Macrogen Corporation, South Korea. Sequencing reactions were performed in both directions separately using M13 Forward and M13 Reverse primer set. Sequence trace viewing and editing were performed with the BioEdit Sequence Alignment Editor Software (Hall, 1999).

3.4.4 Phylogenetic analysis of clonal sequences

The cloned sequences were checked for Vector contamination using the VecScreen software from NCBI (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.htm). The CHECK-CHIMERA program (http://rdp.cme.msu.edu/) of the Ribosomal Database Project (Maidak *et al.*, 2001; Cole *et al.*, 2003) was used to check the sequences for presence of possible chimeric artifacts. Sequence data was analyzed with RDP online software package Release 10.13 (Cole *et al.*, 2009) offered by the Centre for Microbial Ecology, Michigan State University, USA. The new sequences were added to the *my*RDP space of the database (Cole *et al.*, 2007), aligned in sync with the RDP Public database sequences using the SeqMatch Tool and tested for anomalies with the Pintail online software trained on the infernal secondary structure aware alignment tool (Cole *et al.*, 2009; Ashelford *et al.*, 2005). The 16S rRNA gene sequences were compared to sequences in the public database using the nucleotide Basic Local Alignment Search Tool (BLASTn) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) for similarities to sequences in the GenBank (Altschul *et al.*, 1990; Shayne *et al.*, 2003). GenBank sequences with highest similarities to the query sequences in the study were retrieved and used in the construction of phylogenetic trees. Phylogenetic analyses were conducted using MEGA Version 4 (Tamura *et al.*, 2007). The Maximum composite likelihood model was used for Neighbour joining tree constructions (Saitou & Nei, 1987) with bootstrap confidence of 1000 replicate samplings (Tamura *et al.*, 2004; Felsenstein, 1985).

CHAPTER FOUR

RESULTS

4.1 Enumeration of microbial isolates

All colonies from the KMM1, AGSA and PDA plate cultures were counted and calculation of CFU/ml determined as follows using the formula: CFU/ ml = CFU/ plate x dilution factor x 1/aliquot (Table 1)

Table 1: Enumeration of Microbial culture colonies from *E. saccharina* larval gut homogenates

e of *E. saccharina*	Culture plate counts per 10^{-5} dilution					
	KMM1		ASGA		PDA	
	Mean Plate count	CFU/ml	Mean plate count	CFU/ml	Mean plate count	CFU/ml
us rhizomes	178	$1.78x10^5$	17	$1.7x10^4$	9	$9.0x10^3$
cane stalks	226	$2.26x10^5$	17	$1.7x10^4$	9	$9.0x10^3$
eared control	90	$9.0x10^4$	0	Nil	5	$5.0x10^3$

KMM1culture isolates from larval gut homogenates of *E. saccharina* collected from infested sugar cane host plants had the highest number of colony forming units for 10^{-5} dilution followed by isolates from papyrus, while isolates from laboratory reared controls yielded two and half fold lower CFUs/ml. AGSA and PDA isolation media gave the same number of CFUs/ml of isolates from papyrus and sugarcane host plants. Regression analysis on the effect of host plant on microbial counts in larval guts *Eldana saccharina*

showed an overall significantly higher abundance of microbial isolates from papyrus and sugarcane host plants compared to laboratory reared control larvae (p - value < 0.0001; Table 2). However, there was no significant difference in number of isolates between papyrus and sugarcane host plants (p-value = 0.1851; Data set 2, Table 2).

Table 2: Poisson regression analysis on the effect of host plant on microbial abundance in guts of *Eldana saccharina* larvae

Dataset 1

Factor	Risk ratio(RR)	95% CI for RR	*p*-value
Intercept	85.17	(75.17 - 95.76)	< 0.00001
Host plant			
Papyrus	2.15	(1.87 - 2.47)	< 0.00001
Sugarcane	2.65	(2.31 - 3.04)	< 0.00001
Isolation Medium			
PDA	0.05	(0.04 - 0.06)	< 0.00001
AGSA	0.07	(0.06 - 0.08)	< 0.00001

Papyrus Vs sugarcane: Chi-square = 14.9, df = 1, p-value = 0.00011

Significant overall host plant effect: X2 = 197.6, df = 2, p-value< 0.0001
Significant overall isolation medium effect: X2 = 1236.8, df = 2, p-value=0.0001

Dataset 2

Significant effect of the isolation medium on the number of counts found in each host plant: p-value = 0.005349 (by Fisher exact test)

Within AGSA the proportions for papyrus and sugarcane were significantly higher than in Lab control, p-value=0.00000002373, but no significant difference between papyrus and sugar, p-value = 0.1851
Within KMM1, there was no significant difference among the two host plants, p-value = 0.2231
Within PDA, both sugarcane and papyrus were both significantly higher than the Lab control (p-values equal 0.002935 and 0.04654, respectively), but not significantly so from one another (p-value= 0.4328)

4.2 Morphological Characterization of Microbial Isolates

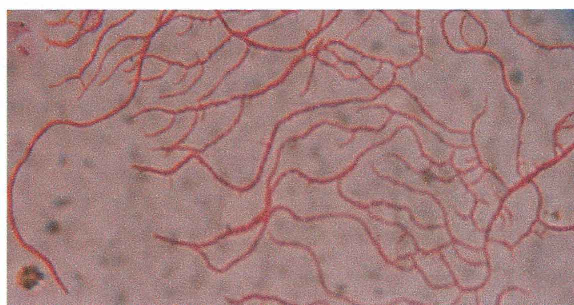
Morphological features of majority of the isolates obtained from the pour plate culture characterization were similar to those of members of the Actinobacteria (Plates 6-18). All the 41 colonies isolated in Arginine Glycerol Salts Agar medium showed rapid growth and sporulation at a temperature range of 28-32°C under aerobic culture conditions. GAESP culture isolates revealed a diversity of colored pure colonies with a smooth to tough, leathery and filamentous appearance, and production of colored pigments, in the form of secretions into the culture medium within a few days of incubation. The colonies also produced the characteristic fresh earthy odour which is attributed to Geosmin production that is commonly associated with most members of the Actinobacteria (Wood *et al.*, 2001).

Several colonies had secretory structures on the aerial mycelia which secreted droplets of metabolites onto the surface of colonies prior to sporulation (Plates 9, 10, 13 and 17). Gram stained slide cultures of the isolates revealed a branched network of mycelia with characteristically shaped sporophores forming at the apexes of aerial mycelia (Plates 7b-13b). Some isolates had spores that stained dark red with Gram stain (Plate 6b and 9b); while a few had spore vacuoles which remained colorless in the presence of the Gram stain (Plates 10b and 12b).

Isolates GAESS 1-16 were obtained from *E. saccharina* larvae from sugarcane host plants. Like their Papyrus counterparts, the isolates had varied unique morphological characteristics that differentiated them from one another but they showed colony characteristics and growth patterns similar to those of the actinobacteria (Plates 13-18).

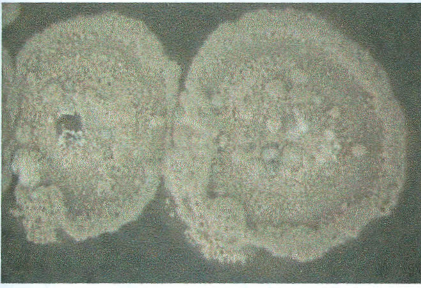
Morphological examination of the KMM1 culture isolates revealed bacterial colonies that had cream and yellow pigments (Plates 19-23). Microscopic examination showed unicellular bacteria, majority of which were Gram negative bacilli and Cocci (Plates 19-21), while two isolates, KMESP-5 and KMESS-2 had gram positive Cocci and bacilli respectively (Plates 22 and 23). PDA isolated colonies mostly had non filamentous colonies, characterized by white to cream-yellow colony forming units, which were generally larger than the KMM1 isolates (Plates 24-28). Some of the isolates had a network of root-like growths on the colony margin extending into the culture medium (plates 25a and 26a). The colonies had a raised elevation with a smooth surface though few had a flaky surface appearance. Microscopic examination revealed Gram positive, single yeast cells, with shapes varying from round, ovoid to rods. The cell sizes were larger than bacteria, with some cells joining to form pseudo-hyphae (Plates 24 and 27). One isolate had a brown secretion on the colony surface (Plate 24).

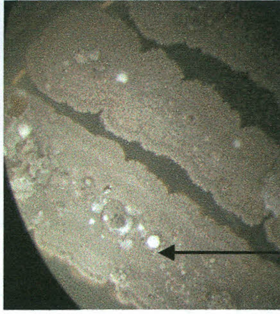
6a



6b

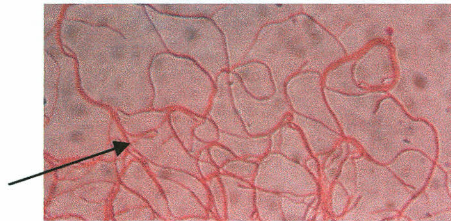
Plate 6: a) Isolate GAESP-1 single bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium, showing cream substrate mycelial growth with 'halo'-ring like formation in the medium and white aerial mycelia bearing grayish spore structures. 6b) Gram stained slide culture of GAESP-1 showing tributary-like mycelial branching and y-shape of sporophores at the tips of the mycelia network.





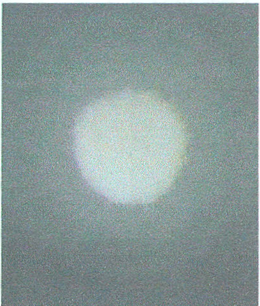
7a 7b

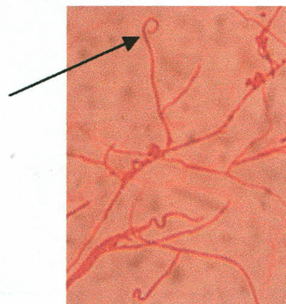
Plate 7: a) Isolate GAESP- 2 bacterial colonies on Arginine Glycerol salts agar (AGSA) culture medium, showing gray aerial sporulation on white surface mycelia. 7b) Original streak culture colony showing spots of secondary aerial vegetative growth covered with white mycelia (arrow).



7c

Plate 7: c) Isolate GAESP- 2 Gram stained slide culture showing mycelial branching. Arrow indicates position and the characteristic convoluted pattern of the sporophores at the tips of the mycelia network





8a. 8b.

Plate 8: a) Isolate GAESP- 4 single bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium. Notice the 'halo'-ring formation in the medium around the colony and white aerial mycelia. 8b) Gram stained slide culture showing filament branching. Arrow shows position and curled shape of the sporophores.

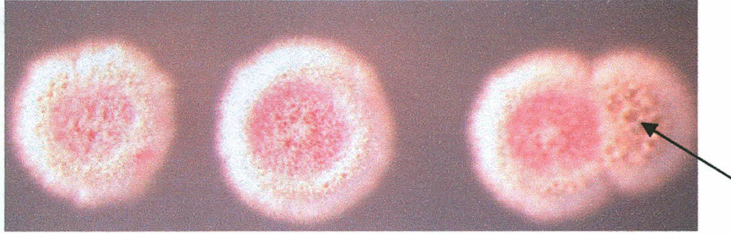

9a

Plate 9: a) Isolate GAESP- 5 bacterial colonies on Arginine Glycerol salts agar (AGSA) culture medium, showing white aerial sporulation on pink mycelia. Colonies on the right show different stages of growth, with one colony showing secretory structures on the aerial mycelia (indicated by an arrow).

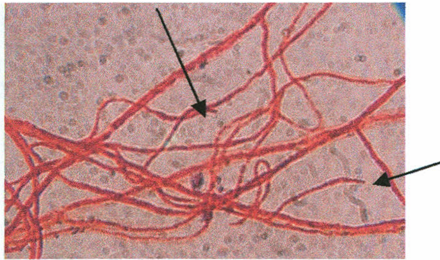

9b

Plate 9: b) Isolate GAESP-5 Gram stained slide culture showing characteristic near perpendicular alternate mycelial branching. Arrows indicate position and shape of the sporophores at the tips of the mycelia network.

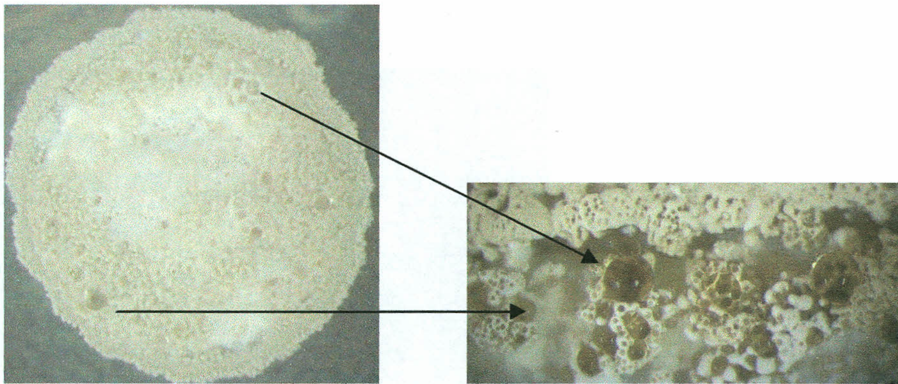
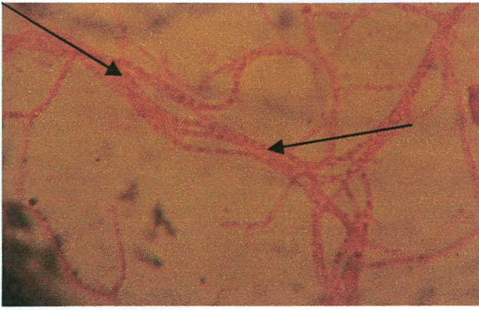
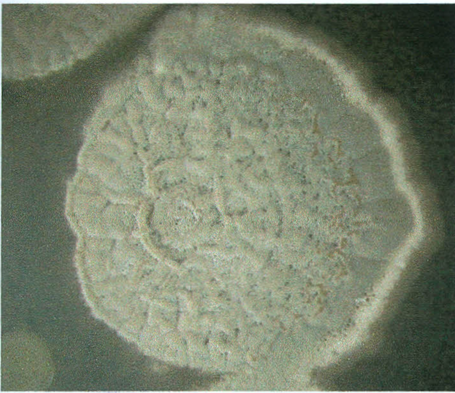


Plate 10: a) Isolate GAESP-10 bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium, showing white sporulation on beige aerial mycelia. Arrows show straw coloured secretory products on the aerial hyphae of an order colony.



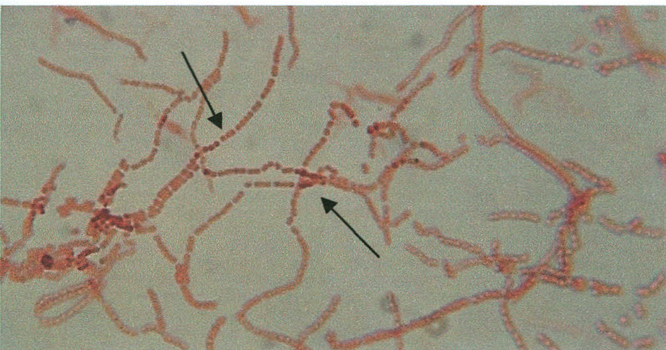
10b

Plate 10: b) Isolate GAESP-10 Gram stained slide culture showing filament branching. Arrows indicate rod-shaped chain of the sporophores with Gram variable spore vacuoles.



11a

Plate 11: a) Isolate GAESP-11 bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium, showing gray aerial sporulation on white surface mycelia.



11b

Plate 11: b) Isolate GAESP-11 Gram stained slide culture showing mycelial branching and Gram positive segmented spore chains.

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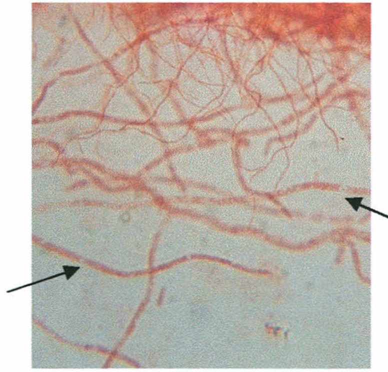
12a



12b

Plate 12: a) Isolate GAESP-15 bacterial colonies on Arginine Glycerol salts agar (AGSA) culture medium, showing white aerial sporulation centrally located on purplish-gray colony mycelia. Colonies show a rounded shape with a serrated margin. 12b). Gram stained slide culture showing mycelial branching. Arrows indicate position of sporophores with Gram variable (colorless) spores.

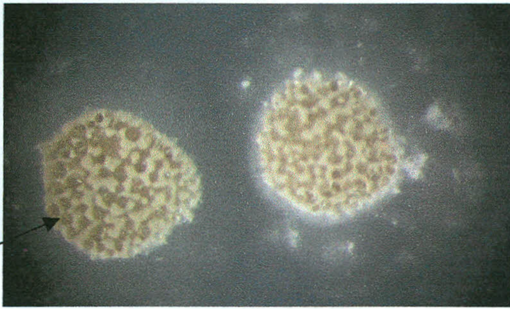


Plate 13: Isolate GAESS -1 bacterial colonies on Arginine Glycerol salts agar (AGSA) culture medium, showing cream aerial mycelia with white sporulation scattered peripheral to the colony. Colonies have straw coloured secretory products (arrow).

Plate 14: Isolate GAESS-2 bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium, showing beige dry-looking aerial mycelia surrounded by radial rings of beige sporulation. Colony shows a generally rounded shape with a serrated margin.

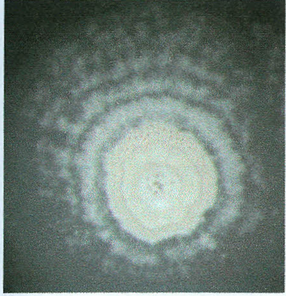


Plate 15: Isolate GAESS-3 bacterial colony on Arginine Glycerol salts agar (AGSA) culture medium, showing a slightly raised colony with cream leathery aerial mycelia, surrounded by curls of white sporulation.

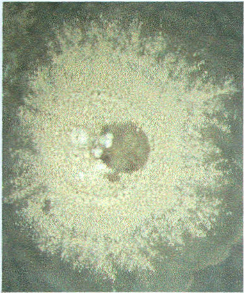


Plate 16: Isolate GAESS-6 single colony, on Arginine Glycerol salts agar (AGSA) culture medium showing beige dry-powdery looking aerial mycelia spreading outwards to form an irregular colony margin. In the middle of the colony is development of vegetative structures from the substrate mycelia.

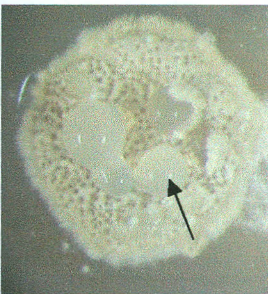


Plate 17: Isolate GAESS-12; mature colony on Arginine Glycerol salts agar (AGSA) culture medium showing beige-cream aerial mycelia with white sporulation and an irregular colony margin. Notice cream secretion on colony (arrow).

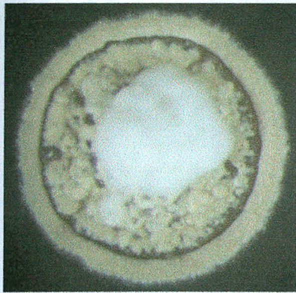
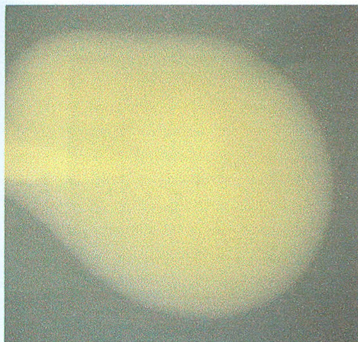
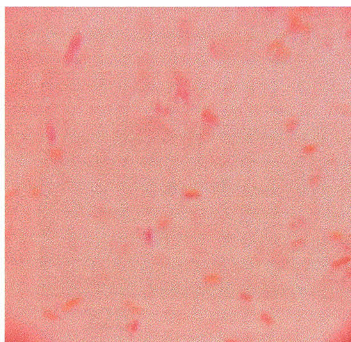


Plate 18: Isolate GAESS-14 single colony on Arginine Glycerol salts agar (AGSA) culture medium showing a round colony with cream aerial mycelia and well defined entire margin. In the middle is a white mass of white fluffy hyphae, raised above the aerial mycelia as secondary growth.



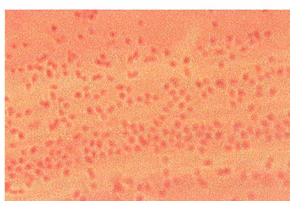
19a



19b

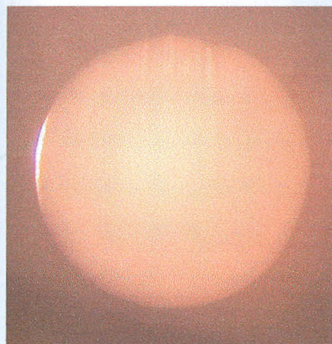
Plate 19: a) Isolate KMESP-1 single bacterial colony on Kenya Minimal culture medium (KMM1), showing yellow, raised, round colony, with an entire margin. 19b). Gram stained slide of KMESP-1, showing Gram negative bacilli (X1000magnification)

20a



20b

Plate 20: a) Isolate KMESP-2 bacterial colonies on Kenya Minimal culture medium (KMM1), showing cream colored, raised, oval colonies, with an entire margin. 20b) Gram stained slide of isolate KMESP-2 showing Gram negative cocco-bacilli (X1000Magnification)


21a

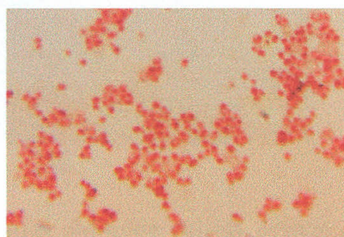

21b

Plate 21: a) Isolate KMESC-2 bacterial colony on Kenya Minimal culture medium (KMM1), showing cream colored, raised, oval colonies, with an entire margin. 21b). Gram stained slide of isolate KMESC-2 showing Gram negative Cocco-bacilli(X 1000 magnification)

22a

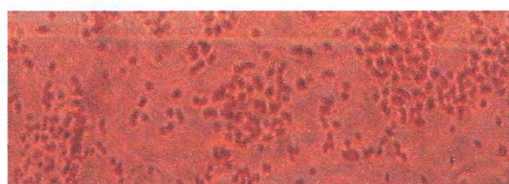
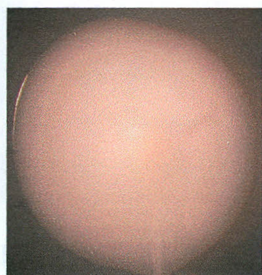

22b

Plate 22: a) Isolate KMESP-5 bacterial colonies on Kenya Minimal culture medium (KMM1), showing cream colored, raised, round colonies, with an entire margin. 22b). Gram stained slide of isolate KMESP-5 showing Gram positive Cocco-bacilli (X1000 magnification).


23a

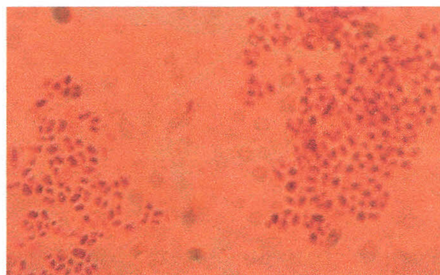

23b

Plate 23: a) Isolate KMESS-2 bacterial colony on Kenya Minimal culture medium (KMM1), showing cream colored, raised, round colonies, with an entire margin. 23b is a slide showing Gram positive bacilli (X1000 magnification)

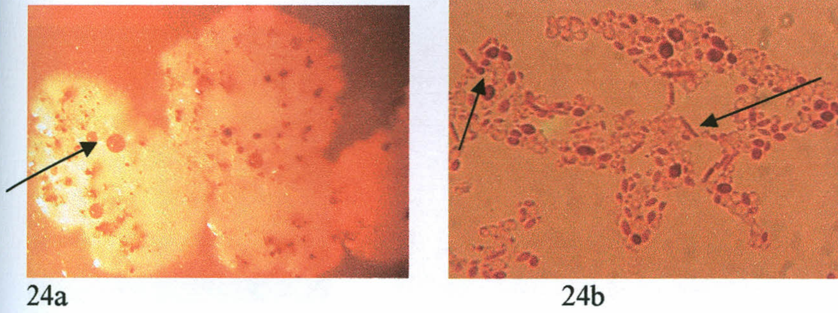

24a 24b

Plate 24: a) Isolate PDA ESP-2 plate colonies showing raised, cream coloured colonies with an irregular margin. Notice the orange–brown coloured secretion from the colony surface. 24b). Gram stained slide showing Gram positive round-ovoid yeast cells. Arrows show cells joined to form pseudo-hyphae (X1000 magnification).

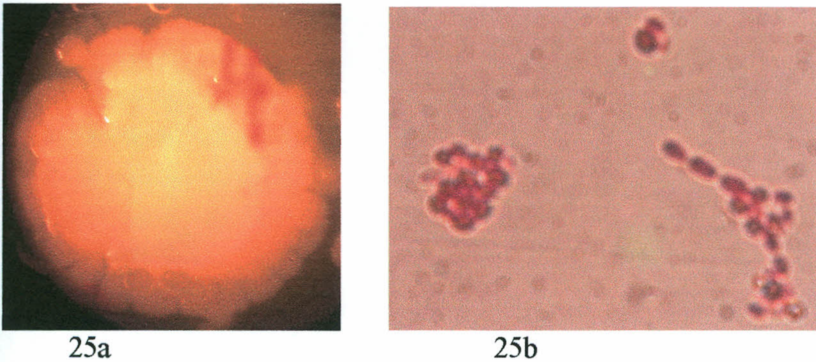

25a 25b

Plate 25: a) PDA ESP-4 plate colony showing raised, cream coloured colony with a serrated margin. 26b is a gram stained slide showing Gram positive cocco-bacilli with cells joining to form chains or clusters.
(X1000 magnification).

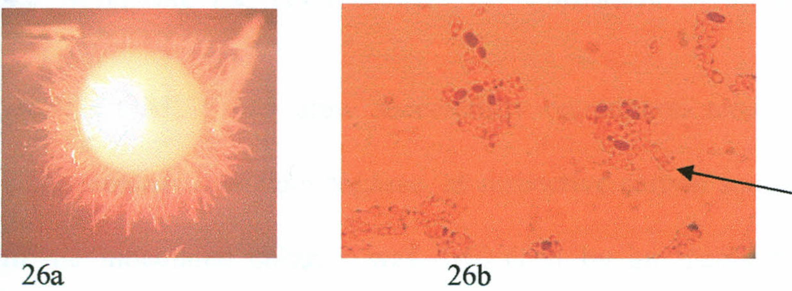
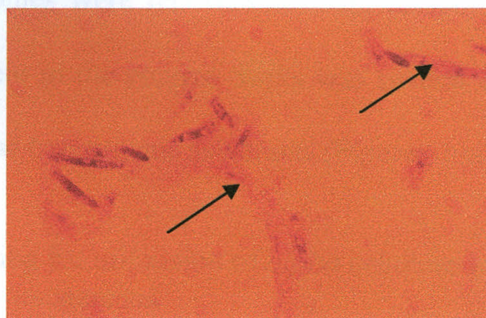
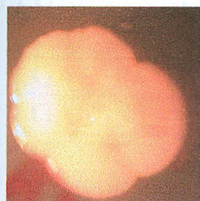

26a 26b

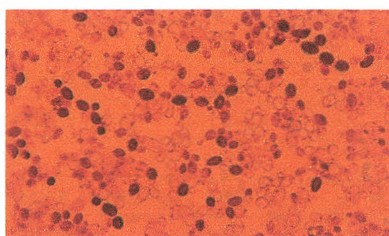
Plate 26: a) PDA ESS-2 plate colony showing raised, cream coloured, round colony with an entire margin. Notice the peripheral root-like growth in to the medium. 26b). Gram stained slide of isolate PDA ESS-2 showing Gram positive rod shaped yeast cells with some dividing Gram variable cells. (X1000 magnification).



27a 27b

Plate 27: a) Isolate PDA ESS5 colony showing a yellowish-cream, raised, round colony with an entire margin. 27b is a Gram stained slide showing Gram variable rod shaped yeast cells. Arrows show cells joined to form pseudo-hyphae.





28a 28b

Plate 28: a) PDA ESS-7 plate colony showing a yellowish-cream, raised, round colony with a serrated margin. 28b is a Gram stained slide of PDA ESS-7 showing Gram positive ovoid yeast cells. (X1000 magnification).

4.3 Screening bacterial isolates for utilization of alpha cellulose and sugarcane bagasse

Isolates GAESS1-15 grew successfully on mineral medium in the presence of alpha cellulose and sugarcane bagasse, as respective carbon sources (Tables 2 and 3). Growth in the inoculated cultures was evidenced by an increase in turbidity in shake flask cultures, gas production, and secretion of pigments in the liquid cultures; and recovery of viable isolates after 14 days of fermentation (Parekh, *et al.*, 1999; Novitsky and Morita, 1976). As shown in Figures 1 and 2, all isolates showed a cumulative increase in cell biomass in the two substrates, with sugarcane bagasse giving a higher level of cellular

biomass. No viable colonies were recoverable from control flasks after 14 days. The cumulative cellular protein in the control flasks was several orders of magnitude lower than the test samples in the two organic substrates (Figures 1 and 2).

Table 3: Bioassay screening for utilization of *alpha* cellulose by bacterial isolates from *E. saccharina* larval gut homogenates

Isolate	Mean Cellular Protein after 14 days (μg/ml)	Culture Pigmentation	Cell viability after 14 days fermentation CFUs / plate	Gas bubbling /Geosmin production	Turbidity
GAESS1	13.8	Cream	168	***	++++
GAESS2	9.5	Brown	70	**	+++
GAESS3	10.8	Purple/Brown	139	**	++++
GAESS4	11.8	Light brown	143	***	++++
GAESS5	8.6	Off white	69	***	+++
GAESS6	12.2	Cream/light brown	165	***	++++
GAESS7	11.8	Dark brown	147	****	++++
GAESS8	9.04	Off white	73	***	++
GAESS9	14.4	Cream/pink	75	***	++++
GAESS10	8.2	Cream	160	***	++++
GAESS11	13.8	Off white	170	***	+++++
GAESS12	8.7	Cream/light brown	100	***	+++
GAESS13	9.4	Dark brown	120	**	+++++
GAESS14	14	Light brown	150	**	++++
GAESS15	12	Light brown	135	***	++++
NC	1.76	- -	--	--	---

Score Key:
**** High gas bubbling; strong Geosmin odour
*** Moderate gas bubbling / Geosmin odour
** Low gas bubbling
++++ Opaque supernatant > 100 viable CFUs/plate recovered after 14 days.
+++ Translucent supernatant 50-100 viable CFUs / plate
++ Slightly cloudy suspension 50 CFUs / plate
- No gas formation, clear suspension; No viable colonies

Table 4: Bioassay screening for utilization of sugarcane bagasse by bacterial isolates from *E. saccharina* larval gut homogenates

Isolate	Mean cellular protein after 14 days(BCA) (µg/ml)	Culture Pigmentation.	Cell viability after 14 days fermentation CFUs / plate	Gas bubbling / Geosmin production	Turbidity
GAESS1	44.3	Cream	80	***	+++
GAESS2	48.3	Brown	125	****	++++
GAESS3	45.6	Purple/Brown	100	****	+++
GAESS4	55.7	Light brown	140	***	+++
GAESS5	57.3	Off white	150	****	++++
GAESS6	53.7	Cream	145	****	+++
GAESS7	59.7	Dark brown	130	****	++++
GAESS8	48.7	Off white/cream	54	**	++
GAESS9	57.3	Cream	150	***	++++
GAESS10	53	Cream	130	****	+++
GAESS11	48	Off white	110	****	++++
GAESS12	64.7	Cream	185	****	+++
GAESS13	37.7	Dark brown	130	***	++++
GAESS14	66	Light brown	200	***	++++
GAESS15	47.7	Light brown	85	****	+++
GAESS16	61.7	Light brown /cream (secretory)	150	****	+++++
NC	5	--	--	--	--

Score Key:

**** High gas bubbling; strong Geosmin odour

*** Moderate gas bubbling / Geosmin odour

** Low gas bubbling

++++ Opaque supernatant > 100 viable CFUs/plate recovered after 14 days.

+++ Translucent supernatant 50-100 viable CFUs / Plate

++ Slightly cloudy suspension 50 CFUs / plate

- No gas formation, clear suspension; No viable colonies

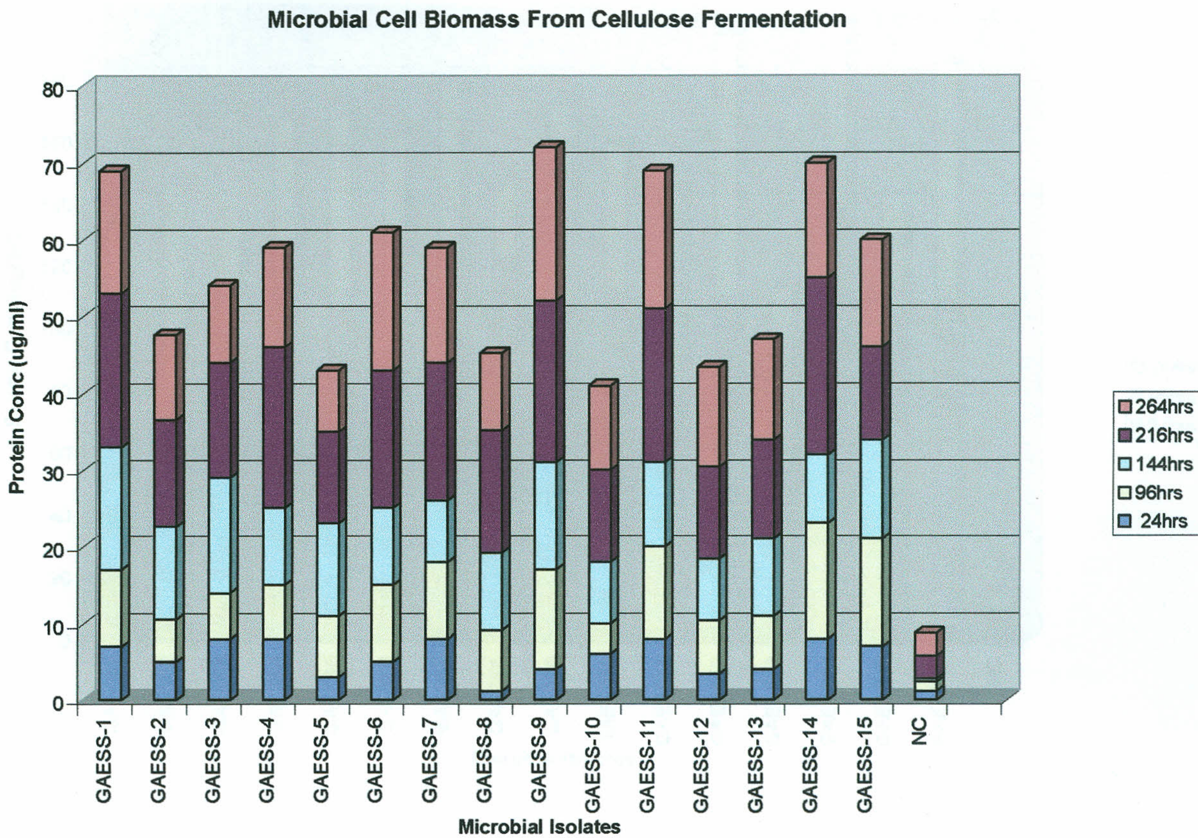


Figure 1: Cumulative total cellular protein of bacterial isolates from *E. saccharina* gut homogenates cultured in liquid mineral medium in the presence of alpha cellulose as sole source of organic carbon (GAESS1-15). Control culture (NC) contained mineral medium but no cellulose. The chart shows a rapid increase in cell biomass after 24hrs of fermentation.

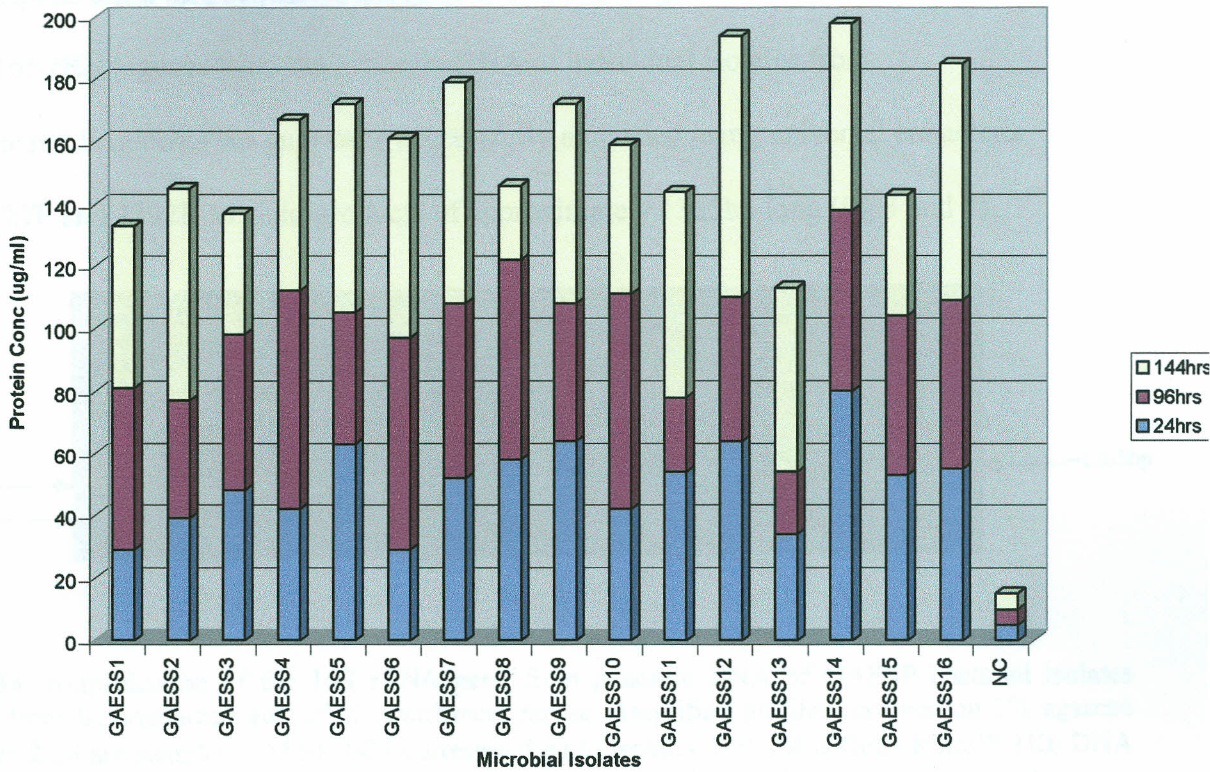


Figure 2: Cumulative total cellular protein of bacterial isolates from *E. saccharina* gut homogenates cultured in liquid mineral medium in the presence of sugarcane bagasse as sole organic carbon source (GAESS1-15). Control culture (NC) contained mineral medium but no sugarcane bagasse. The chart shows a rapid increase in cell biomass after 24hrs of fermentation

4.4.0 Molecular Characterization of Bacterial Isolates

4.4.1 Amplification and sequence analysis of 16S rRNA gene

16S rRNA genes from the genomic DNA of individual isolates from homogenized *E. saccharina* guts were successfully amplified using universal eubacteria primers 27F and 1392R to yield products of approximately 1365bp long (fig.3 and 4).

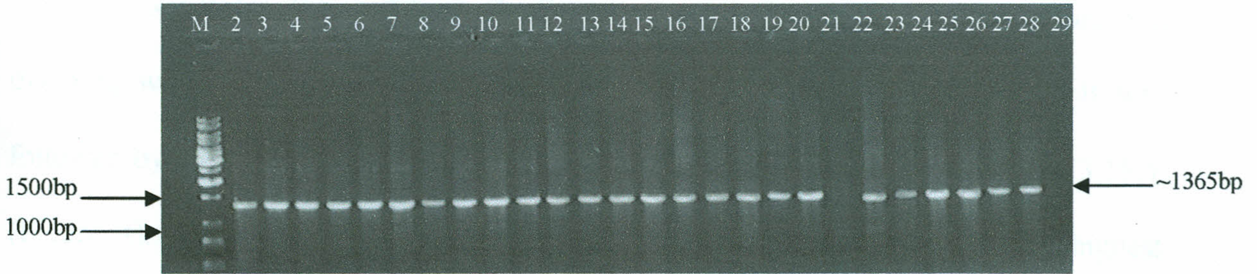


Figure 3a: Amplification of the 16S rRNA gene from genomic DNA of GAESP bacterial isolates obtained from homogenized guts of *E. saccharina* larvae. Amplified profiles resolved on 1% agarose gel. Lanes 2-28 are samples GAESP 1-25 corresponding to isolates 1-25. M is Gene Ruler™ 1Kb DNA ladder (Fermentas Inc, Germany) used as size marker. Lanes 21 and 28 are controls.

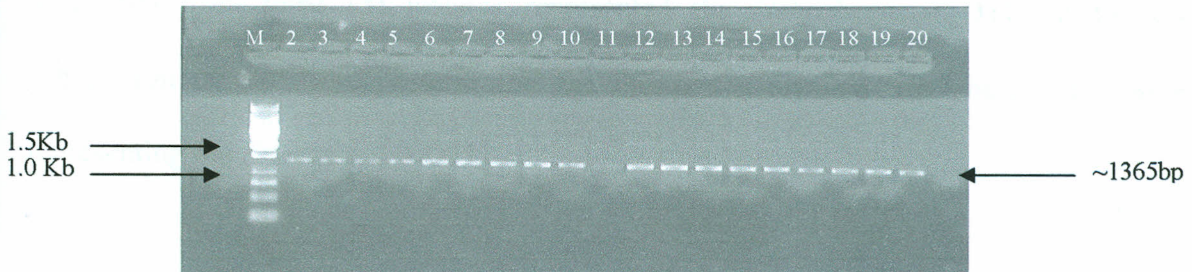


Figure 3b: 1% agarose gel profile representative of purified 16S rRNA fragments amplified from GAESP bacteria isolates from gut homogenates of *E. saccharina* larvae. M is Gene Ruler™1kb DNA ladder (Fermentas Inc, Germany), used as size marker. Lanes 2-20 show purified fragments of approximately 1365bp for isolates GAESP 1-19.

Alignment of the sequences with the SeqMatch online software of the Ribosomal Database project and BLAST search of the 16S rRNA gene sequences confirmed that all the isolates belonged to the Eubacteria domain. Query sequences that had the highest BLASTn search scores compared to GenBank sequences were assigned to operational taxonomic units (OTUs) so that every sequence in a specific OTU had the highest similarity score for the matching GenBank sequence (Table 4). The Actinobacteria division, which represented 71.9% of the isolates, dominated the sequences. It was followed by γ-Proteobacteria (15.6%), α- Proteobacteria (9.4%), and Firmicutes (3.1%), (Figure 4). Eighteen (18) of the isolates in the Actinobacteria division had highest similarity scores to the *Streptomyces* OTU, four (4) with *Microbacterium* while one (1) isolate was placed in the *Cellulosimicrobium* OTU. Two (2) Isolates in the γ- subdivision of the Proteobacteria each represented sequences in the *Enterobacte*r and *Klebsiella* OTUs respectively, while one (1) isolate represented *Stenotrophomonas*. Query sequences from three (3) isolates represented the *Ochrobactrum* OTU in the α-subdivision of the Proteobacteria and the Firmicutes division had one (1) sequence representing the *Enterococcus* OTU.

Table 5: Nucleotide BLAST search results for 16S rRNA gene sequences of bacterial isolates from *E. saccharina* gut homogenates detected by16S rRNA primers UN 27forw and 1392rev

ate ry uence	Closest hit in GenBank (Accession No)	Phylogenetic affiliation	Percentage similarity	E-value
ESP1	*Streptomyces zaomyceticus* (AB184346.1)	Streptomycetaceae	99.0	0.0
ESP3	*Streptomyces zaomyceticus* (AB184346.1)	Streptomycetaceae	99.0	0.0
ESP13	*Streptomyces zaomyceticus* (EU593738.1)	Streptomycetaceae	98.0	0.0
ESP17	*Streptomyces zaomyceticus* (EF178685.1)	Streptomycetaceae	98.0	0.0
ESP23	*Streptomyces zaomyceticus* (EF178685.1)	Streptomycetaceae	99.0	0.0
ESP2	*Streptomyces drozdowiczii* (EF654097.1)	Streptomycetaceae	97.0	0.0
ESP4	*Streptomyces fradiae* (AB184253.1)	Streptomycetaceae	99.0	0.0
ESP5	*Streptomyces rubrogriseus* (AF503501.1)	Streptomycetaceae	99.0	0.0
ESP6	*Streptomyces sampsonii* (AB362247.1)	Streptomycetaceae	99.0	0.0
ESP8	*Streptomyces sampsonii* (AB362247.1)	Streptomycetaceae	99.0	0.0
ESP9	*Streptomyces sampsonii* (AB362247.1)	Streptomycetaceae	99.0	0.0
ESP7	*Streptomyces albidoflavus* (FJ600729.1)	Streptomycetaceae	99.0	0.0
ESP19	*Streptomyces albidoflavus* (FJ600729.1)	Streptomycetaceae	99.0	0.0
ESP10	*Streptomyces celluloflavus* (AB184476.1)	Streptomycetaceae	99.0	0.0
ESP11	*Microbacterium sp.* OUTC 4 (FJ210845.1)	Microbacteriaceae	99.0	0.0
ESP15	*Microbacterium oxydans* (GQ152132.1)	Microbacteriaceae	93.0	0.0
ESP24	*Microbacterium oxydans* (FJ950590.1)	Microbacteriaceae	87.0	0.0
ESP25	*Microbacterium oxydans* (GQ279110.1)	Microbacteriaceae	99.0	0.0
ESP12	*Streptomyces venezuelae* (AY703449.1)	Streptomycetaceae	94.0	0.0
ESP14	*Streptomyces eurocidicus* (AY999748.1)	Streptomycetaceae	99.0	0.0
ESP16	*Streptomyces phaeochromogenes* (EU594476.1)	Streptomycetaceae	94.0	0.0
ESP18	*Streptomyces sp.* (FJ001754)	Streptomycetaceae	99.0	0.0
ESP20	*Cellulosimicrobium cellulans* (EU287931.1)	Promicromonosporacea	89.0	0.0
IESP1	*Ochrobactrum sp.* (EF465412.1)	Brucellaceae	89.0	0.0
IESP11	*Ochrobactrum haematophilum*(T) (AM422370.1)	Brucellaceae	97.0	0.0
IESC5	*Ochrobactrum anthropi* (AM490611.1)	Brucellaceae	94.0	0.0
IESP2	*Klebsiella sp.* (HM462444.1)	Enterobacteriaceae	89.0	0.0
IESP7	*Klebsiella oxytoca* (AJ871862.1)	Enterobacteriaceae	88.0	0.0
IESP4	Bacterium B28. (FJ628394.1)	Unclassified	84.0	0.0
IESC2	*Enterobacter sp.* (EU430751.1)	Enterobacteriaceae	92.0	0.0
IESP5	*Enterococcus casseliflavus* (*FJ915804.1*)	Enterococcacae	97.0	0.0
IESC1	*Stenotrophomonas maltophilia* (AB294557.1)	Xanthomonadacea	99.0	0.0

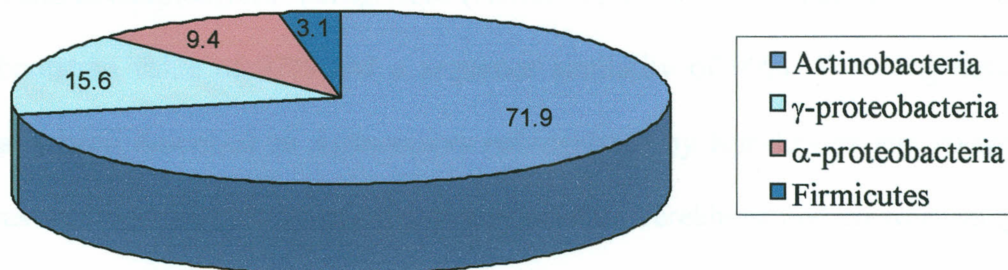


Figure 4: Percentage distribution of Phylogroups from microbial isolates obtained from homogenized guts of *E. saccharina* larvae

4.4.2 Phylogenetic analysis of 16S rRNA sequences from bacterial Isolates

Phylogenetic analysis of the 16S rRNA gene sequences placed the isolates into four (4) main clusters in the Eubacteria domain, namely *Streptomyces*, *Proteobacteria*, *Microbacterium* and *Cellulosimicrobium/Cellulomonas*, as shown in Figure 5. Sequences from culture Isolates GAESP-1, GAESP-3; GAESP-17 and GAESP-23 clustered with *Streptomyces zaomyceticus* Accession numbers AB184346.1 and EF178685.1 respectively, with sequence similarity scores of 98-99%. *Streptomyces zaomyceticus* produces Zaomycin, an acidic lipopeptide antibiotic with activity against Gram positive bacteria (Borders *et al.*, 2006; Hinuma, 1954). Isolates GAESP-6, GAESP8 and GAESP-9 formed a cluster with *Streptomyces sampsonii,* AB362247.1 and had a sequence

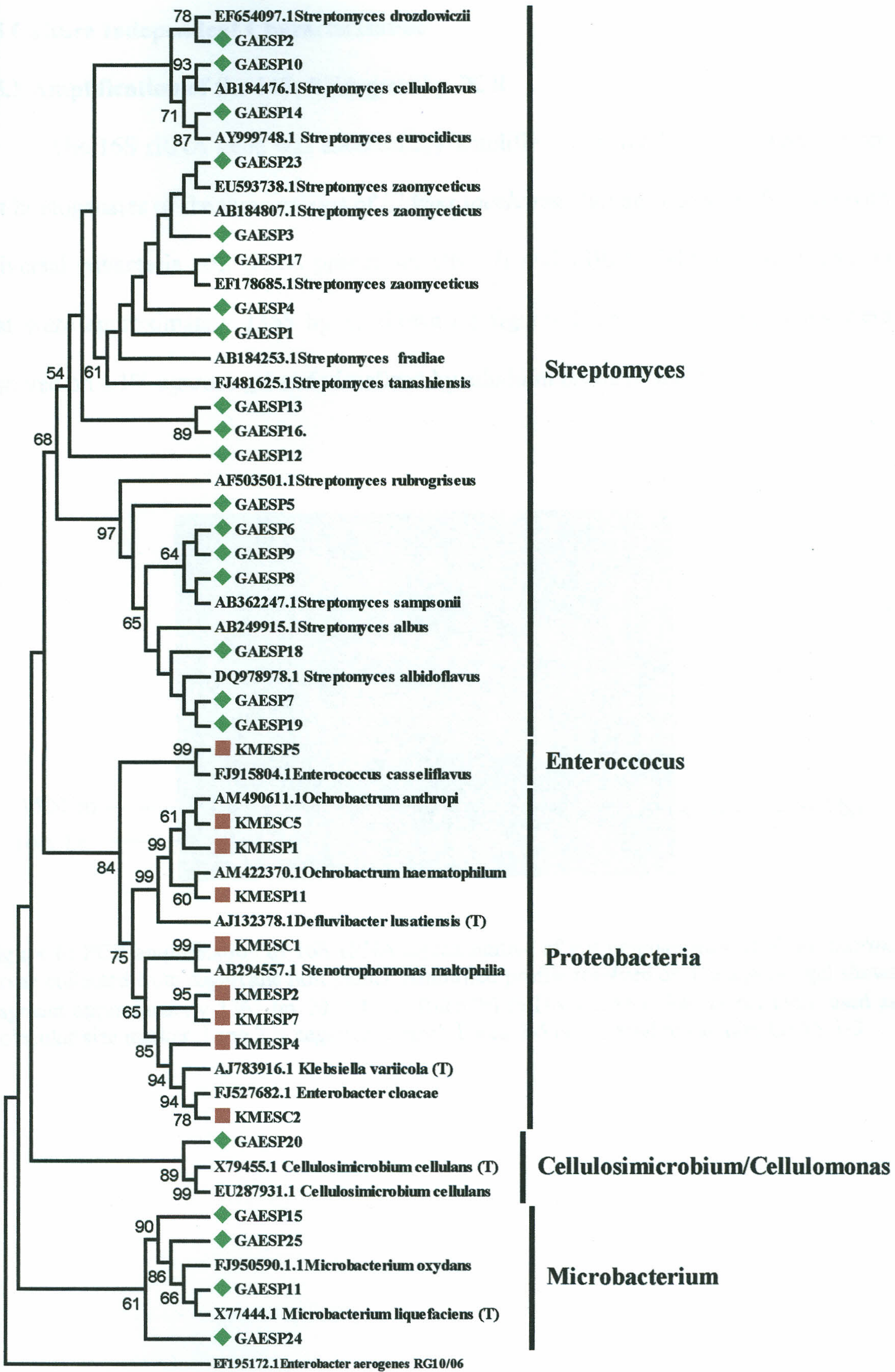
similarity of 99%. Isolate GAESP-2 clustered together with *Streptomyces drozdowiczii*, (EF654097.1) with a similarity score of 97% and bootstrap support of 78%. Isolates GAESP-7 and GAESP-19 both clustered with *Streptomyces albidoflavus* (FJ600729.1) with sequence similarities of 99% to GenBank sequences. Isolate GAESP-5 had close relatedness to *Streptomyces rubrogriseus* (AF503501.1) and the affiliation was supported by a bootstrap value of 97% and a sequence similarity of 99%. *S. rubrogriseus* had originally been described as *Actinomyces rubrogriseus* by Kurylowicz and co-workers and was validated in 1986 as combined species after Terekhova and co-workers (IJSB, 1986; Gause *et al.*, 1983). Isolate GAESP 10 clustered closely with *Streptomyces celluloflavus* (AB184476.1), with a high sequence similarity of 99% and a bootstrap support of 93%. *S. celluloflavus* is a cellulolytic actinomycete that has previously been shown to play a significant role in cellulose degradation and litter feeding by the woodlouse *Porcellio scaber* (Ihnen and Zimmer, 2008).

Isolate GAESP-20 branched separately to form a lone cluster together with *Cellulosimicrobium cellulans* (EU287931.1), with bootstrap confidence and sequence similarity of 89%., respectively.

Isolates GAESP-11, GAESP-15, GAESP-24 and GAESP-25 branched to form the *Microbacterium* cluster, with bootstrap supports of up to 90% and sequence similarity score values ranging from 87 to 99%. Members of this cluster belong to the phylogenetically separate branch in the family of Microbacteriaceae.

Nearly all the unicellular isolates were affiliated to the proteobacteria cluster, with representatives of the α-Proteobacteria and γ- Proteobacteria subdivisions while one isolate, KMESP-5 belonged to the Firmicutes division (Figure 5). Isolates KMESP-1,

KMESC-5 and KMESP-11 were affiliated to the genus *Ochrobactrum*, and had sequence similarities ranging from 87% to 97% with database sequences. Two isolates, KMESP-2 and KMESP-7 formed a cluster with the genus *Klebsiella* with sequence similarities of 87% and 89% respectively, while KMESC-2 showed relatedness to unidentified species in the genus *Enterobacter* (EU430751.1). Isolate KMESC-1 had close sequence relatedness to *Stenotrophomonas maltophilia* (AB294557.1), with bootstrap value and sequence similarity of 99% respectively. The only close affiliate to the Firmicutes division, isolate KMESP-5, showed close sequence relatedness to *Enterococcus sp.* (FJ965843.1) with a high bootstrap value of 99% and a similarity of 97% to database sequences.


EF654097.1Streptomyces drozdowiczii
GAESP2
GAESP10
AB184476.1Streptomyces celluloflavus
GAESP14
AY999748.1 Streptomyces eurocidicus
GAESP23
EU593738.1Streptomyces zaomyceticus
AB184807.1Streptomyces zaomyceticus
GAESP3
GAESP17
EF178685.1Streptomyces zaomyceticus
GAESP4
GAESP1
AB184253.1Streptomyces fradiae
FJ481625.1Streptomyces tanashiensis
GAESP13
GAESP16.
GAESP12
AF503501.1Streptomyces rubrogriseus
GAESP5
GAESP6
GAESP9
GAESP8
AB362247.1Streptomyces sampsonii
AB249915.1Streptomyces albus
GAESP18
DQ978978.1 Streptomyces albidoflavus
GAESP7
GAESP19
KMESP5
FJ915804.1Enterococcus casseliflavus
AM490611.1Ochrobactrum anthropi
KMESC5
KMESP1
AM422370.1Ochrobactrum haematophilum
KMESP11
AJ132378.1Defluvibacter lusatiensis (T)
KMESC1
AB294557.1 Stenotrophomonas maltophilia
KMESP2
KMESP7
KMESP4
AJ783916.1 Klebsiella variicola (T)
FJ527682.1 Enterobacter cloacae
KMESC2
GAESP20
X79455.1 Cellulosimicrobium cellulans (T)
EU287931.1 Cellulosimicrobium cellulans
GAESP15
GAESP25
FJ950590.1.1Microbacterium oxydans
GAESP11
X77444.1 Microbacterium liquefaciens (T)
GAESP24
EF195172.1Enterobacter aerogenes RG10/06
Streptomyces
Enteroccocus
Proteobacteria
Cellulosimicrobium/Cellulomonas
Microbacterium

4.5 Culture Independent Characterization

4.5.1 Amplification of the 16SrRNA gene by PCR

The 16S rRNA gene was successfully amplified from total DNA extracted from gut homogenates of the three groups of *Eldana saccharina* larvae. The amplification with universal eubacteria 16S rRNA primer set UN 27f and 1392R yielded PCR fragments that were approximately 1365 bp as shown on figures 6 and 7. The amplicons were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

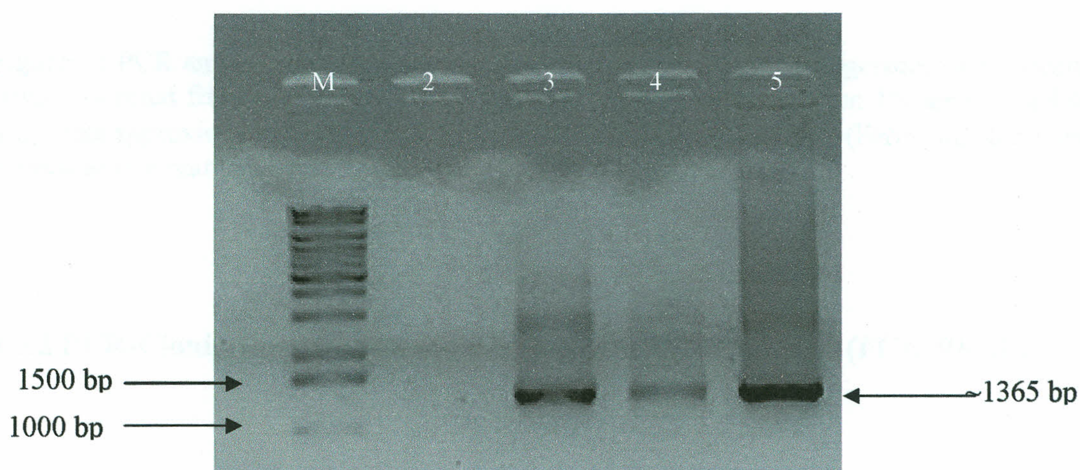


Figure 6: PCR amplification of 16S rRNA representative of gut homogenates of *E. saccharina* larvae collected from sugarcane host plants. Amplified profile resolved on 1% agarose gel shows fragment approximately 1365 bp. M is Gene Ruler™1kb DNA ladder (Fermentas Inc,), used as molecular size marker. Lane 2 is negative control. Lanes 3-5 correspond to samples GESS 1-3.

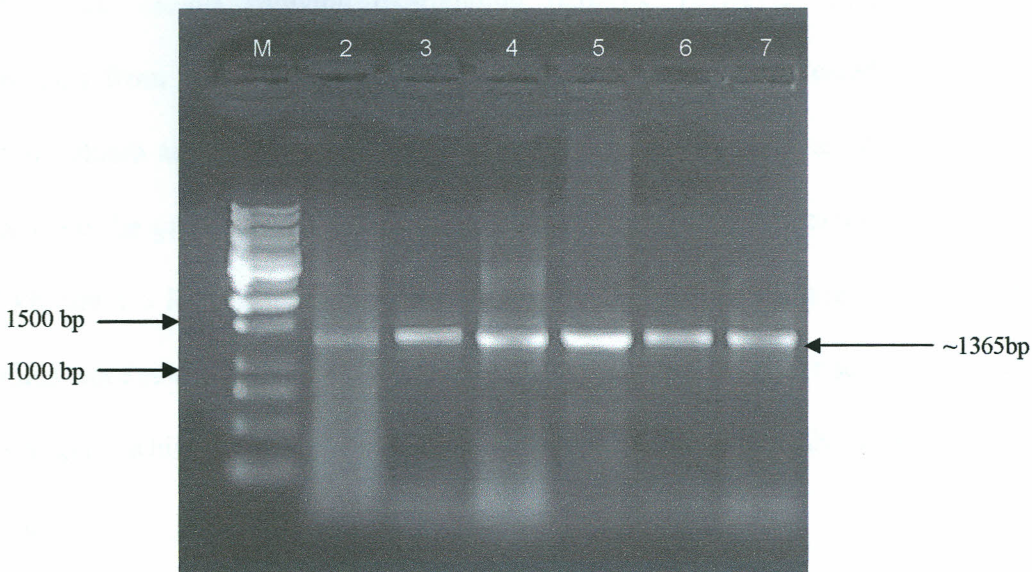


Figure 7: PCR amplification of 16S rRNA representative of gut homogenates of *E. saccharina* larvae collected from papyrus host plants. Amplified profile resolved on 1% agarose gel shows fragments approximately 1365 bp. M is Gene Ruler™ 1kb DNA ladder (Fermentas Inc,), used as molecular size marker. Lanes 2-7 are samples GESP 1-6

4.5.2 PCR-Cloning and Restriction Fragment Length Analysis (PCR-RFLP)

Gel purified 16SrRNA genes from the PCR products were successfully cloned into the 2.1 pCR® -TOPO vector and the transformation efficiency of the chemically competent DH5α-T1 strain of *E.coli* cells confirmed by growth of a large number of white colonies on LB medium in the presence of X-gal. Restriction digestion analysis of the positive clones from the three populations of *E. saccharina* with a cocktail of five restriction enzymes revealed a high degree of polymorphic bands as shown in figures 8a and 8b. The plasmids bearing the PCR construct had an approximate molecular size of 5.2kb. The restriction digestion generated fragments ranging from 200 bp to approximately 1.3 kb,

with a few clones showing overlapping patterns. However, digestion of plasmid constructs from the laboratory reared *E. saccharina* larvae revealed a lower level of polymorphism as shown in Figure 8c. Restriction digestion of the uncloned 16S rRNA gene from the gut homogenates produced five fragments of approximately 750 bp, 1 kb, 1.2 kb and 1.3 kb (Figure 8c). Thirty five (35) clones with unique restriction fragment profiles were selected from the Papyrus group of *E. saccharina* for sequencing of the 16S rRNA gene while sixteen (16) clones were selected from the laboratory reared control group.

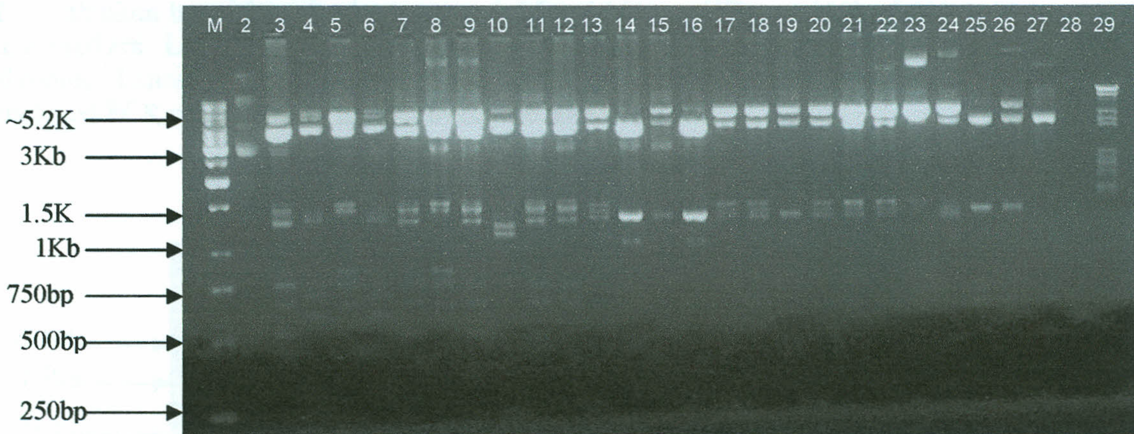


Figure 8a: RFLP profiles of PCR inserts cloned into pCR® 2.1 plasmid and digested with a cocktail of five Restriction enzymes, *AvaI*, *BamHI*, *Hind* III, *KpnI* and *XbaI*. Digested clones from *E. saccharina* larval gut homogenates derived from papyrus host plants. Fragments resolved on 1.2% TAE ethidium bromide stained agarose gel. M is 1kb DNA ladder used as size marker. Lanes 2 and 27 are unligated plasmid controls. Lanes 3 - 26 represent digested constructs; lane 28 is blank while lane 29 represents ʎ DNA digested with the same enzyme cocktail.

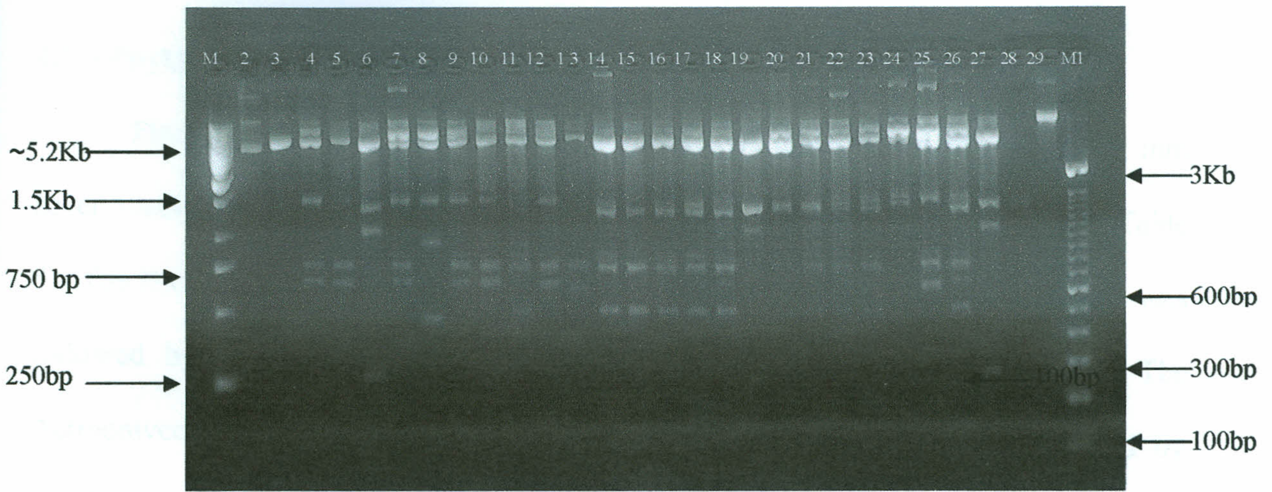


Figure 8b: RFLP profiles of PCR inserts cloned into pCR® 2.1 plasmid and digested with a cocktail of five Restriction enzymes, *Ava*I, *BamH*I, *Hind* III, *Kpn*I and *Xba*I. Digested clones from *E. saccharina* gut homogenates derived from sugarcane plants. Fragments resolved on 1.2% TAE ethidium bromide stained agarose gel. M and M1 are 1Kb and 100bp DNA ladders used as size markers. Lane 2 is undigested plasmid while lanes 3 and 29 show linearized, un-ligated plasmids. Lanes 4-27 represent digested GESS clones while lane 28 shows digestion of the uncloned PCR product using the same enzyme cocktail.

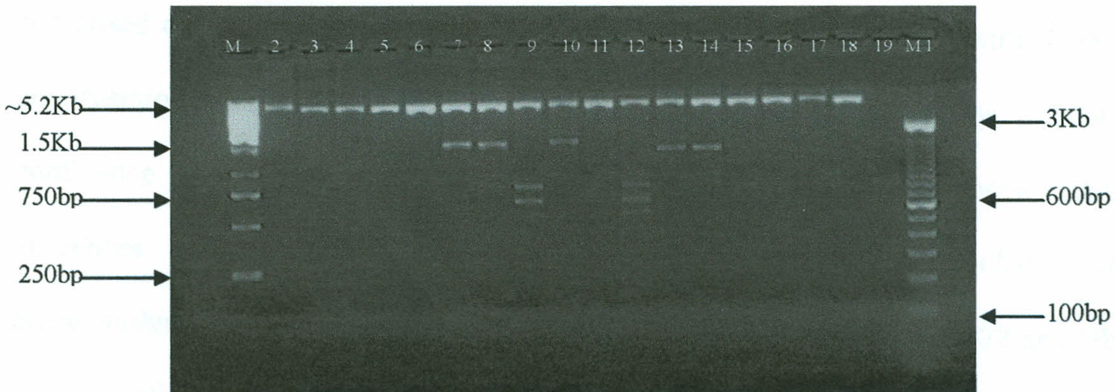


Figure 8c: RFLP profiles of PCR inserts cloned into pCR® 2.1 plasmid and digested with a cocktail of five Restriction enzymes, *Ava*I, *BamH*I, *Hind* III, *Kpn*I and *Xba*I. Digests of clones derived from larval gut homogenates of laboratory reared *E. saccharina.* Fragments resolved on 1.2% TAE, ethidium bromide stained agarose gel. Lanes 2-18 are GESC 1-17 clonal digests while lane 19 shows digested 16S rRNA gene from the gut homogenates with five fragments of approximately 750 bp, 1000 bp, 1.2 kb and 1.3 kb.

4.5.3 Phylogenetic analysis of 16S rRNA gene sequences

Phylogenetic analysis of the cloned 16S rRNA gene placed the sequences into seven main clusters covering ten orders in the Eubacteria domain (Figure 10 and Table 5). The Rickettsiales order was largest with 27.7 % of the clonal sequences and it was followed by the Enterobacteriales with 21.3% of the sequences (Figure 10). The Actinomycetales and Lactobacillales had 10.7% of the sequences each, followed by Bacillales, Xanthomonadales, Caulobacterales, Rhizibiales, Burkholderiales and Pseudamonadales orders. The Proteobacteria division was the largest Phylogroup with members from the alpha and βeta subdivisions.

The seven identified clusters represented five Phylogenetic sub-divisions of the Eubacteria domain, namely the Actinobacteria, alpha-Proteobacteria, βeta-Proteobacteria and gamma-Proteobacteria and the Firmicute*s* (Figure 10). The α-*Proteobacteria* comprised of seventeen clones, with 13 of the clones clustering closely with *Wolbachia endosymbiont* taxa (EU096232.1). This affiliation was supported by a bootstrap confidence of 99% and a similarity score of 94 to 99% to the closest GenBank sequences. Clones GESP-2 and GESP-4 clustered together with *Ochrobactrum haematophilum* (GenBank accession number AM422370.1), while GESP-32 and GESP-33 were affiliated to the *Caulobacter* genus. Both affiliations had a bootstrap value of up to 99% and the sequences shared similarities of 90-98% with the closest known GenBank sequence. The Actinobacteria sub-division was represented by Clones GESP-8, GESP-29, GESP-31, GESP-35 and GESC-6 all of which were closely affiliated to the Actinobacteria cluster, with all the nodes being supported by bootstrap values of 95-99%. Clone GESP-8 clustered closely with the GenBank sequence of *Gordonia*

polyisoprenivorans (*DQ154925.1*), with a bootstrap confidence of 99% and a sequence similarity of 93% to the nearest neighbour in the GenBank and RDP databases. Clones GESP-29, GESP-31, GESP-35 and GESC-6 showed close sequence relatedness with high G+C content Gram positive bacterial sequences for *Microbacterium aurum, Nocardioides hankookensis, Plantibacter flavus* and *Propionibacterium acnes* respectively.

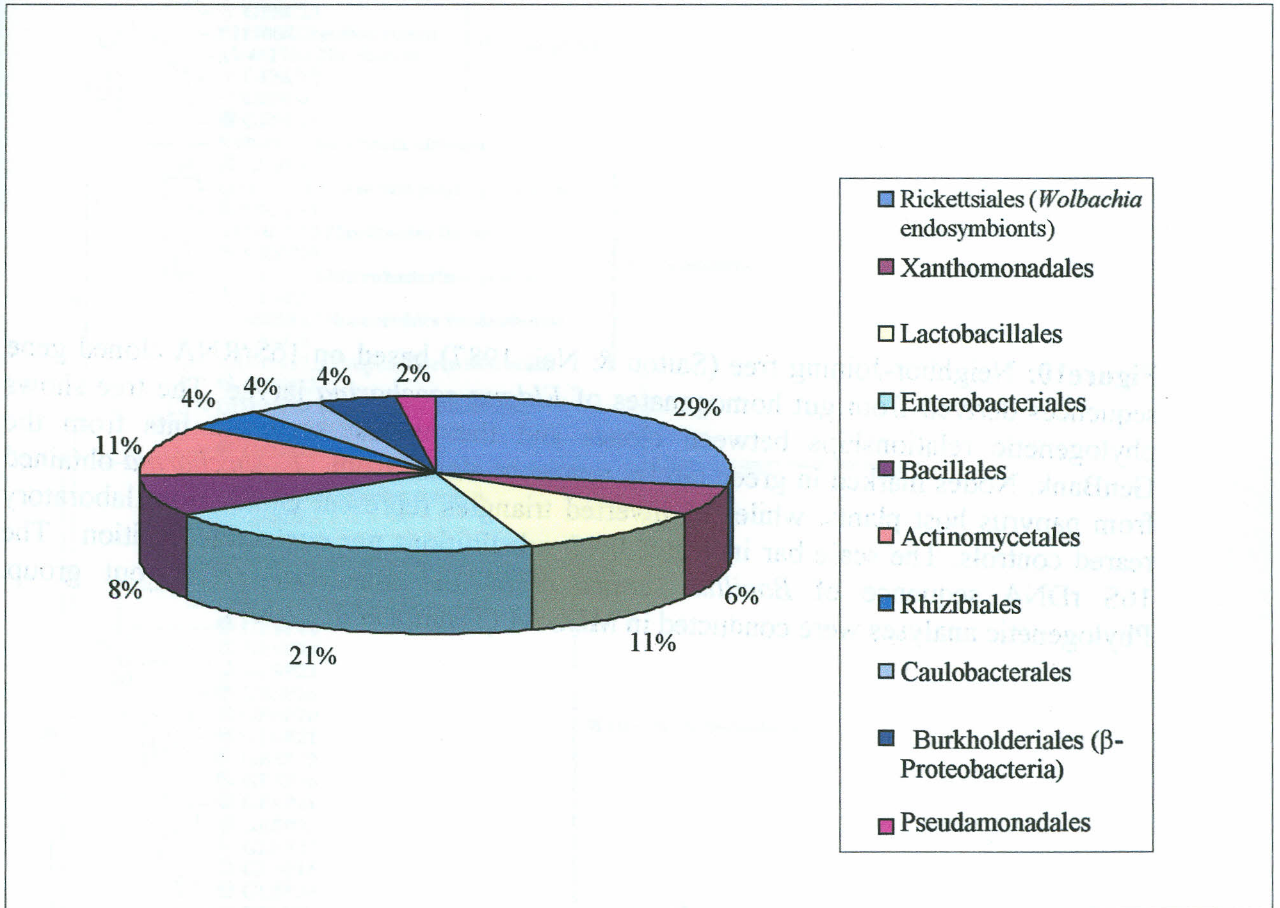
The Firmicutes sub-division had representatives in the *Bacillus* and *Enterococcus clusters*. Clone GESC-4 and GESC-15 were identical and they clustered closely with *Bacillus sp.* (AY461752.2), with bootstrap value of 54% and sequence similarities of 95% and 96 % respectively. Clones GESC-14, GESP-12, GESP-14 and GESP-17 clustered closely with members of the genus *Enterococcus* and were supported by a bootstrap value of 99% and sequence similarities greater than 98%, except for GESP-13, which had a similarity of 92% to the nearest relative both in the GenBank and the Ribosomal Databases. In the γ- sub division of the Proteobacteria were clones closely affiliated to the *Klebsiella, Serratia* and *Stenotrophomonas* taxa. Clones GESP-1, GESP-5, GESP-7, GESP-9, GESP-10, GESP-28 and GESP-34 clustered under *Klebsiella pneumoniae* (EU301772) and had sequence similarities of 97% -99% to their respective closest relatives in the GenBank data base. Clones GESC-2, GESC-11 and GESC-12 branched alone to form a cluster under *Stenotrophomonas* taxon (AY 162068.1) with a bootstrap support of up to 98% and sequence similarities of 89-98%. Clones GESC-3 and GESC-16 were affiliated to *Achromobacter xylosoxidans* with bootstrap values of 99% and 93% respectively and sequence similarities of 98%. These two clones were the only representatives of the β-Proteobacteria sub-division.

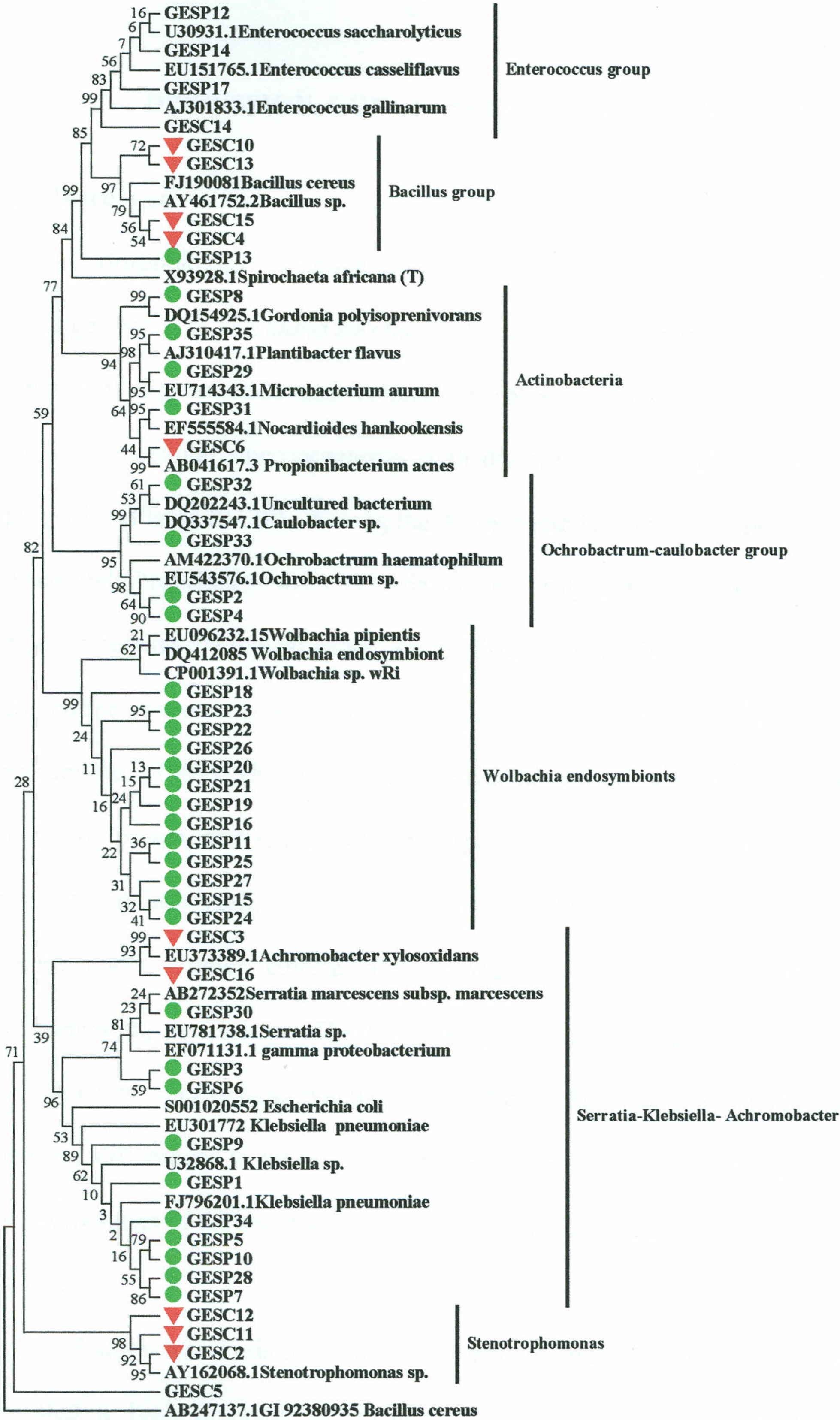
Table 6: Nucleotide BLAST search results for 16S rRNA cloned gene sequences from *Eldana saccharina* gut homogenates detected by16S rRNA primers UN 27forw and 1392rev

Query sequence	Closest GenBank Sequence hit and Accession Number	Phylogenetic affiliation	Percent similarity	E-value
GESP1	*Klebsiella pneumoniae* (GI:206564770)	Enterobacteriales	99.0	0.0
GESP5	*Uncultured bacterium* (EU301772)	Enterobacteriales	99.0	0.0
GESP7	*Klebsiella pneumoniae* (FJ796201.1)	Enterobacteriales	98.0	0.0
GESP9	*Klebsiella pneumoniae* 342 (CP000964.1)	Enterobacteriales	97.0	0.0
GESP10	*Uncultured bacterium clone* (EF509951.1)	Enterobacteriales	98.0	0.0
GESP28	*Klebsiella pneumoniae* 342 (CP000964.1)	Enterobacteriales	96.0	0.0
GESP34	*Klebsiella sp. strain zlmy* (U32868.1)	Enterobacteriales	99.0	0.0
GESP2	*Ochrobactrum sp.* (AB490238.1)	Rhizibiales	95.0	0.0
GESP4	*O. haematophilum* (AM422370.1)	Rhizibiales	98.0	0.0
GESP32	*Uncultured bacterium* (DQ202243.1)	Caulobacterales	97.0	0.0
GESP33	*Caulobacter sp.* (DQ337547.1)	Caulobacterales	90.0	0.0
GESP11	*Wolbachia sp.* (CP001391.1)	Rickettsiales	98.0	0.0
GESP 15	*Wolbachia pipientis* (EU096232.1)	Rickettsiales	98.0	0.0
GESP 16	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	99.0	0.0
GESP 18	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	98.0	0.0
GESP 19	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	98.0	0.0
GESP 20	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	98.0	0.0
GESP 21	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	99.0	0.0
GESP 22	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	94.0	0.0
GESP 23	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	95.0	0.0

Query sequence	GenBank hit / accession number	Phylogenetic Affiliation	Percent similarity	E-value
GESP 24	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	99.0	0.0
GESP 25	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	97.0	0.0
GESP 26	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	99.0	0.0
GESP 27	*Wolbachia sp.* wRi (CP001391.1)	Rickettsiales	99.0	0.0
GESP3	*Serratia marcescens* (EU407551.1)	Enterobacteriales	100.0	0.0
GESP6	*Uncultured γ-* proteobacterium (EF071131.1)	Enterobacteriales	94.0	0.0
GESP30	*Serratia sp.* (EU781738.1)	Enterobacteriales	99.0	0.0
GESP8	*Gordonia polyisoprenivorans* (DQ154925.1)	Actinomycetales (Gordoniacea)	93.0	0.0
GESP29	*Microbacterium aurum* (EU714343.1)	Actinomycetales	92.0	0.0
GESP31	*Nocardioides hankookensis* (EF555584.1)	Actinomycetales (Nocardioidaceae)	86.0	0.0
GESP35	*Plantibacter flavus* (AJ310417.1)	Actinomycetales	99.0	0.0
GESC6	*Propionibacterium acnes* (AB041617.3)	Actinomycetales (Propionibacteriaceae)	96.0	0.0
GESP12	*Enterococcus casseliflavus* (EU151765.1)	Lactobacillales	99.0	0.0
GESP13	*Enterococcus* saccharolyticus (U30931.1)	Lactobacillales	92.0	0.0
GESP14	*Enterococcus casseliflavus* (EU151765.1)	Lactobacillales	99.0	0.0
GESP17	*Enterococcus casseliflavus* (AF039899.1)	Lactobacillales	98.0	0.0
GESC1	*Enterococcus gallinarum* (AJ301833.1)	Lactobacillales	99.0	0.0
GESC2	*Stenotrophomonas sp* (AY162068.1)	Xanthomonadales	98.0	0.0
GESC3	*Achromobacter xylosoxidans* (EU373389.1)	Burkholderiales	98.0	0.0
GESC5	*Uncultured bacterium*(DQ675028.1)	Pseudamonadales	92.0	0.0
GESC11	*Stenotrophomonas sp.*(AY162068.1)	Xanthomonadales	98.0	0.0
GESC12	*Stenotrophomonas sp* (AY162068.1)	Xanthomonadales	89.0	0.0
GESC16	*Ralstonia sp.* (AY162057.1)	Burkholderiales	98.0	0.0
GESC10	*Bacillus cereus* (AB247137.1)	Bacillales	98.0	0.0
GESC4	*Bacillus cereus* (FJ685763.1)	Bacillales	95.0	0.0
GESC13	*Bacillus sp.*(AY461752.2)	Bacillales	96.0	0.0
GESC15	*Bacillus cereus*(CP001407.1)	Bacillales	96.0	0.0

Figure 9: Relative abundance (%) of bacterial phylogroups from cloned 16S rRNA genes derived from homogenized guts of *E. saccharina* larvae.




GESP12
U30931.1Enterococcus saccharolyticus
GESP14
EU151765.1Enterococcus casseliflavus
GESP17
AJ301833.1Enterococcus gallinarum
GESC14
Enterococcus group
GESC10
GESC13
FJ190081Bacillus cereus
AY461752.2Bacillus sp.
GESC15
GESC4
Bacillus group
GESP13
X93928.1Spirochaeta africana (T)
GESP8
DQ154925.1Gordonia polyisoprenivorans
GESP35
AJ310417.1Plantibacter flavus
GESP29
EU714343.1Microbacterium aurum
GESP31
EF555584.1Nocardioides hankookensis
GESC6
AB041617.3 Propionibacterium acnes
Actinobacteria
GESP32
DQ202243.1Uncultured bacterium
DQ337547.1Caulobacter sp.
GESP33
AM422370.1Ochrobactrum haematophilum
EU543576.1Ochrobactrum sp.
GESP2
GESP4
Ochrobactrum-caulobacter group
EU096232.15Wolbachia pipientis
DQ412085 Wolbachia endosymbiont
CP001391.1Wolbachia sp. wRi
GESP18
GESP23
GESP22
GESP26
GESP20
GESP21
GESP19
GESP16
GESP11
GESP25
GESP27
GESP15
GESP24
Wolbachia endosymbionts
GESC3
EU373389.1Achromobacter xylosoxidans
GESC16
AB272352Serratia marcescens subsp. marcescens
GESP30
EU781738.1Serratia sp.
EF071131.1 gamma proteobacterium
GESP3
GESP6
S001020552 Escherichia coli
EU301772 Klebsiella pneumoniae
GESP9
U32868.1 Klebsiella sp.
GESP1
FJ796201.1Klebsiella pneumoniae
GESP34
GESP5
GESP10
GESP28
GESP7
Serratia-Klebsiella- Achromobacter
GESC12
GESC11
GESC2
AY162068.1Stenotrophomonas sp.
Stenotrophomonas
GESC5
AB247137.1GI 92380935 Bacillus cereus
0.1

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMENDATIONS

5.1 Discussion

Morphological examination of the KMM1 and PDA culture isolates from homogenized guts of *Eldana saccharina* larvae revealed bacterial and yeast colonies with variety of pigmentation and colonial morphologies while microscopic examinations showed unicellular microorganisms with diverse cell morphologies and Gram staining properties (Plates 19-.28). Among the Actinobacteria, all the 41 culture isolates produced extensively branched primary mycelia which transformed during the life cycle into aerial mycelia bearing the typical spore. Some of the colonies formed a secondary growth zone around the primary colony, a feature that is common among the *Streptomyces* (Manteca and Sanchez, 2009). The most common mycelial colours were grey and beige in addition to cream, brown, and white, while the spores had white, grey, pink and beige colours above the aerial mycelial network (Plates 6-18). This diversity of colony morphology and colour under similar culture media conditions provided a preliminary basis for differentiating the isolates. The morphologic differentiation of the isolates was further supported by microscopic examination of slide cultures of the different isolates, which revealed differences in mycelial branching patterns, spore chain morphology and Gram stain properties of the sporophores and spores for the Actinobacteria (Plates 7-13).

Sequence analysis of the microbial isolates and the cloned 16S rRNA gene further revealed a high diversity of bacterial communities as identified by the different

characterization methods used. Several phylogenetic divisions of the Kingdom Bacteria were represented among the sequences, in which most of the cloned sequences were closely affiliated to the Proteobacteria division. The highest number of clones represented the α-Proteobacteria subdivision while a few members of the β-Proteobacteria were also detected (Figure 10). Members of Actinobacteria sub-division, spanning five major families of the Actinomycetales were prevalent among the sequences from clones and culture isolates, with the rest representing γ-Proteobacteria, α-Proteobacteria, and the Firmicutes (Figures 4, 5 and 10). These findings closely concur with previous reports on microbial associations in the larval guts of the long horn Cerambycid beetles (Grünwald *et al.*, 2009), larval midguts of the Gipsy moth (Broderick *et al.*, 2004) and oral secretions of bark beetles (Cardoza *et al.*, 2009) but with differences at the genus and species profiles.

In this study, thirteen clones in the α-Proteobacteria subdivision had closest sequence matches to the *Wolbachia* operational taxonomic unit (Table 4). Previous occurrence of *Wolbachia* species in insects has been reported among the pyralid *Paraponyx sp.* (Werren *et al.*, 1995), as well as the Mediterranean flour moth *Ephestia kuehniella* (Fujii *et al.*, 2001). This is an interesting observation owing to the fact that the larvae of *Paraponyx sp.*, like those of *E. saccharina* feed on plants found in aquatic habitats which could have been a possible source of endosymbiont acquisition. *Wolbachia* species are unculturable, maternally-transmitted obligate intracellular bacteria that are known to invade and maintain themselves in numerous arthropods and nematodes through the induction of reproductive parasitism and the establishment of a mutualistic symbiosis. The role of *Wolbachia* in *E. saccharina* is not clear though members of the *Wolbachia* genus are

estimated to occur in nearly 70% of all insects and other arthropods, where they have been implicated in cytoplasmic incompatibility, parthenogenesis, feminization and male killing (Vera, *et al.*, 2009; Lo *et al.*, 2007; Werren and Windsor 2000). It is worthy to note that no *Wolbachia* species were detected from the culture isolated sequences, a finding that affirms the importance of using a combination of detection methods in studying microbial diversity (Bramucci *et al.*, 2003).

Other OTUs detected in the α-Proteobacteria included *Ochrobactrum* and *Caulobacter*. The *Ochrobactrum genus* is recognized as a highly versatile *proteobacterium* with ability to colonize an exceptionally wide variety of habitats. Strains of *Ochrobactrum anthropi* have previously been reported to support larval development of *Musca domestica* (Zurek *et al.*, 2000) and the same metabolic role could be possible for *E. saccharina*. *Caulobacter* species are known to possess the ability to receive and transfer plasmids (Ely, 1979), a phenomenon that could play both defensive and nutritive roles for *E. saccharina* larvae (Feldhaar and Gross, 2009).

The second largest Phylogroup from the cloned and culture isolated sequences was γ- Proteobacteria, being represented by the *Enterobacter, Klebsiella, Serratia* and *Stenotrophomonas* taxa. These phylotypes are quite ubiquitous in nature, occurring in fresh water, soil, plants and animals, including insect guts (Feldhaar and Gross, 2009; Oliveira *et al.*, 2000, Zayed and Bream 2004). Members of the *Stenotrophomonas* genus have previously been reported to colonize midguts of divergent insect lineages including the sand fly, *Lutzomyia longipalpis* (Gouveia *et al.*, 2008) and the Diamondback moth, *Plutella xylostella* (Indiragandhi, *et al.*, 2007). The detection of these members of the

Enterobacteriaceae family in *E. saccharina* concurs with previous reported presence of this group as endosymbionts in several other insect species (Rani *et al.*, 2009; Gouveia *et al.*, 2008; de Vries *et al.*, 2008; Andreote *et al.*, 2004). This presence is further supported by the reported characteristic of most members of the *Enterobacteriaceae* to utilize a wide variety of carbohydrate substrates (Holt *et al.*, 1994; Ngugi *et al.*, 2007), such as those associated with feeding habitats of *E. saccharina*.

The Firmicutes Phylogroup had a notably higher representation among the cloned sequences as compared with the culture derived sequences. Six (6) clones shared close sequence relatedness with the genus *Enterococcus* while four (4) clones represented *Bacillus cereus*. Only one culture-isolated sequence (KMESP-4) had a close match with the genus *Enterococcus*. Diverse *Enterococcus* phylotypes have previously been identified from the larval guts of the gypsy moth, *Lymantria dispar*, a polyphagous Lepidopteran insect whose larval midguts have been recorded to exhibit typically high pH profiles, ranging from 8.0 to 10.0 (Brinkmann *et al.*, 2008; Broderick *et al.*, 2004).

Analysis of the 16S rRNA gene sequences placed the culture isolates into three main phylotypes in the Actinobacteria division, namely *Streptomyces* (18 isolates), *Microbacterium* (4 isolates) and the *Cellulosimicrobium* (1 isolate). On the other hand, the cloned sequences, GESP 29, GESP 31, GESP 35 and GESC 6 were closely related to five different actinobacteria taxa, namely *Gordonia polyisoprenivorans*, *Microbacterium aurum*, *Nocardioides hankookensis*, *Plantibacter flavus* and *Propionibacterium acnes* respectively (Tables 4 and 5). Previous studies have reported *Gordonia polyisoprenivorans* to exhibit capabilities to degrade complex organic polymeric compounds including Polyisoprene rubber (Linos *et al.*, 1999). *G. polyisoprenivorans* has

also been reported to secrete exopolysaccharides which have emulsifying activity for mono-aromatic petroleum hydrocarbons like Benzene and Toluene (Fusconi *et al.*, 2010). Such attributes demonstrate the potential for biotechnological application of *G. polyisoprenivorans* in the breakdown of environmental contaminants (Arenskötter *et al.*, 2004), and further affirm the importance of insects as potential sources of novel microbial resources of industrial importance (Kaltenpoth, 2009). Similar occurrences of the genera *Gordonia, Microbacterium and Nocardioides* have been reported in the larval gut systems of the long-horned Cerambycid beetles (Grünwald *et al.*, 2009) and the Longicorn beetle (Park *et al.*, 2007). The *E. saccharina* larvae used for this study were collected from humid sites with a lot degrading vegetative matter, especially the papyrus rhizomes, and this may have provided ideal habitats for the proliferation and acquisition of the coryneform bacteria *Plantibacter* and the *Propionibacterium*. Members of the two genera have previously been reported to be involved in energy metabolism in insects and the higher animals (Cruden & Markovetz, 1987). Detection of the presence of these two members in *E. saccharina* gut homogenates could suggest a possible role in mobilizing energy for the late stage larvae from cellulosic matter and also from fatty body reserves in preparation for pupation and eclosion (Wu *et al.*, 2009; McFarlane and Alli, 1995; Jarvis *et al.*, 1998).

Results from sequence analysis showed dominance of the *Streptomyces* genus in the culture isolated sequences both in abundance and species diversity. Several reasons may have been responsible for this finding. Firstly, the culture method and isolation medium used in this study were selective for streptomycetes due to their relative rapid growth over other actinobacteria under similar conditions (Lazzarini *et al.*, 2000).

Streptomycetes are also known to abound in agricultural soils and aquatic habitats especially in environments that are rich in organic matter such as were the sites from where the larvae for this study were sampled. Thus the acquisition of streptomycetes by *E. saccharina* and the successful development of a symbiotic association may be reflected in the diversity and abundance of the *Streptomyces* genus as observed in this study. Their possible role in the nutrition of *E. saccharina* larvae especially in enabling survival of the insect on nutritionally poor diets like those found in the papyrus rhizomes could also partly explain the relatively higher diversity of *Streptomyces* isolates in the gut micro flora of *Eldana saccharina*. The larvae of *E. saccharina* are known to ingest large amounts of plant fibres as they tunnel through the sugarcane stalks or papyrus rhizomes (Plate 2). The microbial digestion of this cellulosic biomass would play an important role in the survival of *E. saccharina,* especially those living on high fibre, low nutrient papyrus rhizomes. Fungi and certain Actinobacteria are the only microorganisms that have developed a strategy for nutrient recycling through enzymatic breakdown of lignocellulose (McCarthy, 1987). Diverse Actinomycetes with ability to degrade hemicellulose (xylan) and pectin have also been isolated from guts of Longicorn beetles (Park *et al.,* 2007). *Streptomyces* are the most successful genus in the Actinomycetales order. They are metabolically diverse, with capabilities to digest many organic compounds, including the more complex recalcitrant components of plant fibres, through production of extracellular hydrolytic enzymes (Goodfellow & Williams, 1983; McCarthy & Williams, 1992). Diverse species of *Streptomyces* with cellulolytic activity have previously been isolated from agricultural and forest soils (Semêdo *et al.*, 2004).

Streptomycetes are also of medical and industrial importance because they synthesize antibiotics, enzymes and a host of other metabolites (Nakashima *et al.*, 2005; Omura *et al.*, 2001). Several theories have been suggested to explain antibiotic and other secondary metabolites production. The most widely accepted theory is that antibiotics help the host insect to compete with other organisms in the relatively nutrient-depleted environment of the soil by antagonizing deleterious competitors (Kaltenpoth, 2009).

Recent findings indicate that symbiotic microorganisms that produce a large variety of secondary metabolites (Kaltenpoth, 2009) and several novel compounds with antimicrobial activity have been isolated from mutualistic bacteria, particularly those that protect their hosts against pathogens (Piel, 2009). For example, Cardoza and co-workers (2006) reported isolation of antifungal compounds from *Streptomyces* and a number of other actinobacteria in oral secretions of the spruce beetle, which were active against antagonistic fungi that invade the beetles' ovipositional and larval feeding galleries.

Besides *Streptomyces*, other Actinobacteria taxa identified from the isolates included the *Microbacterium* and *Cellulosimicrobium*. The genus *Microbacterium* was unified with the genus *Aureobacterium* with which it forms a monophyletic association (Takeuchi and Hatano, 1998). *Microbacterium species* have previously been isolated from midguts of wild *Culex quinquefasciatus* mosquitoes (Pidiyar, *et al.,* 2004), oral secretions of the Spruce bark beetle, *Dendroctonus ponderosae* Hopkins (Cardoza *et al.*, 2009), and from phyllosphere of grasses and mulch swards (Behrendt, *et al.,* 2002). Cellulolytic strains of *Cellulosimicrobium* have previously been isolated from the guts of larvae and adults of the red Turpentine beetle, *Dendroctonus valens* Laconte (Morales-Jiménez *et al.*, 2009); and in the hind gut of the Australian termite *Mastotermes*

darwiniensis Froggatt, (Bakalidou *et al.*, 2002). Strains of *Cellulosimicrobium cellulans* have also been shown to exhibit yeast-lytic β-1, 3-glucanase activity (Ferrer, 2006), a feature that would find useful applications in screening for anti-fungal agents. *Cellulosimicrobium* belongs to the Promicromonosporacea family of actinomycetes.

In this study it was observed that several isolates consistently exhibited secretory structures in plate cultures, which secreted products on the colonies (Plates 9, 10, 13, 17 and 24). This interesting phenomenon is the subject of further investigation into the biochemical properties of the secretions and their functions first at the insect physiology level and then their potential application in biotechnology. Similarly, all the Actinobacteria isolates tested in this study were able to grow in α-cellulose and sugarcane bagasse for up to 14 days and to produce cellular biomass (Figures 1 and 2). Streptomycetes are well recognized for their role in the degradation of lignocelluloses in decomposition of plant litter in diverse environments (Williams, 1978). Therefore, the observed abundance and diversity of *Streptomyces* in the guts *E. saccharina* larvae is of considerable metabolic significance. Moreover, the finding of Actinobacteria in *E. saccharina* from sugarcane and papyrus host plants further supports the nutritional versatility of this bacterial Phylogroup, enabling the host insect to thrive on nutrient- rich and nutrient poor substrates, the ability to degrade complex compounds and the potential to antagonize competitors through production of antibiotics and other metabolites (Kaltenpoth, 2009; Wood *et al.*, 2001). These attributes are of considerable significance to the survival of the host insect due to the fact the natural habitats for *E. saccharina* are also inhabited by a vast diversity of microorganisms many of which would be pathogenic to the insect. Production of antibiotics and other metabolites could also protect *E.*

saccharina from parasitoids and predators (Kaltenpoth *et al.*, 2005). The ability of the Actinobacteria isolates to utilize cellulose and sugar cane bagasse further suggests a possible role of these bacteria in the energy metabolism for *E. saccharina.* Similar associations and role have been observed in the spruce bark beetles *Dendroctonus ponderosae* (Cardoza *et al.*, 2009) and the litter feeding woodlouse *Porcellio scaber* (Ihnen and Zimmer, 2007), among others.

Culture isolation using selective PDA medium yielded yeast isolates from the three groups of *E. saccharina* larvae (Plates 26-30) and only one fungal isolate from the sugarcane group of *E. saccharina* larvae. Yeasts isolated from xylophagous insects have been evaluated for their ability to degrade xylan and assimilate D-xylose (Grünwald *et al.*, 2009; Nguyen *et al.*, 2006).

For control experiment, 16th generation larvae of *E. saccharina* were used in this study. Our inability to isolate Actinobacteria from these laboratory reared control *E. saccharina* larvae coupled with a relatively low microbial diversity and abundance by culture dependent or sequence based approaches (Table 2; Figure 8c) may partly be due to controlled anti-bacterial treatment of the artificial diet at the *icipe* insect mass rearing unit (Onyango and Ochieng'-Odero, 1994; McCarthy *et al.*, 1978). Moreover, the fact that no *Wolbachia* species were identified from clonal sequences of laboratory reared control *E. saccharina* (Table 4) similarly suggests a possible loss of the symbionts from founder colonies through a succession of generations of laboratory rearing.

From available literature there is no previous recorded in-depth study report on the microbial diversity in the intestinal tracts of *E. saccharina* or any of the African graminivorous stem borers using a combination of culturing and culture independent

approaches. The information obtained in this study will therefore be a valuable resource in furthering our understanding of the interaction between *E. saccharina* and its symbionts in the design and implementation of Integrated Pest Management strategies (Conlong and Rutherford, 2009; Gounou *et al.*, 2008; Conlong, 2001).

5.2 Conclusions

In summary, cultivation dependent and sequence-based profiling of the 16S rRNA gene used in this study has revealed a high microbial diversity from gut homogenates of *E. saccharina* with the dominance of the Proteobacteria and Actinobacteria phylogroups. These methods have the potential to explore complex insect gut metagenomes and to investigate interactions between insects and microorganisms as they take place in nature. In this study, 32 culture isolates and 48 clonal sequences from the intestinal tracts of *E. saccharina* larvae were characterized to genus and species level on the basis of sequence homology search results from the GenBank. The observed polymorphism in the 16S rRNA RFLP profiles from the papyrus and sugarcane-derived *E. saccharina* larvae compared with the laboratory controls suggest that the insects' habitat and diet had an influence on species composition and diversity of the gut microbial communities. The study also revealed significant differences in abundance between microbial isolates from wild and laboratory reared *E. saccharina* larvae. Thirty two (32) of the tested Actinobacteria isolates were able to grow on cellulose and sugarcane bagasse respectively, as sole carbon sources. In addition the study revealed bacterial and yeast isolates with secretory products as potential microbial candidate isolates for further investigation and application of the metabolites secreted. The search and development of

natural products in diverse insect microbiomes remain an indispensable and unparalleled source of biologically active compounds. Thus, research into the diversity of symbiotic microbiota in the larval guts of *E. saccharina* has helped to shed some light on the extant microbial communities associated with this economically important and biologically versatile insect. Microorganisms are currently accepted as the best renewable source for bioactive compounds, and the exploration of yet under-explored sources, such as the insect symbiotic systems and associations, has a great potential for discovery of novel bioactive producing microbial genes that would be useful for further development as enzymes and drug candidates.

5.3 Recommendations

Further work will be necessary to:

1. Complete characterization to sequence level of the isolates derived from *E. saccharina* larvae feeding on sugarcane stalks as well as the yeast isolates in order to assign identities to the isolates and thus provide a more complete view of the microbial diversity in *E. saccharina*.
2. Employ archaea specific primers to screen for members of the Archaea domain in order to give further insights into the complete microbial community and species structures in the guts of *E. saccharina*.
3. Explore the *Wolbachia* phylogenetic super groups present in *E. saccharina* using *Wolbachia* specific primers.
4. Characterize the metabolites produced in the observed microbial secretions and to elucidate the sequence motifs, pathways and functions of the genes responsible.

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